Clock genes and biological rhythms

Effects of psychotropic drugs

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CONTENTS

Scientific environment

The work presented in this thesis was initiated while I was a research student in the Medical School Research Program (Forskerlinjen), during my medical studies at the University of Bergen (2002-2009). Subsequently, I was a full-time PhD student and completed the thesis in the spring of 2012. The studies were carried out in Dr. Einar Martens Research Group for Biological Psychiatry at the Center for Medical Genetics and Molecular Medicine, Haukeland University Hospital, and the Department of Clinical Medicine, University of Bergen, Norway. Professor Vidar M. Steen acted as main supervisor, with Dr. Johan Fernø serving as co-supervisor.

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Bergen, August 2012

Teresa Maria Osland

Abbreviations

When applicable, the notation refers to mouse genes. In line with the International Committee on Standardized Genetic Nomenclature for Mice (Eppig*, et al.*, 2007), gene symbols are italicized and begin with an uppercase letter, followed by lowercase letters, whereas protein symbols use all uppercase letters, and are not italicized.

Acaca	Acetyl-Coenzyme A carboxylase α
Actb	β−actin
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
ASPS	Advanced Sleep Phase Syndrome
B2M	β-2 microglobulin
Bmal1	Brain and muscle aryl hydrocarbon receptor nuclear translocator like 1 (*Arntl*)
C6	Rat glioma cell line
cDNA	complementary DNA
Clock	Circadian locomotor output cycles kaput
Cry1	Cryptochrome 1
Cry2	Cryptochrome 2
Csnk1ε	Casein kinase 1, ε (*Ck1ε*)
Dbp	D site albumin promoter binding protein
DMSO	Dimethyl sulfoxide
DSPD	Delayed Sleep Phase Disorder
E4bp4	E4 promoter binding protein 4 (*Nfil-3*)
Fads2	Fatty acid desaturase 2
Fasn	Fatty acid synthase
FASPS	Familial Advanced Sleep Phase Syndrome
GABA	γ-aminobutyric acid

GaMg	Human glioma cell line
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase
GRP	Gastrin-releasing peptide
Gsk-3β	Glycogen synthase kinase 3β
GWAS	Genome-wide association study
H-35	Rat hepatoma cell line (Reuber's)
HepG2	Human hepatoma cell line
HK-2	Human kidney cell line
Hlf	Hepatic leukemia factor
Hmgcr	3-hydroxy-3-methylglutaryl-CoA reductase
MEQ	Horne-Östberg Morningness Eveningness Questionnaire
mRNA	messenger RNA
Mvk	Mevalonate kinase
NIH-3T3	Mouse embryonic fibroblast cell line
NKA	Na^+/K^+-ATPase
Nthy-ori 3-1	Human thyroid cell line
PACAP	Pituitary adenylate cyclase-activating polypeptide
Per1	Period homolog 1 (Drosophila)
Per2	Period homolog 2 (Drosophila)
Per3	Period homolog 3 (Drosophila)
Pgc-1α	Peroxisome proliferator activated receptor γ coactivator 1α (*Ppargc1α*)
Ppar-α	Peroxisome proliferator activated receptor α
Ppar-γ	Peroxisome proliferator activated receptor γ
PS	Preferences Scale
qRT-PCR	Quantitative real-time polymerase chain reaction
Rev-Erb-α	Reverse viral erythroblastis oncogene product α (*Nr1d1*)
RHT	Retinohypothalamic tract
Ror-α	Retinoic acid-related orphan receptor α
RORE	Retinoic acid-related orphan receptor response element
Rplp0	Ribosomal protein, large, P0

Scd1	Stearoyl-Coenzyme A desaturase 1
Scd2	Stearoyl-Coenzyme A desaturase 2
SCN	Suprachiasmatic nucleus
shRNA	Small hairpin RNA
SNP	Single-nucleotide polymorphism
SREBP	Sterol regulatory element-binding protein
SSRI	Selective serotonin reuptake inhibitor
Tef	Thyrotroph embryonic factor
VIP	Vasoactive intestinal peptide
VNTR	Variable number tandem repeat
VP	Vasopressin

Summary

The sleep-wake cycle, lipid metabolism and hormone levels are examples of circadian rhythms, which are endogenously generated cycles that reach both maximum and minimum values once in the course of 24 hours. In mammals, circadian rhythms are regulated by a set of clock genes that are functionally linked, and polymorphisms in these genes may be associated with variations in circadian rhythms. Disruption of the circadian clock has been associated with poor physical health, including metabolic disturbances such as obesity and dyslipidemia, as well as mental illness, including bipolar disorder, a severe chronic affective disorder. The mood stabilizer lithium is widely used in the pharmacological treatment of bipolar disorder, and has been shown to affect circadian rhythms, although its molecular mechanisms of action remain largely unknown.

In this study, we investigated cultured mice fibroblasts (NIH-3T3 cells) for effects of lithium on the expression of genes that regulate the circadian clock. Robust circadian oscillations of rhythmic clock genes were observed in control and lithium-treated samples in this model of the circadian clock. A main effect of lithium was to differentially alter the amplitude of expression of several clock genes, including an increase in the peak amplitude of *Per2* and *Cry1*, and a reduction of the maximal amplitude of *Per3*, *Bmal1* and *Rev-Erb-α* transcription, indicating a possible role for alteration of oscillation amplitudes of clock genes in the mechanisms of lithium action on biological rhythms. Additional mood stabilizers, antipsychotics and antidepressants are also used in the treatment of bipolar disorder; hence we investigated whether they too affect the transcription of key circadian clock genes. Interestingly, clock gene expression was differentially up- or down-regulated, such as a reduction of *Per2* transcription by clozapine and imipramine, and an increase in the expression of *Rev-Erb-α* by clozapine. The observed drug-induced effects

could reflect shared regulatory mechanisms that contribute to their psychotropic action. Since numerous antipsychotics and antidepressants are known to induce lipogenic gene expression, and given the suggested link between the circadian clock and lipid metabolism, we also examined their effects on lipid metabolism-related genes. The antipsychotics and antidepressants increased the transcription levels of lipid metabolism genes (which did not oscillate), whereas the mood stabilizers had no such effect, indicating that no evident regulatory link between the circadian clock and lipid metabolism is present in this model system, although it cannot be excluded.

Genetic factors are likely to influence diurnal preference (chronotype), and in search of associations between clock gene variants and chronotype, several studies have investigated the role of the clock gene *PER3*, and found an association between a variable number tandem repeat (VNTR) in *PER3* and diurnal preference. However, conflicting findings have been reported, and our replication study on 432 healthy Norwegian students did not confirm the association, indicating that variation in the *PER3* VNTR does not appear to be associated with self-report measures of chronotype in our study sample, and suggesting that further studies are needed to clarify the proposed role of *PER3*.

It is not the strongest of the species that survives, nor the most intelligent that survives. It is the one that is the most adaptable to change.

Charles Darwin

1 Introduction

1.1 Circadian rhythms and physiological processes

Rotation of the earth around its axis creates a repetitive cycle of day and night. Most living organisms, spanning from humans and animals to plants, fungi and bacteria, anticipate the daily changes in their environment by having circadian rhythms, which reflects the strong pressure in natural selection in favoring organisms with a built-in clock. The circadian clock is evolutionary conserved and is estimated to be around 700 million years old, originating from the time when plants diverged from the common lineage with animals and fungi (for review, see Dunlap, 1999; Lowrey and Takahashi, 2004; von Schantz, 2008). In comparison, *Homo sapiens* emerged only 200,000 years ago.

The circadian clock is a cellular mechanism that generates rhythmic output, with temporal organization, allowing biological processes to occur at the most opportune time, while preventing incompatible reactions from taking place simultaneously (reviewed by Gachon*, et al.*, 2004). The term circadian derives from the Latin *circa diem*, meaning "about one day", which is roughly the period length of circadian rhythms. Examples of physiological parameters that display circadian rhythms include the sleep-wake cycle, feeding behavior, core body temperature, hormone levels, blood pressure and metabolism (for reviews on circadian rhythms, see Dardente and Cermakian, 2007; Takahashi*, et al.*, 2008; Hastings*, et al.*, 2008). The human circadian rhythms of core body temperature, physical activity and cognitive functions all peak in the afternoon, while in contrast triglyceride levels and secretion of the hormone melatonin both peak during the subjective night (Redman*, et al.*, 1983; Rajaratnam and Arendt, 2001), see Figure 1.1.

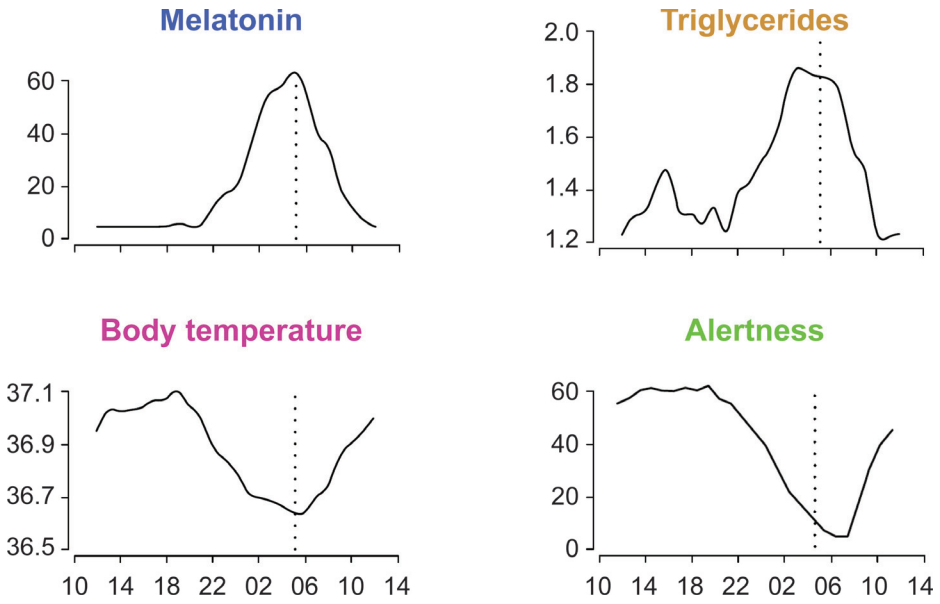

Figure 1.1. Circadian rhythms of melatonin, triglycerides, core body temperature and subjective alertness in human beings. Plasma melatonin and triglyceride levels peak during the night. They have similar phases and oscillate in antiphase with core body temperature and alertness levels, which reach their minimum values (troughs) at night. The parameters were measured in humans kept in controlled conditions, and are given as functions of circadian time (in hours). Plasma melatonin level is given in pg/mL, plasma triglycerides in mmol/L, body temperature in degrees Celsius and alertness was estimated with a subjective scale (0 = not alert and 100 = very alert). Illustration modified from Rajaratnam and Arendt and reproduced with permission of the authors (Rajaratnam and Arendt, 2001).

Circadian oscillations are characterized by their period, amplitude and phase. The rhythms are endogenously generated, but may be entrained by external time cues (*Zeitgebers*). The most important cue is light, although feeding time and ambient temperature may also set the clock (Damiola*, et al.*, 2000; Stokkan*, et al.*, 2001; reviewed in Gachon*, et al.*, 2004). Circadian rhythms are still existent in the absence of external Zeitgebers and remain invariant within a physiological range of temperatures (temperature compensation), even in cell culture (Tsuchiya*, et al.*, 2003; Izumo*, et al.*, 2003).

1.2 The mammalian circadian pacemaker and peripheral clocks

Since the endogenous period commonly deviates slightly from 24 hours, the clock needs continuous adjustment to the external 24-hour cycle. This takes place in the circadian pacemaker, which in mammals consists of a population of neurons in the suprachiasmatic nuclei (SCN) of the brain. The SCN are bilateral structures located in the anterior hypothalamus, situated just above the optic chiasm, which in mice consist of around 10,000 neurons each (Abrahamson and Moore, 2001). It was demonstrated that bilateral ablation of the SCN in rats permanently destroyed their circadian rhythms (Figure 1.2A) (Stephan and Zucker, 1972; Moore and Eichler, 1972) and it was later shown that surgically implanting intact SCNs in hamsters restored rhythmicity (Lehman*, et al.*, 1987). Moreover, functional SCN transplants in genetically arrhythmic mice generated a circadian rhythm with a period similar to that of the donor animal, and not the host (Ralph*, et al.*, 1990; Sujino*, et al.*, 2003).

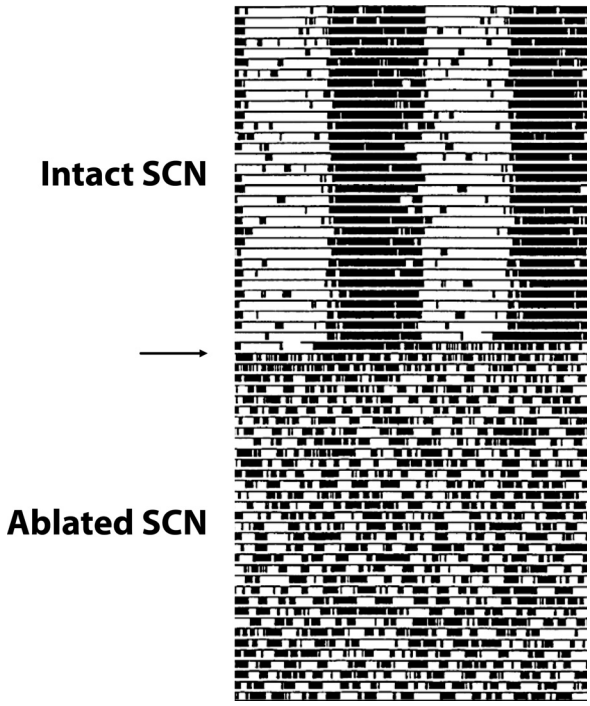

Figure 1.2A. Ablation of the SCN causes loss of circadian rhythms. The locomotor activity recording of a rat kept in a light-dark cycle is depicted on horizontal lines, with two days per line. Locomotor activity displays a circadian pattern of activity (*black bars*) and rest (*white bars*). The data has been double-plotted to facilitate visualization, with each line showing the preceding day and the following day. Following bilateral SCN ablation (as indicated by the arrow), activity is randomly distributed, indicating loss of circadian rhythms. Modified from Moore (Moore, 1999).

The circadian clock is characterized by three components: input pathways that reset the time, a pacemaker that generates the rhythm, and output signals that control circadian gene expression to promote circadian physiology and behavior (for review, see King and Takahashi, 2000; Lowrey and Takahashi, 2004). Light is a powerful Zeitgeber, and entrainment of the SCN clock is achieved by means of light-sensitive neurons in the retina that project directly to the SCN via the retinohypothalamic tract (RHT) (Figure 1.2B) (Moore and Lenn, 1972; Sadun*, et al.*, 1984). These retinal ganglion cells express the photopigment melanopsin (Gooley*, et al.*, 2001; Hannibal*, et al.*, 2002; Hattar*,*

et al., 2002; Hannibal*, et al.*, 2004). Melanopsin transduces light, and electrical signals are sent through the RHT to the SCN where the neurons release the transmitters glutamate and pituitary adenylate cyclase-activating polypeptide (PACAP) (Hannibal*, et al.*, 2000; reviewed in Reppert and Weaver, 2002; Hannibal, 2002), initiating a cascade of reactions that leads to adjustment of the circadian clock.

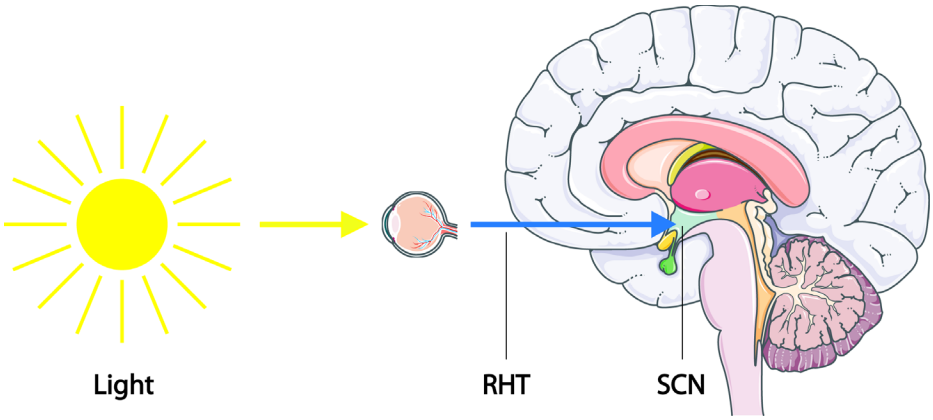

Figure 1.2B. The retinohypothalamic tract. Light is a powerful Zeitgeber, and is transduced into neural signals by specific light-sensitive neurons in the retina of the eye. In turn, these retinal ganglion cells project directly to the SCN via the RHT, where they serve to entrain the SCN clock. Illustration by Osland TM, with the use of Servier Medical Art.

Distinct cell populations in the SCN contribute to the clock, and based on their neurochemical properties they form two functional subregions, where a venterolateral core contains neurons expressing several neurotransmitters, including vasoactive intestinal peptide (VIP) and gastrin-releasing peptide (GRP), whereas a dorsomedial shell is characterized by neurons that express vasopressin (VP) (Abrahamson and Moore, 2001; reviewed in Moore*, et al.*, 2002; Hastings and Herzog, 2004). In addition, in most SCN neurons, the above-mentioned neuropeptides are colocalized with γ-aminobutyric acid (GABA) (Moore and Speh, 1993). To adjust the SCN clock by light, retinal input entrains the core neurons of the SCN, before the signal is conveyed from

the light-induced cells in the core to the rhythmic cells in the shell, which then set the clock (reviewed in Antle and Silver, 2005).

Circadian rhythms are found throughout the body, including peripheral organs such as lungs, kidneys, liver and skin (Zylka*, et al.*, 1998; Yamazaki*, et al.*, 2000; Bjarnason*, et al.*, 2001; Yoo*, et al.*, 2004). SCN neurons mainly project to neighboring areas in the hypothalamus, but also to the surrounding thalamus and other areas of the brain (Berk and Finkelstein, 1981; Abrahamson and Moore, 2001). Circadian output signals from the SCN are transmitted via neural connections (Ueyama*, et al.*, 1999; la Fleur*, et al.*, 2000; for review, see Kalsbeek*, et al.*, 2006) and humoral pathways (Silver*, et al.*, 1996; Oishi*, et al.*, 1998b; McNamara*, et al.*, 2001) to synchronize local clocks in peripheral organs. The pacemaker is self-sustained, whereas the oscillation of peripheral clocks will dampen markedly after a few cycles without input from the SCN (Yamazaki*, et al.*, 2000; reviewed in Hirota and Fukada, 2004). Circadian clocks consist of numerous, autonomous single cell oscillators that are synchronized to generate coordinated output (Welsh*, et al.*, 1995), and certain cell cultures grown *in vitro* may also display circadian rhythms (Balsalobre*, et al.*, 1998; Akashi and Nishida, 2000; Allen*, et al.*, 2001). Although there is a delay of 4-6 hours in the expression patterns of circadian clock genes and proteins between the SCN and peripheral clocks (Lopez-Molina*, et al.*, 1997; Balsalobre*, et al.*, 1998; Zylka*, et al.*, 1998; Allen*, et al.*, 2001; reviewed in Balsalobre, 2002), the clock mechanisms are highly similar (reviewed in Ko and Takahashi, 2006), which allows us to use cell culturing as a model for peripheral circadian clocks.

1.3 Clock genes and molecular mechanisms of the mammalian circadian clock

Clock genes encode proteins that are necessary for the generation or regulation of the circadian clock. Numerous clock genes have oscillating expression patterns, such as period homolog 1 (*Per1*), period homolog 2 (*Per2*), period homolog 3 (*Per3*), cryptochrome 1 (*Cry1*), cryptochrome 2 (*Cry2*) and brain and muscle aryl hydrocarbon receptor nuclear translocator like 1 (*Bmal1*) (Tei, *et al.*, 1997; Balsalobre, *et al.*, 1998; Oishi, *et al.*, 1998a; Zylka, *et al.*, 1998; Fustin, *et al.*, 2009), whereas others are expressed at constant levels, including circadian locomotor output cycles kaput (*Clock*) (Oishi, *et al.*, 1998a; Shearman, *et al.*, 1999; Shearman, *et al.*, 2000) (Figure 1.3A). Interestingly, mice with a clock gene mutation may have abnormal rhythms or may even be arrhythmic (Vitaterna, *et al.*, 1994; Bae, *et al.*, 2001; reviewed in King and Takahashi, 2000; Ko and Takahashi, 2006).

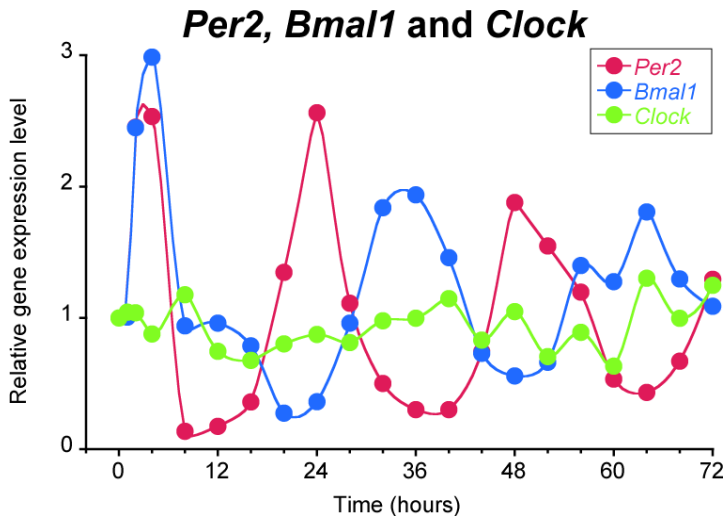

Figure 1.3A. Gene expression profiles of *Per2*, *Bmal1* and *Clock* in cultured mouse fibroblasts (NIH-3T3) cells following a serum shock. NIH-3T3 cells were transiently exposed to a high dose (50%) of serum (serum shock) to synchronize circadian rhythms. After an initial surge of transcription, robust oscillations of *Per2* (*red symbols*) and *Bmal1* (*blue symbols*) expression levels were observed, in antiphase with each other. In contrast, the expression of *Clock* (*green symbols*) did not oscillate. Cell cultures were serum-shocked at time t=0 and the mean value of n=3 replicates is given for each time point. The expression values were normalized to glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and the value at time t=0 was arbitrarily set to 1. Experiment performed by Osland TM.

Clock genes are linked together by several positive and negative transcriptional-translational feedback loops, ensuring stable oscillations in the levels of both messenger RNAs (mRNAs) of clock genes and their protein levels. The causes and effects in the feedback loops are circularly linked, and include the proteins CLOCK and BMAL1, forming a heterodimer that binds to regulatory sequences (E-boxes) to activate the transcription of several other clock genes, including *Per1, Per2*, *Per3, Cry1* and *Cry2* (King, *et al.*, 1997; Gekakis, *et al.*, 1998; Jin, *et al.*, 1999; Bunger, *et al.*, 2000; reviewed in Reppert and Weaver, 2001). Following translation in the cytoplasm, PER and

CRY proteins dimerize and enter the nucleus, where the PER-CRY complex inhibits the actions of the CLOCK-BMAL1 dimer, thus indirectly repressing their own transcription via a negative feedback loop (Kume*, et al.*, 1999; Vitaterna*, et al.*, 1999; Griffin*, et al.*, 1999; Shearman*, et al.*, 2000) (Figure 1.3B). Subsequently, PER and CRY proteins are degraded, and when their protein levels are sufficiently low, the cycle is complete and *Per* and *Cry* mRNA levels may rise again. Another feedback loop involves *Bmal1* transcription, which is positively regulated by retinoic acid-related orphan receptor-α (*Ror-α*) (Sato*, et al.*, 2004), and negatively regulated by reverse viral erythroblastis oncogene product α (*Rev-Erb-α*) (Preitner*, et al.*, 2002), where the proteins REV-ERB-α and ROR-α compete to bind at retinoic acid-related orphan receptor response elements (ROREs) in the promoter of *Bmal1* with opposite effects (Preitner*, et al.*, 2002; Sato*, et al.*, 2004; Guillaumond*, et al.*, 2005). In turn, the transcription of both *Ror-α* and *Rev-Erb-α* is modified by the CLOCK-BMAL1 dimer (Preitner*, et al.*, 2002; Sato*, et al.*, 2004; reviewed in Ko and Takahashi, 2006). The interacting feedback loops contribute to tight regulation of the clockwork, necessary to maintain a period of close to 24 hours. Furthermore, post-translational mechanisms, including phosphorylation, dimerization, nuclear import and export, regulation of transcriptional activity and chromatin modification all participate in fine-tuning the oscillations and contribute to the precision of the clock (Lee*, et al.*, 2001; reviewed in Ko and Takahashi, 2006; Dardente and Cermakian, 2007). As an example, phosphorylation of PER proteins by casein kinase 1, ε (CSNK1ε) contributes to regulating protein stability (Vielhaber*, et al.*, 2000; Lowrey*, et al.*, 2000; Vanselow*, et al.*, 2006).

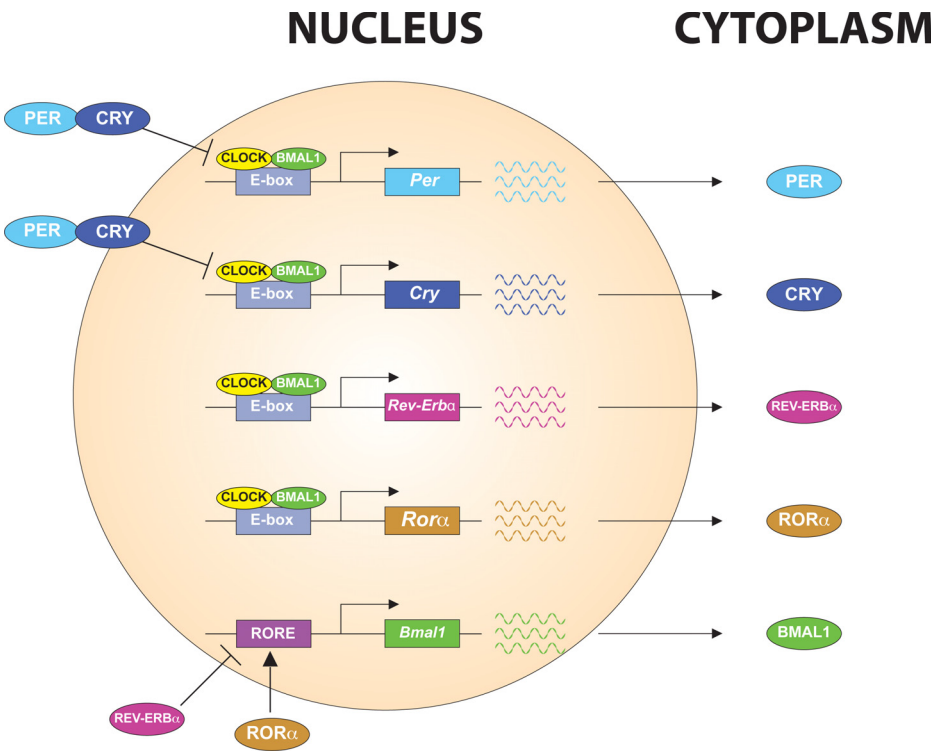

Figure 1.3B. Gene expression and feedback mechanisms in the mammalian circadian clock. The expression of the mammalian clock genes *Per*, *Cry*, *Rev-Erb-α*, *Ror-α* and *Bmal1* is tightly regulated to ensure proper circadian oscillations. The CLOCK-BMAL1 complex drives a positive forward loop by binding to E-boxes and promoting transcription of target genes, whereas the heterodimer PER-CRY inhibits transcription mediated by CLOCK-BMAL1. An additional feedback loop involves REV-ERB-α and ROR-α, that compete to bind to ROREs to regulate the expression of *Bmal1* with opposite effects. Illustration by Fernø J and Osland TM (modified from Hirota and Fukada, 2004).

The circadian clock regulates the expression of numerous target genes, including D site albumin binding protein (*Dbp*), hepatic leukemia factor (*Hlf*), thyrotroph embryonic factor (*Tef*) and E4 promoter binding protein 4 (*E4bp4*). These transcription factors exhibit circadian cycling in the SCN and peripheral tissues (Lopez-Molina*, et al.*, 1997; Balsalobre*, et al.*, 1998; Mitsui*, et al.*, 2001), and regulate downstream target genes involved in neurotransmitter metabolism and fatty acid metabolism (for review, see Staels, 2006).

Disruption of the circadian clock may have impact on many aspects of physiology and behavior, and has been associated with complications such as sleep disturbances, mental illness (including bipolar disorder), metabolic complications and cancer (for review, see Gachon*, et al.*, 2004; Lamont*, et al.*, 2007).

1.4 Diurnal preference and clock genes

Diurnal preference (chronotype) indicates the preference of an individual for morning versus evening. Morning types tend to rise early and are energetic in the first part of the day, whereas evening types generally get up later and have a correspondingly delayed activity peak. There is large variation in chronotype between individuals, also among people living in the same environmental conditions (reviewed in Roenneberg*, et al.*, 2007). These differences may be described by a scale of morningness-eveningness, as evaluated with standard questionnaires such as the Horne-Östberg Morningness Eveningness Questionnaire (MEQ, Horne and Ostberg, 1976) and the Preferences Scale (PS, Smith*, et al.*, 2002). Circadian rhythm sleep disorders are found at the two extremities of the chronotype scale. Subjects with advanced sleep phase syndrome (ASPS) suffer from drowsiness in the late afternoon (or early evening) followed by persistent early onset of sleep and spontaneous early morning awakening, both occurring before the conventional or desired time (ICSD-2, American Academy of Sleep Medicine, 2005). Delayed Sleep Phase Disorder (DSPD) is found at the other extremity, with subjects typically having a stable sleep schedule, with onset of sleep later than preferred (often between 2 a.m. and 6 a.m.) and great difficulty rising at the desired time in the morning (ICSD-2, American Academy of Sleep Medicine, 2005).

Several clock genes have been examined for influence on diurnal preference, and an association between a single-nucleotide polymorphism (SNP) in the

clock gene *CLOCK* and chronotype has been found (Katzenberg*, et al.*, 1998), but conflicting results have also been reported (Robilliard*, et al.*, 2002; Pedrazzoli*, et al.*, 2007). In addition, mutations in *PER2* may lead to a heritable autosomal dominant subtype of ASPS known as Familial Advanced Sleep Phase Syndrome (FASPS) (Toh*, et al.*, 2001). FASPS patients have advanced sleep-wake cycles, and their oscillations of core body temperature and melatonin are correspondingly advanced by 4-5 hours. Sequence variations of the human *PER3* gene have been extensively studied for vulnerability to circadian rhythm traits, and a variable number tandem repeat polymorphism (VNTR) in exon 18 of *PER3* was reported to be associated with DSPD (Ebisawa*, et al.*, 2001). The VNTR exists as a short allele of 4 tandem repeats of 54 base pairs and a long allele of 5 such repeats. The authors found an association between DSPD and the short allele in a Japanese population. Several replication studies have since been performed in both DSPD patients and the general population, with diverging results (Archer*, et al.*, 2003; Pereira*, et al.*, 2005; Viola*, et al.*, 2007), indicating a need for additional studies.

1.5 The circadian clock, bipolar disorder and lithium

1.5.1 Clinical aspects of bipolar disorder and circadian rhythms

Bipolar disorder is a serious mental illness, characterized by extreme mood swings with alternating periods of mania and depression. It is thought that circadian rhythms may play a role in the pathophysiology, as irregular sleep patterns are commonly seen in bipolar patients (Wehr*, et al.*, 1985; Kasper and Wehr, 1992; Klemfuss, 1992; reviewed in Manji and Lenox, 2000; Jackson*, et al.*, 2003), and subjects may present with unstable cycling of other circadian parameters, e.g. body temperature, blood pressure and melatonin secretion (Atkinson*, et al.*, 1975; Kripke*, et al.*, 1978; or see Lenox*, et al.*, 2002; McClung, 2007 for reviews).

1.5.2 Clock genes and the heritability of bipolar disorder

The lifetime risk of bipolar disorder is around 1% (Merikangas*, et al.*, 2007). It is a potentially disabling condition with a significantly elevated suicide rate, which together with an increased occurrence of cardiovascular disease and metabolic disorders, leads to a mortality rate that is 2-3 times higher among patients than in the general population (Osby*, et al.*, 2001; Laursen*, et al.*, 2007; reviewed in Muller-Oerlinghausen*, et al.*, 2002; Fagiolini*, et al.*, 2008). Both environmental and genetic factors are thought to be involved in the etiology of the condition, with a complex mode of inheritance (reviewed by Barnett and Smoller, 2009). Family studies have revealed a markedly higher prevalence of bipolar disorder in first-degree relatives of patients, and the concordance between monozygotic twins is around 40%, with an estimated heritability of around 80% (McGuffin*, et al.*, 2003; Kieseppa*, et al.*, 2004; Edvardsen*, et al.*, 2008; Lichtenstein*, et al.*, 2009; reviewed in Barnett and Smoller, 2009). Numerous genome-wide association studies (GWAS) have been performed to screen for genes involved in the heritability of bipolar disorder (Purcell*, et al.*, 2009), and interestingly, several clock genes have been implicated in the pathophysiology. Variants in *PER2, PER3*, *CLOCK*, *BMAL1* and *REV-ERB-α* (*NR1D1)* have been suggested to be associated with bipolar disorder, although replication studies have not always confirmed the findings (Mansour*, et al.*, 2006; Nievergelt*, et al.*, 2006; Shi*, et al.*, 2008; Mansour*, et al.*, 2009; Kishi*, et al.*, 2009).

1.5.3 Pharmacological treatment of bipolar disorder

Drug therapy of bipolar disorder aims at attenuating mood fluctuations and preventing the occurrence of new episodes. The mood stabilizers lithium, valproic acid and carbamazepine are cornerstones in the pharmacological treatment (for guidelines, see Sachs*, et al.*, 2000; American Psychiatric Association, 2000; Baldessarini*, et al.*, 2010), and lithium has long been

considered the gold standard drug (Gershon*, et al.*, 2009; Hirschowitz*, et al.*, 2010; for a review on the history of lithium treatment, see Schou, 2001).

Manic phases of bipolar disorder typically present with elevated or irritable mood, distractibility, increased impulsivity and decreased need for sleep (American Psychiatric Association, 2000). Acute manic phases may be treated with a mood stabilizer, but also antipsychotic drugs such as olanzapine, risperidone, quetiapine or haloperidol are effective (reviewed in Fountoulakis and Vieta, 2008; Cipriani*, et al.*, 2011). Often a combination of drugs from the two categories is used for optimal effect, with an underlying reasoning that the drugs have different and potentially complementary mechanisms of action (reviewed in Goodwin*, et al.*, 2009). Main features of depressive episodes are depressed or irritable mood, apathy, psychomotor retardation and increased need for sleep (American Psychiatric Association, 2000). Depressive episodes may be treated with antidepressants in addition to a mood stabilizer (Sachs*, et al.*, 2007; see consensus guidelines in Sachs*, et al.*, 2000), although the risk of switching from depression to mania is present (reviewed in Fountoulakis*, et al.*, 2008). Being a chronic condition, bipolar disorder may require lifelong pharmacological treatment, which underlines the importance of optimizing the drug therapy. Lithium is widely used in long-term treatment, even though it has several limitations, including a narrow therapeutic window of serum concentration of 0.6-1.2 mM, where lower doses are ineffective, and patients may experience toxic effects if the serum concentration exceeds 1.5-2.0 mM (reviewed in Muller-Oerlinghausen*, et al.*, 2002; Grandjean and Aubry, 2009). Another drawback is that not all patients respond to lithium treatment (for review, see Grof*, et al.*, 2009). Still, since lithium may be highly effective in both acute manic and depressive phases, as well as in long-term prophylaxis, it is frequently the drug of choice (reviewed in Coryell, 2009; Gershon*, et al.*, 2009).

1.5.4 Lithium and the circadian clock

It is intriguing that the alkali metal lithium, one of the most efficient drugs in the treatment of bipolar disorder, has been shown to modify circadian rhythms in humans and animals. Lithium prolonged the circadian rhythms in manic depressive patients (Kripke*, et al.*, 1978) and in healthy controls (Johnsson*, et al.*, 1983). Interestingly, the volunteers in the latter study were kept under constant conditions on the Norwegian island of Spitsbergen during the arctic midsummer, with little variation in light intensity and no additional external Zeitgebers. They displayed prolonged circadian oscillations of body temperature, locomotor activity and sleep-wake cycle with lithium administration (Johnsson*, et al.*, 1983). Lithium has also been shown to prolong circadian rhythms in rats (McEachron*, et al.*, 1982; Subramanian*, et al.*, 1998) and yet other studies have demonstrated that lithium prolonged the locomotor activity period in fruit flies (Padiath*, et al.*, 2004; Dokucu*, et al.*, 2005).

The main hypotheses for lithium action include its inhibition of inositol monophosphatase activity (the inositol depletion hypothesis) (Hallcher and Sherman, 1980; Berridge*, et al.*, 1982; reviewed in Quiroz*, et al.*, 2010) and its inhibition of the enzyme glycogen synthase kinase 3β (GSK-3β) (Klein and Melton, 1996; Padiath*, et al.*, 2004; reviewed in O'Brien and Klein, 2009). However, the exact underlying mechanisms of lithium action, and in particular how it modifies the circadian clock, have yet to be fully established.

1.6 Circadian rhythms, energy metabolism and lipogenesis

The circadian clock influences a large number of genes both directly and indirectly, and global gene expression studies have revealed that 2-10% of the mammalian transcriptome oscillates with circadian rhythmicity both *in vivo* in rodent SCN, liver and heart (Ueda*, et al.*, 2002; Storch*, et al.*, 2002; Panda*, et*

al., 2002) and also *in vitro* in cell cultures (Grundschober*, et al.*, 2001; Duffield*, et al.*, 2002). Interestingly, transcriptome studies further demonstrated that numerous genes involved in metabolic pathways oscillated (Akhtar*, et al.*, 2002; Miller*, et al.*, 2007), indicating that the circadian clock participates in regulating energy metabolism. In fact, it has been shown that the two processes are closely connected, and new links between the circadian clock and lipid metabolism are gradually being discovered (reviewed in Staels, 2006; Green*, et al.*, 2008). Circadian oscillations of metabolic factors are also observed at the functional level, as exemplified by the robust circadian rhythms of serum triglyceride levels (Figure 1.1) (Rajaratnam and Arendt, 2001), and the circulating amount of the appetite-regulating hormone leptin (Sinha*, et al.*, 1996; Licinio*, et al.*, 1997). Interestingly, the diurnal rhythm of leptin has been shown to be SCN-dependent (Kalsbeek*, et al.*, 2001), even though food intake and anticipation of food may influence rhythmicity in metabolic factors, including triglycerides and leptin (Ribeiro*, et al.*, 1998; Ahren, 2000).

Triglycerides, phospholipids and cholesterol are essential for energy storage, but they are also structural components of cell membranes and act as signaling molecules. Lipids are obtained in part from the diet, and in part synthesized endogenously *de novo* (reviewed in Lafontan, 2008). Excess carbohydrates in the diet are degraded to pyruvate and converted to fatty acids, which are subsequently synthesized to triglycerides. The liver is the main site for converting acetyl-CoA (an intermediate in the carbohydrate and lipid metabolism) via fatty acids to triglycerides (Figure 1.6), a process known as lipogenesis (for reviews, see Lafontan, 2008; Wakil and Abu-Elheiga, 2009; Voshol*, et al.*, 2009).

Major regulators of lipid biosynthesis gene transcription are the sterol regulatory element-binding proteins (SREBPs) (Figure 1.6) (reviewed in Bengoechea-Alonso and Ericsson, 2007; Raghow*, et al.*, 2008). The SREBP family consists of three isoforms, SREBP-1a, SREBP-1c and SREBP-2, where

the two splice variants SREBP-1a and SREBP-1c are encoded by the same gene. To a large extent, the different isoforms of SREBP modulate different aspects of lipid synthesis. SREBP-1c preferentially regulates synthesis of triglycerides and phospholipids, controlling transcription of genes encoding key enzymes in lipogenesis, including acetyl-coenzyme A carboxylase α (*Acaca*), fatty acid synthase (*Fasn*), fatty acid desaturase 2 (*Fads2*), stearoyl-Coenzyme A desaturase 1 (*Scd1*) and stearoyl-Coenzyme A desaturase 2 (*Scd2*) (Nakamura*, et al.*, 2004; reviewed in Shimano, 2009). The isoform SREBP-2 primarily regulates genes involved in cholesterol synthesis, such as 3-hydroxy-3-methylglutaryl-CoA reductase (*Hmgcr*) and mevalonate kinase (*Mvk*), whereas the isoform SREBP-1a modifies the transcription of genes in both pathways, but primarily promotes fatty acid synthesis (reviewed in Bengoechea-Alonso and Ericsson, 2007; Raghow*, et al.*, 2008).

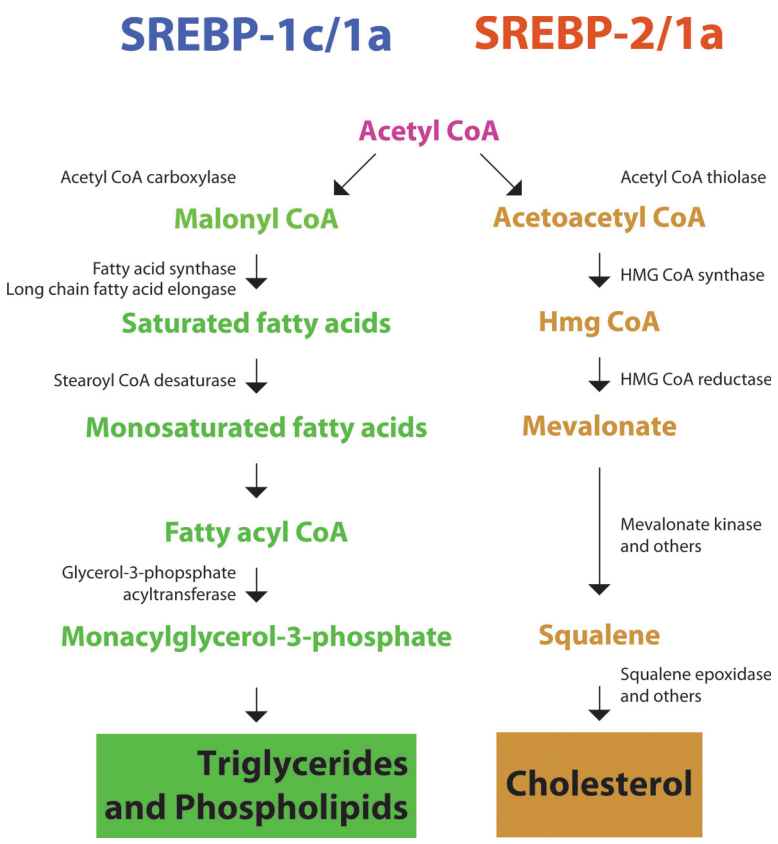

Figure 1.6. SREBP pathways and synthesis of triglycerides, phospholipids and cholesterol. The isoform SREBP-1c preferentially regulates transcription of genes involved in the synthesis of triglycerides and phospholipids, whereas SREBP-2 preferentially activates genes involved in cholesterol synthesis. SREBP-1a modifies the transcription of genes in both pathways. Diagram by Osland TM (modified from Horton, *et al.*, 2002).

1.7 Psychotropic drugs and SREBP activation

Bipolar patients are vulnerable to weight gain and metabolic disturbances, and pharmacological treatment involving drugs associated with weight gain is one of the leading explanations for the associated obesity (McElroy, *et al.*, 2002; Correll, 2007; for review, see Fagiolini, *et al.*, 2008). Many antipsychotic drugs, such as clozapine and olanzapine, as well as some antidepressants,

including imipramine, have been shown to stimulate transcription of cholesterol and triglyceride genes *in vitro* in cell cultures (Ferno*, et al.*, 2006; Raeder*, et al.*, 2006b; Yang*, et al.*, 2007) and *in vivo* in rats (Minet-Ringuet*, et al.*, 2007; Ferno*, et al.*, 2009). These genes are controlled by SREBP transcription factors (reviewed in Shimano, 2009; Sato, 2010), and the SREBP system has been proposed to be involved in the weight gain often associated with antipsychotics and antidepressants (Le Hellard*, et al.*, 2009). The degree of SREBP activation varies between the drugs, and among the potent SREBP activators we find the antipsychotics clozapine, olanzapine and haloperidol, and the antidepressants imipramine and amitriptyline (Ferno*, et al.*, 2006), whereas among the weaker SREBP activators we find the antipsychotic ziprasidone. The mood stabilizers lithium, valproic acid and carbamazepine appear not to activate SREBP (Ferno*, et al.*, 2006; Raeder*, et al.*, 2006a; Raeder*, et al.*, 2006b).

Schizophrenia is a major mental illness, characterized by positive symptoms, such as delusions, hallucinations (auditory or visual) and disorganization of speech, as well as negative symptoms, which include flattening of affect and poverty of speech or thought (reviewed in Freedman, 2003; Tamminga and Holcomb, 2005; American Psychiatric Association, 2000). Patients may also present with cognitive dysfunction, including impairment of memory, executive function and motor skills (Bilder*, et al.*, 2000). Antipsychotics are considered the cornerstone of schizophrenia treatment, and include first-generation antipsychotics, which mainly act by reducing positive symptoms, such as haloperidol, and second-generation antipsychotics, including clozapine and olanzapine, that may improve both positive and negative symptoms (reviewed in Freedman, 2003). In the clinical setting, clozapine has been associated with substantial weight gain and dyslipidemia (Cohen*, et al.*, 1990; Leadbetter*, et al.*, 1992) and severely low white blood cell count (agranulocytosis) (Alvir*, et al.*, 1993). *In vitro*, clozapine causes pronounced SREBP activation with subsequent lipogenesis (Ferno*, et al.*, 2005; Raeder*, et*

al., 2006b). Despite the possible complications of the drug, it remains a potent antipsychotic that may be effective in treatment-resistant schizophrenia (Kane*, et al.*, 1988; reviewed in McIlwain*, et al.*, 2011). Noteworthy, clozapine may also be used to improve symptoms of bipolar disorder (for review, see Ertugrul and Meltzer, 2003).

Imipramine was the first tricyclic antidepressant to be developed in the 1950s (Azima and Vispo, 1958). It is a potent drug, but has to a large extent been replaced by newer classes of antidepressants, in particular selective serotonin reuptake inhibitors (SSRIs) that are better tolerated and have fewer side effects (reviewed in Wood*, et al.*, 2002). Nevertheless, it remains an important tool for the investigation of antidepressant drugs. Imipramine has been associated with marked weight gain in patients (Berken*, et al.*, 1984; Fernstrom*, et al.*, 1986), and in cell culture it causes pronounced activation of the SREBP system, followed by lipogenesis (Raeder*, et al.*, 2006a; Raeder*, et al.*, 2006b). A meta-analysis reported that although imipramine prevents depressive episodes in bipolar patients, it is little used in the clinic due to its side effects (Beynon*, et al.*, 2009).

2 Aims of the study

The overall aim of this study was to explore the role of clock genes in psychotropic drug effects and on selected biological functions (lipid metabolism and sleep). Specifically, we had the following objectives:

1. Use a cell culture model of the circadian clock to explore the rhythmicity of clock gene expression, and investigate the effects of the mood stabilizer lithium on the transcription of clock genes (paper I)
2. Identify and compare the effects of different psychotropic drugs, including the antipsychotic clozapine and the antidepressant imipramine, on the transcription of genes involved in the control of circadian rhythms and lipid metabolism (paper II)
3. Examine and genotype a population of Norwegian students to investigate the influence of a VNTR in the clock gene *PER3* on diurnal preference (paper III)

3 List of publications

Paper I

Osland TM, Ferno J, Havik B, Heuch I, Ruoff P, Laerum OD and Steen VM (2011a). Lithium differentially affects clock gene expression in serum-shocked NIH-3T3 cells. J Psychopharmacol 25: 924-33

Paper II

Osland TM, Skrede S, Ferno J and Steen VM. Transcriptional effects of psychotropic drugs on circadian clock genes and lipid biosynthesis genes in cultured NIH-3T3 cells. *Manuscript submitted to BMC Neuroscience*

Paper III

Osland TM, Bjorvatn B, Steen VM and Pallesen S (2011b). Association study of a variable-number tandem repeat polymorphism in the clock gene PERIOD3 and Chronotype in Norwegian university students. Chronobiol Int 28: 764-770

4 Summary of results

4.1 Paper I

We used an *in vitro* model of the circadian clock to perform a comprehensive study of the gene expression profiles of the clock genes *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2*, *Bmal1*, *Clock*, *Rev-Erb-α*, *Ror-α*, *Gsk-3β*, *Csnk1ε*, *E4bp4* and *Dbp* for three consecutive days. Serum-shocked cultures of mouse fibroblasts (NIH-3T3 cells) displayed rhythmic gene expression patterns of clock genes with a variety of phases and amplitudes, with peaks of expression levels distributed throughout the cycle. We examined the effects of lithium on clock gene expression, and found that lithium increased the peak amplitude of expression of *Per2* and *Cry1*, whereas the peak amplitudes of expression of *Per3, Cry2, Bmal1, E4bp4, Rev-Erb-α* and *Ror-α* were reduced. Moreover, the period of *Per2* was apparently prolonged by lithium. In light of the proposed role of circadian rhythm disturbances in bipolar disorder, these differential effects on clock gene expression could reflect a mechanism for the effects of lithium on circadian rhythms that may be relevant for its therapeutic effects in the treatment of bipolar disorder.

4.2 Paper II

The effects of lithium on the circadian clock have been suggested to be therapeutically relevant for the treatment of bipolar disorder. Additional mood stabilizing agents, antidepressants and antipsychotics have also been implicated in the pharmacological treatment; hence we explored and compared their effects on clock gene transcription. Since several antipsychotics and antidepressants are known to induce lipogenic gene expression, and given the accumulating evidence for a link between the circadian clock and lipid metabolism, we also investigated drug effects on the expression of lipid

biosynthesis genes. Lithium, imipramine and clozapine were investigated for effects on serum-shocked NIH-3T3 cells in a time course experiment. Clock genes displayed robust circadian rhythms, in contrast to lipid biosynthesis genes, suggesting that circadian oscillations in clock genes were not conveyed to the lipid metabolism genes in this model system. The effects of clozapine and imipramine differed partly from the effects of lithium, including reduced expression of *Per2* by imipramine and clozapine, and increased *Rev-Erb-α* expression levels by clozapine, indicating that the drugs act on the circadian clock via different mechanisms. Lithium had no effect on the expression of lipid metabolism genes, in contrast to the marked up-regulation caused by imipramine and clozapine. A panel of additional psychotropic drugs was examined at one time point in a separate experiment. The investigated antipsychotics and antidepressants had similar effects to clozapine and imipramine, suggesting drug class effects. The observed drug effects could reflect some shared regulatory mechanisms that contribute to their psychotropic action.

4.3 Paper III

Polymorphisms in clock genes could be associated with differences in circadian rhythms, and interest in their impact on circadian parameters, including diurnal preference, is growing due to potential relevance for physical and mental health. For instance, the circadian clock heavily influences timing of sleep, and associations between sleep and various complications have been reported, including obesity and psychiatric disorders. Several studies have investigated the role of a VNTR in the clock gene *PER3*, where the short allele of the VNTR has been associated with diurnal preference and with DSPD, but conflicting findings have also been reported. We performed a replication study to explore the role of this *PER3* polymorphism on diurnal preference in a sample of 432 Norwegian university students. The widely used Horne-Östberg

Morningness Eveningness Questionnaire (MEQ) and the Preferences Scale (PS) were used to assess chronotype. We found no association between the *PER3* VNTR and diurnal preference, indicating that the proposed role of *PER3* needs further clarification.

It is the theory which decides what we can observe.

Albert Einstein

5 Discussion

5.1 Selected methodological aspects

5.1.1 Serum-shocking of cultured cells

In this study, we explored circadian rhythms by examining the transcription of selected clock genes in cell culture. Circadian rhythms in cultured cells may be entrained by several factors, including exposure to a high dose (50%) of horse serum for two hours (serum shock) (Balsalobre*, et al.*, 1998; Allen*, et al.*, 2001), the glucocorticoid hormone dexamethasone (Balsalobre*, et al.*, 2000a), tissue plasminogen activator (Akashi and Nishida, 2000) and forskolin (Yagita and Okamura, 2000), indicating that various pathways are involved in the synchronization of circadian rhythms in peripheral clocks (Balsalobre*, et al.*, 2000b). We chose to use serum-shocking for entrainment, as serum contains a large number of signaling factors, and might be thought to stimulate several of the involved pathways for circadian entrainment.

It has been shown that a serum shock may synchronize the circadian rhythms in several cell lines, including Reuber's rat hepatoma cells (H-35) and mouse fibroblasts (NIH-3T3) (Balsalobre*, et al.*, 1998; Akashi and Nishida, 2000). Nevertheless, we screened numerous cell types in search of an additional cell line (derived from a target organ of lithium treatment) which might be more relevant for bipolar disorder and the known side effects of lithium, including human glioma cells (GaMg), human hepatoma cells (HepG2), human kidney cells (HK-2), human thyroid cells (Nthy-ori 3-1), rat glioma cells (C6), H-35 and NIH-3T3 cells. However, only the latter two cell lines displayed robust oscillations in clock gene expression after serum-shocking (Osland, *et al.*, unpublished data), in agreement with previous studies (Balsalobre*, et al.*, 1998; Akashi and Nishida, 2000). In contrast to the H-35 cells, NIH-3T3 cells were highly contact-inhibited, which is essential in serum shock experiments to

avoid potential biases caused by cell division; hence we chose the latter cell line for our studies.

Circadian rhythms of NIH-3T3 cells were synchronized by serum-shocking confluent cultures. As predicted from the literature, robust oscillations were generated of rhythmic clock genes (e.g. *Per2, Cry1* and *Rev-Erb-α*) with a variety of different phases (Balsalobre*, et al.*, 1998; Oishi*, et al.*, 1998a; Mitsui*, et al.*, 2001; reviewed in Hastings and Herzog, 2004), whereas other clock genes were expressed at constant levels (e.g. *Clock*, *Csnk1ε* and *Ror-α*) (Shearman*, et al.*, 1999; Shearman*, et al.*, 2000; Preitner*, et al.*, 2002; Maywood*, et al.*, 2003; Guillaumond*, et al.*, 2005; for review, see Ko and Takahashi, 2006). In line with previous studies, we found a gradual damping over time of the oscillations (Yamazaki*, et al.*, 2000; reviewed in Hirota and Fukada, 2004), which was attributed to lack of feedback, causing the cells to gradually fall out of phase with each other. This may, at least in part, be due to the individual cells containing autonomous clocks, each with their own period, causing the cells to require continuous entrainment in order to remain synchronized to one another. The damping effect limited the observation time, but nevertheless we observed robust oscillations for three consecutive days (*i.e.* three full circadian cycles). We observed only minor biological and technical variation, such as effects of the number of times the cells had been subcultured (passage number), and on the whole we found relatively little variation between the experiments. In summary, cell culturing provided a simplified and robust model to study clock gene expression, and the model could provide results that are relevant for circadian clocks *in vivo*.

5.1.2 Selection of psychotropic drugs and dosage

NIH-3T3 cell cultures were exposed to psychotropic drugs in order to investigate potential effects on the expression of genes involved in the circadian clock or lipid biosynthesis. We aimed at using drug doses that were

sufficiently high to produce effects *in vitro* on the target genes in question, but not so high as to cause cell toxicity. Hence, the investigated drug doses often markedly exceeded relevant serum concentrations in patients, but it is common to study high drug doses *in vitro* (Iitaka*, et al.*, 2005; Kaladchibachi*, et al.*, 2007; Wu*, et al.*, 2007). Lithium is known to affect the circadian clock by mechanisms that remain largely unknown, and we therefore studied its effects on clock gene expression. As mentioned in the Introduction, lithium has a narrow therapeutic plasma concentration range of 0.6-1.2 mM in humans (reviewed in Muller-Oerlinghausen*, et al.*, 2002; for review on lithium toxicity, see McKnight*, et al.*, 2012), although much higher drug concentrations (20 mM) are tolerated *in vitro* in cell culture (Iitaka*, et al.*, 2005; Yin*, et al.*, 2006; Wu*, et al.*, 2007). Since we found dose-dependent effects of lithium on clock genes, with no significant toxicity up to 20 mM lithium chloride as determined by flow cytometry, we used this latter concentration to obtain pronounced effects of the drug (paper I and paper II).

In addition to mood stabilizers, antipsychotics and antidepressants are effective in the treatment of bipolar episodes (Goodwin*, et al.*, 2009; Cipriani*, et al.*, 2011), and we therefore explored their effects on the transcription of clock genes. Antipsychotic and antidepressant drugs have often been associated with weight gain, dyslipidemia and type 2 diabetes (reviewed in Allison*, et al.*, 2009; Raedler, 2010). Since new links between the circadian clock and lipid metabolism are continuously emerging (reviewed in Asher and Schibler, 2011), we also studied drug effects on the transcription of lipid biosynthesis genes, and whether drug-induced effects on clock genes and lipogenic genes corresponded (paper II). We investigated effects of imipramine, a prototypical tricyclic antidepressant, and the second-generation antipsychotic clozapine, both potent SREBP activators (Ferno*, et al.*, 2005; Raeder*, et al.*, 2006a) that have been associated with substantial weight gain in patients (Berken*, et al.*, 1984; Fernstrom*, et al.*, 1986; Cohen*, et al.*, 1990; Leadbetter*, et al.*, 1992; Lamberti*, et al.*, 1992; for review, see Allison*, et al.*, 1999; Asenjo Lobos*, et*

al., 2010). We examined the effects of 15 μM imipramine, which is within the range of concentrations where activation of the SREBP system is expected, without toxic drug effects in the cells (Sukma*, et al.*, 2003; Raeder*, et al.*, 2006a; Vik-Mo*, et al.*, 2009). Correspondingly, based on previous studies (Ferno*, et al.*, 2006; reviewed in Ferno*, et al.*, 2011), we exposed the cell cultures to 30 μM clozapine. The selected concentrations are considerably higher than the recommended target plasma concentrations in patients (0.6-1.1 μM imipramine and 1.1-1.8 μM clozapine) (Baumann*, et al.*, 2004), but the use of high doses may in part be justified by the lipophilic properties of the drugs, which cause them to have large volumes of distribution in the body, with a tendency towards drug-accumulation in the brain and adipose tissues. In line with this, the concentration of clozapine has been found to be over 15 times higher in the rat brain than in serum (Weigmann*, et al.*, 1999).

To explore whether the transcriptional changes observed for lithium, imipramine and clozapine on circadian gene expression could be generalized to hold for their respective psychotropic drug classes, we investigated a panel of representative mood stabilizers, antidepressants and antipsychotics for effects on clock genes and lipid metabolism genes. The mood stabilizers valproic acid and carbamazepine, the antidepressants amitriptyline (tricyclic antidepressant) and fluoxetine (SSRI), the first-generation antipsychotic haloperidol, and the second-generation antipsychotic drugs clozapine, olanzapine, quetiapine, aripiprazole and ziprasidone were included in the panel. Hence, both potent activators of the SREBP system, such as clozapine, olanzapine and haloperidol, along with drugs with low lipogenic potential, including aripiprazole and ziprasidone, were represented in the panel. We investigated drug effects at one time point, 24 hours after a serum shock, based on the time course experiments with lithium, imipramine and clozapine, since several clock genes are expected to peak at that time point, and drug effects should also be around their maxima (Ferno*, et al.*, 2005; Raeder*, et al.*, 2006a). We used a fixed concentration of 25 μM of each drug, which based on earlier studies was expected to be well

tolerated in cell culture, and be sufficiently high to produce effects (Koch*, et al.*, 2003; Ferno*, et al.*, 2005; Levkovitz*, et al.*, 2005; Raeder*, et al.*, 2006a; Kato*, et al.*, 2008; Vik-Mo*, et al.*, 2009; Lauressergues*, et al.*, 2010). Of note, these drugs have very different target plasma concentrations in patients, but we used a single drug concentration to make a simple comparison between the agents. Drug effects on gene expression levels were compared to the effects of 20 mM lithium chloride, which had previously been studied comprehensively (paper I). We used 0.05% dimethyl sulfoxide (DMSO) as a solvent due to the low solubility of certain psychotropics, and although it might have stimulated lipogenesis due to its lipophilic compound and influenced our results, we found no significant difference between controls with or without DMSO (data not shown).

5.1.3 Quantitative real-time PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) is a sensitive technique to quantify mRNA levels of specific genes. In complementary DNA (cDNA) synthesis, mRNA is reverse transcribed to cDNA, before targeted DNA strands are repeatedly amplified and quantified in qRT-PCR. The procedure is widely used, as it only requires small amounts of test material and yields reproducible results. TaqMan chemistry or the fluorescent dye SYBR Green may be used in the qRT-PCR to measure fluorescence intensity (in real-time) after each PCR cycle. SYBR Green detects all double-stranded DNA, meaning it can be used with any given primer pair, but it may create false positive signals due to nonspecific amplification products. On the other hand, TaqMan probes only detect specific amplicons, but a different probe is required for each primer pair. Since we obtained consistent results for our primer pairs with SYBR Green, and since TaqMan chemistry is far more expensive, we preferentially used SYBR Green, although TaqMan probes were used for some of the assays.

The mRNA levels of the genes of interest are usually normalized to the amount of one or more endogenous reference genes (so-called housekeeping genes) in the same sample. However, in our experiments we observed that one of the reference genes was affected by the serum shock (β-actin (*Actb*)), and others were sensitive to drug exposure (*Gapdh* and β-2 microglobulin (*B2M*)). Drug response was observed when cell cultures were exposed to clozapine or imipramine, hence to remedy for this, we used the mean of several endogenous controls (ribosomal protein, large, P0 (*Rplp0*), *Actb* and *B2M*) for normalization in the experiments involving antipsychotics and antidepressants (paper II). It is difficult to find appropriate reference genes that remain invariant to all aspects of experimental conditions, but in support of our selection, none of the used reference genes displayed circadian oscillations, and moreover, the results were highly similar with and without normalization to the reference genes.

The qRT-PCR data was analyzed using both the standard curve method and the comparative $\Delta\Delta$ C_t method. A dilution series of cDNA lies at the base for quantification of the samples with the standard curve method. Alternatively, corresponding dilutions at the RNA level may be used to create a standard curve, but high RNA concentrations might cause inhibition during cDNA synthesis, which would result in a flattening of the standard curve, and could bias the results. For the comparative $\Delta\Delta C_t$ method, no standard curve is needed, but it is required that the amplification efficiencies of the primers for the gene of interest and the reference gene are similar. Both methods calculate the fold change in expression of a gene of interest relative to a reference gene, in a treated sample compared to one or several calibrator samples (generally controls or untreated samples are used). Moreover, both methods assume that the amplification efficiency remains constant for each PCR cycle and in all samples. However, this may not always be the case and could influence the outcome. We observed minor differences in the results using the two methods of analysis, with the standard curve method estimating slightly greater

differences between controls and drug-exposed samples, compared to the $\Delta\Delta C_t$ method. Nevertheless, the two methods essentially coincided, and we switched from the standard curve method (paper I) to the $\Delta\Delta C_t$ method for the experiments with antipsychotics and antidepressants (paper II), due to the large sample size in paper I, since the $\Delta\Delta C_t$ method minimizes the number of reactions required, as a standard curve is not necessary for each run. However, results at the gene expression level cannot be extrapolated directly to functional effects, and confirmation of the results at the protein level would have been valuable for the interpretation.

5.1.4 Mathematical and statistical analysis

We applied several methods to describe the circadian oscillations and analyze drug effects. In the experimental setup in paper I, with serum-shocked cells exposed to lithium or vehicle, a squared sine function was used to approximate the oscillations and detect changes in gene expression caused by lithium. Parameters such as period, amplitude and phase were calculated, and we found significant effects of lithium on several genes, mainly on the amplitude of expression. In addition to approximating the data with the mathematical model, we performed a two-way analysis of variance (ANOVA) for each gene, with main effects for time and treatment (drug versus control), along with an interaction term between the two. The mathematical model was more restrictive than ANOVA and the two techniques did not always give fully concordant results. Discrepancies were attributed to limitations of the model in describing the observed oscillations. Due to the large variation in the ability of the functions in the mathematical model to approximate the oscillations, we based our conclusions on two-way ANOVA results for the data from the time course experiments comparing effects of lithium, imipramine and clozapine (paper II). In cases where the interaction term was significant in addition to the main effects, it was less straightforward to interpret drug effects, and the analysis was followed up with Dunnett's post-hoc test. Treatment groups were

subsequently compared using one-way ANOVA at each single time point, followed by Dunnett's post-hoc test of pairwise comparison of the treated samples with the controls. Finally, to examine individual time points for significant effects in the drug panel experiment, we performed t-tests (paper II).

5.2 Circadian rhythms in clock genes and lipid metabolism genes

5.2.1 Circadian oscillations in clock gene expression

Exposing confluent cell cultures of mouse fibroblasts (NIH-3T3 cells) to a serum shock, represents a useful technique for studying peripheral circadian clocks *in vitro*. The serum shock generated robust circadian oscillations of approximately 24 hours in the transcription levels of the majority of the clock genes we investigated. The rhythmic clock genes displayed a variety of phases and amplitudes, and were in agreement with previous reports (Balsalobre*, et al.*, 1998; Mitsui*, et al.*, 2001; for review, see Hastings and Herzog, 2004). For instance, *Per2* expression peaked around 24 and 48 hours, and was in antiphase with *Bmal1* (Oishi*, et al.*, 1998a; reviewed in Hirota and Fukada, 2004), that peaked around 36 and 64 hours (Figure 1.3A). We did not detect oscillations in the expression patterns of some of the clock genes, such as *Clock*, *Csnk1ε* and *Ror-α*, in line with previous studies reporting that they are constitutively expressed (Shearman*, et al.*, 1999; Shearman*, et al.*, 2000; Maywood*, et al.*, 2003; Guillaumond*, et al.*, 2005; reviewed in Ko and Takahashi, 2006). Taken together, the observed expression patterns support the validity of this model system of a peripheral circadian clock.

5.2.2 Links between the circadian clock and lipid metabolism

Obesity represents a major health challenge worldwide, and has become the most common health disorder among young people in Europe (Obesity: preventing and managing the global epidemic, 2000; Doak*, et al.*, 2012; reviewed in Oster, 2010). In light of the numerous proposed links between the circadian clock and lipid metabolism (reviewed in Froy, 2011; Huang*, et al.*, 2011; Delezie and Challet, 2011), it is highly interesting that short sleep is associated with obesity, diabetes and the metabolic syndrome (Grandner*, et al.*, 2011; for review, see Knutson*, et al.*, 2007; Oster, 2010). Furthermore, shift workers have an increased incidence of obesity and diabetes (Karlsson*, et al.*, 2001; reviewed in Huang*, et al.*, 2011). The SCN is entrained by light, but not by food, hence shifts in feeding (as seen in shift workers) may rapidly uncouple peripheral clocks (such as the liver clock) from the SCN clock (Damiola*, et al.*, 2000; Stokkan*, et al.*, 2001), leading to misalignments that may pave the way for metabolic disturbances (reviewed in Delezie and Challet, 2011; Albrecht, 2012). Further emphasizing the impact of food on regulating circadian rhythms, a recent global gene expression study demonstrated that in the absence of food, the number of rhythmic transcripts dropped markedly in mouse liver, while feeding restored cyclic transcription of several hundreds of genes (Vollmers*, et al.*, 2009). In addition to being modified by food intake, the peripheral clock in the liver is synchronized centrally by the SCN. In fact, the circadian clock and lipid metabolism reciprocally influence each other (Figure 5.2.2) (Vollmers*, et al.*, 2009; reviewed in Staels, 2006; Green*, et al.*, 2008).

It has been shown that mutations of the clock genes *Clock* and *Bmal1* lead to adverse metabolic effects in mice, including obesity, diabetes and dyslipidemia (Turek*, et al.*, 2005; Shimba*, et al.*, 2011). Also *Per2* (Yang*, et al.*, 2009a) and *Rev-Erb-*α (Raspe*, et al.*, 2002) have been implicated in the development of metabolic disturbances, emphasizing the crucial role of the circadian clock in lipid homeostasis. Further suggesting a reciprocal link between the circadian

clock and lipid metabolism, obese rats with a mutation in the leptin receptor (Zucker rats) have phase-advances of activity, feeding and body temperature rhythms, and the amplitudes of these circadian oscillations are attenuated (Fukagawa*, et al.*, 1992; Murakami*, et al.*, 1995; Mistlberger*, et al.*, 1998).

The mechanisms that couple these two processes are poorly understood, but numerous genes have been implicated in the interaction at the molecular level (for review, see Green*, et al.*, 2008; Bass and Takahashi, 2010; Asher and Schibler, 2011). Various clock genes contribute to modifying lipid metabolism, such as *Per2* regulating peroxisome proliferator activated receptor γ (*Ppar-γ*) function (Grimaldi*, et al.*, 2010; Schmutz*, et al.*, 2010), *Rev-Erb-α* modulating SREBP target genes (Anzulovich*, et al.*, 2006; Le Martelot*, et al.*, 2009), and *Bmal1* has been implied in glucose and lipid metabolism (Rudic*, et al.*, 2004; Shimba*, et al.*, 2005). Also downstream transcription factors of the circadian clock, such as *Dbp*, *Hlf* and *Tef* are involved in lipid metabolism (reviewed in Staels, 2006). Conversely, several metabolic regulators influence the circadian clock, including AMP-activated protein kinase (AMPK) that modulates circadian rhythms by phosphorylating the clock component CRY1 (Lamia*, et al.*, 2009; reviewed in Fan*, et al.*, 2012), *Ppar-γ* regulating *Bmal1* transcription (Wang*, et al.*, 2008; reviewed in Kawai and Rosen, 2010) and peroxisome proliferator activated receptor γ coactivator 1α (*Pgc-1α*) promoting transcription of *Bmal1* and *Rev-Erb-α* (Liu*, et al.*, 2007).

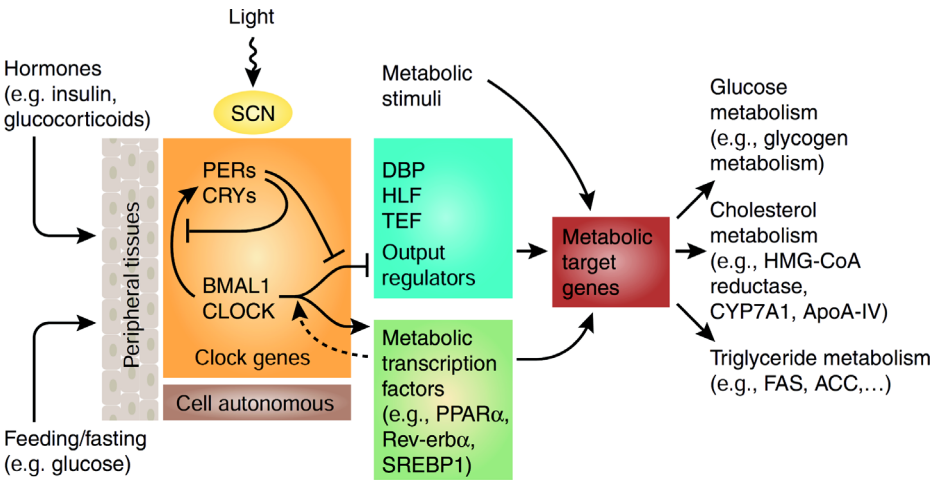

Figure 5.2.2. Interaction between the circadian clock and lipid metabolism. Light synchronizes the pacemaker in the SCN to the environment, whereas circulating hormones and food intake contribute to modifying peripheral oscillators. Output signals from the clock may influence metabolism, and for instance the transcription factors *Rev-Erb-α*, *Dbp*, *Hlf* and *Tef* are among the targets of the circadian clock that in turn regulate metabolism. Reproduced from Staels with permission of the author (Staels, 2006).

To explore the links between the circadian clock and lipid metabolism, we investigated the transcription of several lipid biosynthesis genes and compared them to expression profiles of clock genes. *In vivo*, it has been shown that the expression levels of numerous lipid metabolism genes oscillate, such as peroxisome proliferator activated receptor α (*Ppar-α*) in rats (Lemberger*, et al.*, 1996), or *Fasn, Hmgcr* and *Ppar-α* in mice (Patel*, et al.*, 2001; Oishi*, et al.*, 2005). However, in our model system, a serum shock with subsequent clock gene oscillations was not sufficient to entrain circadian rhythms in the lipid metabolism genes *Fads2*, *Hmgr*, *Scd1* or *Scd2*. Our results are in agreement with previous studies in NIH-3T3 cells under similar experimental conditions, where a serum shock was not sufficient to induce circadian metabolic activity (Allen*, et al.*, 2001; Hughes*, et al.*, 2009). The observed lack of rhythmicity *in vitro* could at least partly be due to the absence of additional factors (Zeitgebers) present *in vivo*, such as hormones or nutritional status, and it

would appear that the mechanisms required for oscillation of clock genes and lipid metabolism genes are independent of each other in this model system. In addition, microarray studies have demonstrated that rhythmicity is highly tissue-specific, with large variation in the set of genes that oscillate in the different tissues, and minimal overlap from study to study (Panda, *et al.*, 2002; Storch, *et al.*, 2002; Zvonic, *et al.*, 2006; Menger, *et al.*, 2007; Miller, *et al.*, 2007), which was proposed to be caused by applying different algorithms to detect oscillation (see Doherty and Kay, 2010 for review). This tissue-specific rhythmicity could also in part explain the lack of rhythmicity in our experimental set-up.

5.3 Psychotropic drug effects on clock genes and lipid metabolism genes

5.3.1 Psychotropic drugs differentially alter the amplitudes of clock gene expression

We investigated the effects of lithium on circadian rhythms in an established *in vitro* model of the circadian clock. Lithium differentially changed the amplitude of several clock genes, including an increase in the oscillation amplitude of *Per2* and *Cry1* expression, whereas the amplitudes of *Per3*, *Cry2*, *Bmal1*, *Rev-Erb-α*, *Ror-α* and *E4bp4* were reduced (paper I). In previous studies on lithium, the focus has mainly been on its lengthening effect on circadian rhythms in humans (Kripke, *et al.*, 1978; Johnsson, *et al.*, 1983) and animals (McEachron, *et al.*, 1982; Subramanian, *et al.*, 1998; Klemfuss, *et al.*, 1992; Padiath, *et al.*, 2004), although lithium has also been reported to reduce the oscillation amplitudes of neurotransmitter receptors (Kafka, *et al.*, 1982). However, only the period of *Per2* was apparently prolonged by lithium in our experimental setup (paper I). In line with our results, a recent study

demonstrated that lithium increased the oscillation amplitude of both gene expression levels and protein amount in mice, in addition to a prolonged period of *Per2* expression *in vivo* and *in vitro* (Li*, et al.*, 2012). Moreover, another study found no alteration in the period of gene expression, and only altered amplitudes of the oscillations were reported, including reduced amplitudes of *BMAL1*, *DBP* and *REV-ERB-α* expression in fibroblasts from bipolar patients compared to controls (Yang*, et al.*, 2009b). Along with our findings, these studies provided new leads for the association between bipolar disorder and circadian rhythms, and suggest a possible role for the oscillation amplitudes of clock genes in the pathophysiology of bipolar disorder. This is further supported by studies demonstrating that subjects suffering from depression (a disorder with many similarities to bipolar disorder) have blunted amplitudes of circadian parameters, including body temperature and plasma melatonin levels, and moreover, the deficient amplitudes are restored in remission (Souetre*, et al.*, 1989; Szuba*, et al.*, 1997).

Additional mood stabilizers, antidepressants and antipsychotics have also been implicated in the treatment of bipolar disorder, and we investigated whether such psychotropic drugs also affect the transcription of key circadian clock genes. Interestingly, we found that clozapine and imipramine also affected the transcription levels of various clock genes, including a down-regulation of *Per2* by both drugs, and an up-regulation of *Rev-Erb-α* expression by clozapine (paper II). Moreover, several other psychotropics and antidepressants also had significant effects on clock gene expression, indicating that alteration of clock gene transcription is not specific for lithium, and may be a common feature of several psychotropic drugs. In fact, it has been reported by others that valproic acid increased *Per2* and *Cry1* expression in mouse and human fibroblasts (Johansson*, et al.*, 2011), fluoxetine differentially augmented or reduced the expression levels of *Clock*, *Bmal1*, *Per1*, *Per2* and *Cry2* in the mouse brain (Uz*, et al.*, 2005) and haloperidol increased *Per1* expression in mouse SCN (Viyoch*, et al.*, 2005), suggesting that modification of circadian rhythms could

represent shared regulatory mechanisms that are relevant for their therapeutic effects.

5.3.2 Transcriptional effects of psychotropic drugs on clock genes and lipid metabolism genes

Although imipramine and clozapine had effects on clock genes, the transcriptional patterns differed from lithium (paper II). This suggests drug-specific regulation of clock genes, which is emphasized by other differences between the drugs, such as the activation of lipogenic gene transcription by clozapine and imipramine, not observed by lithium. Examination of additional mood stabilizers (the antiepileptics valproic acid and carbamazepine), antidepressants (amitriptyline and fluoxetine) and antipsychotics (olanzapine, haloperidol, quetiapine, aripiprazole and ziprasidone) at one time point (24 hours) following a serum shock, revealed similar drug effects to what had been observed for lithium, imipramine and clozapine, respectively, suggesting drug class effects (paper II). In contrast to the antipsychotics and antidepressants, the mood stabilizers only had minor effects on lipid metabolism genes, and it would appear that mood-stabilizing agents regulate clock genes independently of lipid metabolism genes (at least *in vitro*). This indicates that no evident regulatory link between the circadian clock and lipid metabolism is present in this model system, although it cannot be excluded. We found no significant effects by valproic acid or carbamazepine on any gene, and previous studies on the action of these mood stabilizers on circadian rhythms have been few and contradictory (Klemfuss and Kripke, 1995; Dokucu*, et al.*, 2005). It is possible that higher drug concentrations would have had effect on the circadian clock, as has recently been reported for 1 mM valproic acid (Johansson*, et al.*, 2011). However, in support of our negative finding for valproic acid on clock genes, no effect on lipid metabolism genes was found at this concentration either (Raeder*, et al.*, 2006a).

5.3.3 Post-transcriptional modifications of clock proteins

Our main focus on drug effects on the circadian clock has been at the gene expression level, and even though we found several interesting leads to pursue, future experiments should include protein and functional studies, since it has been suggested that post-transcriptional mechanisms represent important circadian control points (Lee*, et al.*, 2001). As an example, the hepatic proteome of mice was examined for rhythmicity. It was demonstrated that 20% of the proteome oscillated, and moreover, for around half of the rhythmic proteins, the levels of the corresponding mRNAs did not display circadian oscillations (Reddy*, et al.*, 2006).

The kinase GSK-3β has been associated with the circadian clock (Padiath*, et al.*, 2004; Kaladchibachi*, et al.*, 2007), and the phosphorylation level of GSK-3β is cyclic, as opposed to the protein amount (Iitaka*, et al.*, 2005), suggesting that important regulation of the circadian clock occurs by post-translational modification. GSK-3β has is a known target of lithium (Klein and Melton, 1996; Stambolic*, et al.*, 1996), and it was recently demonstrated that overexpression of *Gsk-3β* rescued behavioral effects of lithium-treated mice (O'Brien*, et al.*, 2011). We found that lithium treatment did not alter the transcription levels of *Gsk-3β* (paper I), whereas a significant difference at the phosphorylation level was observed (Osland, *et al.*, unpublished data), with lithium augmenting the phosphorylation status of GSK-3β, in line with a previous report (Iitaka*, et al.*, 2005). These authors found that overexpression of GSK-3β caused a phase advance of clock gene expression, whereas inhibition gave a phase delay, and proposed that GSK-3β might contribute to modulating circadian rhythms by regulating the nuclear entry of PER2, since GSK-3β phosphorylates and activates PER2 (Iitaka*, et al.*, 2005). Lithium inhibits GSK-3β by phosphorylation, and could thus delay nuclear translocation of PER2, and consequently prolong the period. In support of this role for PER2 in determining the length of circadian rhythms, it has been

shown that phosphorylation of PER2 at different phosphorylation sites may be used to prolong or shorten the period length in oscillating cell cultures (Vanselow, *et al.*, 2006). The observed effects of lithium on the phosphorylation status of GSK-3β illustrate the value of confirming gene expression results at the functional level. However, for several clock proteins, the available commercial antibodies gave unsatisfactory western blots, and we could not confirm our qRT-PCR findings at the protein level.

5.4 Association of a VNTR polymorphism in *PER3* with chronotype

It has been suggested that a main effect of the clock gene *PER3* is to regulate sleep mechanisms (Archer, *et al.*, 2008; Viola, *et al.*, 2007; reviewed in Dijk and Archer, 2010), and a VNTR in *PER3* has been proposed a role in determining chronotype (Archer, *et al.*, 2003; Pereira, *et al.*, 2005). The short *PER3* allele has been associated with eveningness in healthy controls (Archer, *et al.*, 2003; Pereira, *et al.*, 2005; Lazar, *et al.*, 2012) and in DSPD patients (Ebisawa, *et al.*, 2001; Archer, *et al.*, 2003) (Table 5.4). However, conflicting results have also been reported, such as an association between the long *PER3* allele and DSPD (Pereira, *et al.*, 2005). Finally, several recent studies have reported no association between chronotype and the *PER3* VNTR polymorphism in healthy controls (Viola, *et al.*, 2007; Goel, *et al.*, 2009; Barclay, *et al.*, 2011) (Table 5.4). We performed a replication study in a sample of 432 Norwegian university students, but were not able to confirm previous findings of an association between the VNTR in *PER3* and diurnal preference (paper III).

Table 5.4. Overview over association studies of the VNTR in *PER3* and diurnal preference or DSPD.

Reference	Allele	Association	Tool for assessment of chronotype in healthy controls	Geographic location
(Ebisawa, *et al.*, 2001)	short	DSPD	not applicable	Japan
(Archer, *et al.*, 2003)	short short	DSPD eveningness	not applicable MEQ	Great Britain
(Pereira, *et al.*, 2005)	long long	DSPD morningness	not applicable MEQ	Brazil
(Viola, *et al.*, 2007)		no association	sleep-wake cycle, melatonin and cortisol rhythms	Great Britain
(Goel, *et al.*, 2009)		no association	Composite Scale	U.S.A.
(Viola, *et al.*, 2011)	long	morningness	activity, melatonin rhythm	Switzerland and France
(Barclay, *et al.*, 2011)		no association	MEQ	Great Britain
(Lazar, *et al.*, 2012)	long	morningness	MEQ and Munich Chronotype Questionnaire	Ireland

There may be several explanations for our negative finding. Chronotype has been shown to depend on age, where young adults are often associated with eveningness, and augmenting age is associated with increasing morningness (Carrier, *et al.*, 1997; reviewed in Roenneberg, *et al.*, 2007). In agreement with earlier studies (Archer, *et al.*, 2003; Pereira, *et al.*, 2005), a recent report demonstrated an association between the long allele and a phase advance of certain circadian parameters including melatonin in a sample of older people (Viola, *et al.*, 2011). A limitation of our study was that the sample only consisted of young adults, which could have biased our results. However, the instrument scores for both questionnaires were normally distributed in our study population (paper III). Also, our sample had the advantage of being relatively homogeneous with respect to demographic variables such as age,

education and social environment, and although we did not specifically register the ethnic background of the participants, we estimated that the large majority of the volunteers were ethnically Norwegian. In support of this assumption, the observed genotype frequencies corresponded well with a previous report of *PER3* VNTR genotypes on Norwegians (Nadkarni*, et al.*, 2005). Another possible contributing factor could be the latitude of the geographic locations where the studies were performed, since the latitude varied markedly from study to study (Table 5.4) and many factors, such as temperature, daylight length and intensity vary greatly with latitude; hence it was proposed that latitude and ethnicity might have influenced the associations (Pereira*, et al.*, 2005). However, two studies found no evidence for natural selection of clock gene variants based on latitude (Nadkarni*, et al.*, 2005; Ciarleglio*, et al.*, 2008). It could also be that the role of *PER3* is smaller than previously assumed, which is supported by a recent report on the VNTR in *PER3* having no effect on circadian period (Hasan*, et al.*, 2012), and the question of the role of *PER3* is left unanswered.

6 Concluding remarks and future perspectives

We demonstrated that a main effect of lithium on clock gene expression was to differentially alter the oscillation amplitudes (paper I). Identifying molecular targets of lithium is highly valuable for understanding the pathophysiology underlying bipolar disorder and the molecular mechanisms of lithium action. Animal models of depression and mania may be useful to this purpose, and models of depression have long been established in rodents, such as the forced swim test (Porsolt, *et al.*, 1978) and the tail suspension test (Steru, *et al.*, 1985). Recently, novel animal models of bipolar mania have been proposed, including a model where disruption of the gene *Clock* causes behavior comparable to what is seen in manic patients (Roybal, *et al.*, 2007). This is highly interesting in light of numerous studies demonstrating associations between polymorphisms in clock genes and bipolar disorder (Benedetti, *et al.*, 2003; Nievergelt, *et al.*, 2006; Kripke, *et al.*, 2009). Although contradictory and negative findings have been reported (Mansour, *et al.*, 2009; Kishi, *et al.*, 2009), recent efforts at the genome-wide level still support such an association (McCarthy, *et al.*, 2012). Furthermore, based on studies linking bipolar disorder with the expression of the sodium pump Na^+/K^+-ATPase (NKA) in the brain, such as postmortem findings in brain tissue from patients (Rose, *et al.*, 1998) as well as association studies (Goldstein, *et al.*, 2009), a novel model for mania was recently proposed, involving inactivation of neuron-specific NKA in mice (Kirshenbaum, *et al.*, 2011). In line with the behavioral deficits in animal models of depression being lithium-sensitive (extensively reviewed in O'Donnell and Gould, 2007), lithium also rescued behavioral effects in both models of mania (Roybal, *et al.*, 2007; Kirshenbaum, *et al.*, 2011). In addition to elucidating the pathophysiology of bipolar disorder, such animal models open for the possibility to extensively monitor and manipulate parameters of the circadian clock, for further investigation of the action of lithium and other psychotropic drugs.

In addition to studying the effects of lithium on clock gene expression, we demonstrated that various psychotropic drugs with diverse pharmacological actions, including clozapine and imipramine, also influenced the levels of clock gene transcripts in NIH-3T3 cells, which could be relevant for their therapeutic effects (paper II). This is intriguing since the investigated drugs have main areas of use in distinct, although partly overlapping psychiatric illnesses (reviewed in Craddock, *et al.*, 2006; Lin and Mitchell, 2008; Fountoulakis and Vieta, 2008). The various drugs had diverse effects, indicating that they act on the circadian clock via different mechanisms. However, they may have common targets relevant for their effects on circadian rhythms, and glycogen synthase kinase 3β (GSK-3β) would be a good candidate, as antipsychotics, antidepressants and mood stabilizers have all been shown to regulate GSK-3 (Sutton and Rushlow, 2011; Amar, *et al.*, 2011; Park, *et al.*, 2011; reviewed in Gould and Manji, 2005; Freyberg, *et al.*, 2010), and since GSK-3β has been linked to the circadian clock (Iitaka, *et al.*, 2005; Yin, *et al.*, 2006).

Obesity in the general population represents a major health issue, and even more so in psychiatric patients (McElroy, *et al.*, 2002; Goldstein, *et al.*, 2011; reviewed in Allison, *et al.*, 2009), and metabolic side effects such as weight gain and dyslipidemia represent major concerns in drug-treatment (reviewed in Nasrallah, 2008; Lett, *et al.*, 2011). We confirmed previous findings from our laboratory, demonstrating that antipsychotics and antidepressants increased the transcription of lipogenic genes in cell culture, which may have clinical relevance since these drugs are known to cause weight gain in the clinical setting (Berken, *et al.*, 1984; Fernstrom, *et al.*, 1986; Cohen, *et al.*, 1990; Leadbetter, *et al.*, 1992; reviewed in Allison, *et al.*, 2009; Serretti and Mandelli, 2010), and the SREBP system has been implied in inducing marked weight gain in patients on antipsychotic drugs (Le Hellard, *et al.*, 2009; reviewed in Ferno, *et al.*, 2011). For clozapine and imipramine, we cannot rule out that the effects on clock genes are linked to their effects on lipid biosynthesis genes, even though our data does not clearly indicate this.

However, results from cell culture studies must be interpreted with caution, and animal studies and ultimately clinical trials are required to provide more definitive answers. Nevertheless, *in vitro* studies may provide useful clues for understanding the underlying mechanisms and may generate interesting leads to pursue further. Numerous clock genes and lipid metabolism genes have been proposed as possible mediators for the interplay between the circadian clock and lipid metabolism at the molecular level, including *Per2*, *Bmal1*, *Rev-Erb-α*, *Ror-α*, *Ppar-γ* and *Pgc-1α* (Shimba*, et al.*, 2005; Liu*, et al.*, 2007; Le Martelot*, et al.*, 2009; Grimaldi*, et al.*, 2010; Schmutz*, et al.*, 2010). To provide new insight into the underlying molecular mechanisms of the interaction, the technique of using small hairpin RNA (shRNA) to selectively knock down these genes could be very valuable (Paddison*, et al.*, 2004; Sliva and Schnierle, 2010). Correspondingly, exploring downstream effects after constitutively activating the expression of a given gene *in vivo* in animals and *in vitro* in cell culture could also provide new leads.

The mechanisms by which the circadian clock influences sleep and diurnal preference, have been an increasing focus of attention (Leloup and Goldbeter, 2008; for review, see von Schantz, 2008) since associations between sleep and a range of complications have been reported, including obesity and increased body mass index (Taheri*, et al.*, 2004; Grandner*, et al.*, 2011; Baron*, et al.*, 2011; reviewed in Knutson*, et al.*, 2007; Oster, 2010) and psychiatric disorders (reviewed in Lamont*, et al.*, 2010; Dallaspezia and Benedetti, 2011). Interestingly, it has been shown that bipolar patients are more likely to have a preference for evening compared to healthy controls (Mansour*, et al.*, 2005; Giglio*, et al.*, 2010). Of note, an association between evening chronotype and obesity in bipolar patients has also been reported (Soreca*, et al.*, 2009). Taken together, these findings further highlight the close relationship between the circadian clock, bipolar disorder and lipid metabolism. We studied the impact of the clock gene *PER3* in influencing diurnal preference in healthy students, but could not confirm a significant association between the VNTR

polymorphism in *PER3* and self-report measures of the morningness-eveningness dimension in our study sample (paper III). It seems improbable that a single genetic variant in one gene should be responsible for determining chronotype, and in fact, it has been proposed that several clock genes could each make minor contributions to determining chronotype, whereas specific combinations of these polymorphisms are required to produce a significant association (Pedrazzoli*, et al.*, 2010). In agreement with this, a recent study found relevant effects of several polymorphisms in clock genes on chronotype and adaptation to shift-work (Gamble*, et al.*, 2011). Hence, in future association studies, it would be interesting to explore the effects of combinations of multiple variants on diurnal preference. Of note, the same mechanism might in part explain the hidden heritability of bipolar disorder. It has been suggested that multiple genetic variants could each make minor contributions towards disease vulnerability, which taken together might represent a substantial risk for bipolar disorder (reviewed in Gershon*, et al.*, 2011). Hence, also here it could be interesting to explore the synergy of several genes in conferring risk for bipolar disorder.

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