Photoperiodic Responses on Expression of Clock Genes, Synaptic Plasticity Markers, and Protein Translation Initiators

The Impact Of Blue-Enriched Light

Master report

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Summary

Introduction. Light can have complex effects on circadian rhythmicity, mood, and cognition. Light input synchronizes circadian rhythms in the brain and periphery. In the cell, circadian rhythms are generated by cyclic expression of clock genes. In addition to regulating circadian rhythms, the clock gene BMAL1 was recently found to regulate protein translation thereby influencing neuronal plasticity, a process important for adaptation to changes in the environment. Mechanisms of neuronal plasticity at synapses depend on several immediate early response genes, including Arc, BDNF, and NPAS4. Recent studies have shown that prolonged photoperiod affects clock gene expression and synaptic plasticity markers outside the SCN, like the hippocampus. Here we used a model of prolonged photoperiod (20h light, 4h dark; 20:4 LD) with two different spectral qualities (white and blue-enriched light) to examine photoperiodic responses on the expression of clock genes, synaptic plasticity markers, and protein translation markers in the prefrontal cortex (PFC).

Methods. Rats (n=10/group) were housed in standard 12:12 LD condition, followed by exposure to 7 days of prolonged photoperiod (20:4 LD) in either standard white (267.33 lux; 68.60 W/m^2; 1.96E14 $cm^{-2}s^{-1}$) or blue-enriched (265.6 lux; 186.60 W/m^2; 4.93E14 $cm^{-2}s^{-1}$) light, and recovery in standard 12:12 LD condition. Tissue from PFC was collected at 12 hours after lights on (ZT12) on exposure day 7 (E7), recovery day 3 (R3) and from undisturbed control. PFC tissue was analyzed with qPCR, m^7 GTP (Cap) pull-down analysis, and western blot.

Results. Prolonged photoperiod altered RNA expression of clock genes. *Per2* was decreased at E7 and R3 compared to controls in both light conditions (t'(15-16)>2.40, p's<.029; E7 *d*'s>1.69 and R3 *d*'s>1.35). The main effect of time ($F(1,36)= 10.99$, $p<.01$) revealed a larger reduction at E7 than R3 (-51.8%, $p<.01$, $d=1.63$). Compared to controls, *BMAL1* was increased at E7 in the blue-enriched light condition ($t(15)=2.40$, $p<.05$) and at R3 in the white light condition ($t(16)>4.60$, $p<.001$).

As for markers of synaptic plasticity, *Arc* was decreased in after prolonged light exposure ($t(16)=-3.74$, $p<.002$; $d=-2.03$). *Arc, BDNF* and *NPAS4* showed an effect of time (F's(1,35-38)>5.47, p's<.026), revealing an increase from E7 to R3 in white light for *Arc* (+214.3%, $p<.01$, $d=1.45$), in blue-enriched light for *BDNF* (+42.3%, $p<.05$, $d=1.28$) and in both light conditions for *NPAS4* (white light: +89.7%, $p<.05$, $d=1.07$ and blue-enriched light: +125.6%, $p<.05$, $d= 1.18$). Arc was decreased at E7 in the blue light condition ($t(16)=4.06$, $p<.001$, $d=-2.10$). Likewise, *NPAS4* was decreased compared to control in blue-enriched light only ($t(15)=-3.53$, $p=.003$, $d=-1.82$). Expression of *BDNF* increased at R3 compared to control after recovery from blue-enriched light ($t(14)=3.80$, $p=.002$, $d=1.90$).

No clear changes in individual protein translation markers were observed, yet on a group level expression in promoters of translation was higher and in repressors lower in the white light condition compared to the blue light condition.

Conclusion and Discussion. Exposure to prolonged photoperiod altered RNA expression of clock genes and synaptic plasticity markers in the PFC. After 3 days of recovery of prolonged photoperiod, clock gene expression did not normalize, while synaptic plasticity markers normalized, indicating possible differential regulatory pathways in recovery. The found changes in RNA expression of clock genes and synaptic plasticity genes largely followed similar patterns in white and blue-enriched light, except for *BDNF*, which may have a specific function in recovery from prolonged blue-enriched light. Additionally, regulators of protein translation exhibited a weak spectral-specific pattern with higher expression of

promoters and lower expression of repressors of protein synthesis in white light compared to blue light, yet more research is needed to examine this.

For future studies, the (spectral-specific) photoperiodic-induced expression dynamics of clock genes, synaptic plasticity genes, protein translation regulators and their possible different (ipRGC-mediated) pathways need to be characterized, including multiple time-of-day measurements. Knowledge about these photoperiodic responses can be used to develop light-based therapies for mood disorders.

Keywords: clock genes, PFC, prolonged photoperiod, protein translation initiation, synaptic plasticity

Introduction

Exposure to light has an enormous impact on life. The daily light-dark cycle has been a part of our environment since the beginning of life. Organisms are also subjected to different daily light exposure throughout the year, especially near the poles. Due to these daily and yearly changes, organisms on earth have adopted a dynamic lifestyle. Think for example about the daily sleep-wake rhythm corresponding to the dark-light cycle or seasonal fluctuations in depressive symptoms in depressed patients.

To facilitate this dynamic lifestyle, organisms need mechanisms to constantly adapt and alter their behavior and physiology in response to the daily and yearly changes in light (Bradshaw and Holzapfel 2007; Reppert and Weaver 2002). In mammals, the brain is the most important organ in this adaptation to light. To anticipate the daily cycling changes in our environment, organisms produce circadian rhythms (Reppert and Weaver 2002). For synchronization of these circadian rhythms, the brain needs to receive and integrate external light signals. The suprachiasmatic nucleus (SCN) is the key brain region for this adapting and synchronizing to the daily light cycle in mammals (Ralph, Foster, and Davis 1990). The SCN also functions as a central pacemaker of the body and conveys its circadian rhythm to other brain areas and peripheral tissues (Damiola et al. 2000; Gachon et al. 2004). The light signals to the SCN come from specialized cells in the retina. These intrinsically photosensitive retinal ganglion cells (ipRGCs) contain a specific photopigment called melanopsin (Daneault et al. 2016; Provencio et al. 2000). Melanopsin is most sensitive to blue light (~480 nm), making ipRGC-mediated processes maximally sensitive to this part of the light spectrum (Hattar et al. 2003). In addition to its role in circadian rhythmicity, the SCN is also involved in responding to day length (photoperiod) and the production of circannual rhythms (Bradshaw and Holzapfel 2007; Porcu et al. 2018). Altogether, specialized mechanisms in the mammalian retina and brain have evolved to respond to changes in light.

Synchronization of the rhythm of the body to the external environment is mainly regulated through responses to light. Light-induced synchronization affects the SCN on various levels (Welsh, Takahashi, and Kay 2010; Golombek and Rosenstein 2010; Brancaccio et al. 2014). First, light input from ipRGC projections can induce changes in expression of clock genes of SCN neurons (Schwartz et al. 2011; Reppert and Weaver 2002; Evans et al. 2013). A 24-hour cyclic expression pattern of clock genes, generated by a transcriptional-translational feedback loop, generates circadian rhythmicity on a cellular level. The basis of the circadian molecular clock in mammalian cells consists of a pattern of stimulation and inhibition between brain-and-muscle arnt-like protein 1 (BMAL1) and circadian locomotor output cycles kaput (CLOCK), and on the other side Period (Per1, Per2, Per3) and Cryptochrome (Cry1, Cry2)(Ko and Takahashi 2006; Mohawk, Green, and Takahashi 2012). If the clock gene expression of a cell is changed, then the circadian rhythmicity of that cell is also changed. Take for example a SCN neuron, which has its circadian rhythm generated by cyclic clock gene expression. Light-induced changes of clock gene expression in the SCN, change the rhythm of the SCN (Schwartz et al. 2011). Consequently, also the rhythm in the whole body since the SCN acts as the central pacemaker (Hastings and Herzog 2004). Besides the fact changes in clock genes can change the rhythm, loss of clock genes can cause arrhythmic behavior, as for example in BMAL1 mutant mice (Bunger et al. 2000). Clock genes, such as Per2, can also be affected by circadian patterns such as the sleep-wake cycle (Cirelli, Gutierrez, and Tononi 2004). However, the view that light uniformly affects all SCN neurons is too simplistic. The SCN can be divided in a dorsomedial core and ventrolateral shell part, which react differently to light stimuli (Meijer et al. 2010). Coupling signals between SCN core and shell are essential for the SCN's ability of photoentrainment. In addition, interactions between core and

shell may also function as an indicator of day length, which highlights the role of the SCN in photoperiodic responses (Liu et al. 2007; Evans et al. 2013; Porcu et al. 2018). Thus, the SCN requires adaptive processes on a cellular and network level to be able to respond to daily and yearly changes in light conditions, yet synchronizing the circadian and circannual clock is not the only light-induced adaptive process in the brain.

The brain has the ability to adapt to external stimuli by making structural changes in neuronal connections. This structural remodeling at the level of the synapse is called synaptic plasticity and is the basis for many adaptive processes in the brain, including memory and cognitive performance (Neves, Cooke, and Bliss 2008). Next to its synchronization function, the circadian clock is directly linked to synaptic plasticity processes via the clock gene BMAL1 (Lipton et al. 2015). By regulation of the activity of eukaryotic initiation complex 4F (eIF4F), BMAL1 can produce circadian clock-mediated changes in protein synthesis and consequently in the remodeling of synapses (Lipton et al. 2015). Phosphorylation of BMAL1 by ribosomal protein S6 kinase beta-1 (S6K1) can enhance EiF4F activity, thereby increasing translation of proteins involved in synaptic plasticity. In addition, phosphorylation of BMAL1 can be affected by disruptions of circadian rhythmicity (Marti et al. 2017). The eIF4F complex consists of several components including the mRNA m^7GTP cap-binding unit eukaryotic initiation factor 4E, 4G, and 4A (eIF4E, eIF4G, and eIF4A) and its activity is also regulated via various other mechanisms (Bramham and Messaoudi 2005). For example, blocking the formation of eIF4F by interfering with the binding between eIF4E and eIF4G can suppress the initiation of translation. This interference can be done by several proteins including eIF4E-binding proteins (eIF4E-BPs, for example eIF4E-BP2) and a protein complex consisting of cytoplasmic fragile X mental retardation-interacting protein (CYFIP1) and fragile X mental retardation protein (FMRP) (DeRubeis et al. 2013; Bidinosti et al. 2010; Napoli et al. 2008). Synaptic plasticity processes are not only regulated through protein translation, yet involve modulation by a wide variety of neurotrophic factors (Schinder and Poo 2000). One of the main growth factors involved in activity-dependent remodeling of synapses is Brain-Derived Neurotrophic Factor (BDNF) (Leal, Comprido, and Duarte 2014). Most likely responsible for these long-term adaptive neuronal responses is a BDNF-induced transcriptional program including activity-regulated cytoskeleton-associated protein (Arc, also known as Arg3.1), TGFβ-inducible early gene-1 (TIEG1, also known as Klf10), Narp, neuritin (Nrn1), calcium/calmodulin-dependent protein kinase-related peptide (CARP), and ADP-ribosylation factor-like protein-4 (ARL4L) (Wibrand et al. 2006). Also involved in synaptic plasticity is the transcription factor neuronal PAS domain protein 4 (NPAS4), which is thought to stimulate underlying gene programs and influence BDNF transcription (Bloodgood et al. 2013; Maya-Vetencourt 2013). Interestingly, the regulation of protein translation required for BDNF-mediated synaptic plasticity is mediated via eIF4E activity, which also is affected by the clock gene BMAL1 (Bramham and Messaoudi 2005; Lipton et al. 2015). Yet another link between synaptic plasticity genes and the clock is the fact that Arc and BDNF are shown to be affected by sleep and wakefulness, a process largely regulated by the circadian clock (Cirelli, Gutierrez, and Tononi 2004). In sum, the brain's ability to adapt to light changes relies on interactions between clock genes, protein translation, and BDNF-induced synaptic plasticity.

Exposure to a prolonged photoperiod affects several plastic mechanisms in the brain including, SCN network plasticity, changes in clock genes and synaptic plasticity-related genes. In the SCN, long photoperiods affect the coupling between the phase of SCN shell and core (Evans et al. 2013; Inagaki et al. 2007). Evans and colleagues (2013) found that mice exposed to a prolonged photoperiod had a difference between the peak time of Per2 expression between the core and shell, which increased relative to day length. In addition, after mice were released in constant darkness after exposure to

prolonged light exposure (20h light, 4h dark; 20:4 LD) the SCN core and shell were able to resynchronize in within one week via a transient resynchronization process using coupling signals. The effects of prolonged light exposure are not restricted to the rhythmicity in the SCN, but are observed in SCN-coordinated rhythmicity in other brain areas and peripheral tissues (Porcu et al. 2018). Evans et al. (2015) showed that Per2 rhythmicity in brain areas and peripheral tissues followed the phase of the SCN shell after it was uncoupled from the core by exposure to 20:4 LD. Although the rhythmicity in the tested brain regions (cerebellum, hippocampus, olfactory bulb, and septum) stayed in synchrony with the SCN shell, the expression of clock genes was largely attenuated (Evans et al. 2015). To test whether the attenuation of the circadian rhythmicity in the hippocampus could underlie performance on hippocampus-dependent memory tasks, mice were subjected to 20:4 LD for 4 weeks before undergoing spatial and recognition memory tasks (Dellapolla et al. 2017). Indeed, exposure to 20:4 LD increased performance on hippocampus-dependent memory tasks, which was lost after knock-out of melanopsin, indicating the role of ipRGC-dependent pathways. Furthermore, altered expression of plasticity markers (e.g. BDNF and insulin-like growth factor 2; Igf2) after 20:4LD was found. These changes in plasticity markers were proposed as a possible mechanism through which light-induced changes in SCN-coordinated rhythmicity in the hippocampus could affect performance in memory tasks (Dellapolla et al. 2017). Further evidence that hippocampus-dependent memory tasks are affected by long photoperiods is found in rats (MacDonald et al. 2007) and white-footed mice (Walton et al. 2011; Pyter 2005). However, in contrast to Dellapolla and colleagues (2017), these studies compared animals exposed to long and short photoperiods, where long photoperiod animals showed a better in memory performance. In addition, white-footed mice exposed to long photoperiod showed higher dendritic spine density and long-term potentiation in the hippocampus than mice exposed short photoperiods, indicating altered neuronal plasticity processes (Walton et al. 2011; Pyter 2005). Although many ipRGC-mediated effects go via the SCN, several direct ipRGC projections can affect brain areas involved in regulation of mood, memory, and cognition (Hattar et al. 2006; Daneault et al. 2016). Combined, these studies indicate that photoperiodic-induced ipRGC-mediated changes in circadian rhythmicity and plasticity markers in non-SCN brain areas can affect behavior regulated by these brain areas (Porcu et al. 2018).

A brain region known to be affected by light is the prefrontal cortex (PFC), which is important for regulation of cognitive performance and working memory (Kane and Engle 2002; Vandewalle, Maquet, and Dijk 2009). Acute light exposure modulates activity in the PFC in humans, possibly via activation of subcortical areas such as the thalamus and hypothalamus (including the SCN). These effects are largest by blue light exposure, indicating the involvement of ipRGC pathways (Vandewalle et al. 2007; Vandewalle, Maquet, and Dijk 2009). Moreover, disturbances of circadian rhythmicity have been linked to changes in BMAL1-mediated regulation of synaptic plasticity processes in the PFC. Circadian disturbance by simulated night shift work affected time-of-day variation in phosphorylated BMAL1 and its regulatory enzyme S6K1, which are both important for regulating synthesis proteins required for synaptic plasticity (Marti et al. 2017; Lipton et al. 2015). Thus, light can impact PFC-dependent cognition and circadian disturbance alters circadian clock-regulated plasticity processes in the PFC.

Prolonged photoperiod is shown to affect several circadian and plasticity processes in the brain, yet not much is known about these changes in the PFC. Similar to the findings in the hippocampus, the PFC might undergo photoperiodic responses in molecular markers of circadian rhythmicity and neuronal plasticity. Additionally, research has been focused on the effects of exposure to prolonged photoperiod alone while on earth animals are also exposed to changes in photoperiod.

These changes in photoperiod require a transient resynchronization process in the SCN (Evans et al. 2013; Welsh, Takahashi, and Kay 2010). Not much is known about the effects on brain regions outside the SCN after animals return from a prolonged photoperiod to a 'standard' photoperiod of 12:12 LD. Altogether, (changes in) photoperiod may influence the expression of clock genes, protein translation markers, and BDNF-associated markers of synaptic plasticity in the PFC.

Research Objective

Using a rat model of prolonged photoperiod (20:4 LD), I wanted to characterize the long-term effect on expression of clock genes, synaptic plasticity-associated genes and markers of protein translation in the prefrontal cortex. Additionally, I examined molecular changes resulting from a transition from a prolonged photoperiod (20:4 LD) to a normal photoperiod (12:12 LD). Finally, I wanted to quantify the potential impact of blue light by characterizing the differential effects of exposure to and recovery from prolonged polychromatic white light versus blue-enriched light. Therefore, the following questions will be addressed:

1. Are clock genes, synaptic plasticity-associated genes, and markers of protein translation affected by seven days of prolonged photoperiod?
2. How are clock genes, synaptic plasticity-associated genes, and markers of protein translation affected in the transition to normal photoperiod?
3. What is the impact of blue-enriched light on such photoperiodic responses?

Hypotheses

Clock genes

1. Exposure to a prolonged photoperiod requires adaptation of the cellular basis of circadian rhythmicity and extends the rest phase of the rat. Therefore, I expect that exposure to a prolonged photoperiod will induce changes expression in clock gene expression (e.g. reduction in Per2) corresponding to photoperiod-induced alterations in circadian rhythmicity and activity-rest patterns.
2. In recovery, I expect that the SCN coordinates the resynchronization of clock gene expression. Thus, at recovery day 3 I expect a partial normalization of clock gene expression.
3. If the effects on clock genes are mediated by the melanopsin-containing ipRGCs, blue-enriched light can amplify the effects on clock gene expression.

Synaptic plasticity genes

1. Expression of synaptic plasticity markers are affected by changes in external stimuli (e.g. light), but also sleep and wakefulness. Therefore, matching the pattern in the rest phase (reduced exposure to stimuli), I expect a decrease in expression of synaptic plasticity markers during exposure to prolonged photoperiod.
2. I expect an increase (or normalization) of expression of synaptic plasticity markers in recovery, which may facilitate adaptive processes while also following sleep-wake dependent expression patterns.
3. These effects are hypothesized to be enhanced by blue-enriched light if the expression of the measured synaptic plasticity genes is regulated by the melanopsin-containing ipRGCs.

Protein translation markers

1. Due to the connection with the clock gene BMAL1 and the involvement in adaptive processes, expression of protein translation markers are hypothesized to be affected due to exposure to prolonged photoperiod. To adapt to the new photoperiod, increased protein synthesis via higher expression of promoters and reduction of repressors is expected.
2. In recovery, expression of protein translation regulators is expected to change (higher expression of promoters and lower of repressors) since they may be contributing to adaptive processes needed for resynchronization to a normal photoperiod.
3. Currently, there is no known connection between ipRGC signaling and the protein translation initiators, and therefore differential effects of blue and white light on the expression of protein translation initiators will be explored.

Methods

Experimental procedure

Ethics

All animal work has been conducted according to relevant Norwegian and international laws and guidelines (The European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes). The Norwegian Animal Research Authority approved this research project (permit number: 20124636).

Animals

Male Wistar rats (NTac:WH, Taconic, Denmark) were group housed in individually ventilated cages before the start of the experiment (IVC system, Techniplast®, Italy; type IV 480x375x210 mm). During the experiment, animals were housed individually (IVC cages type III 425x266x185 mm). Ventilation of the cages consisted of 75 air changes per hour. The temperature and humidity were maintained stable during the experiment (23±1°C; 40±1%). Food (Rat and mouse no. 1, Special Diets Services, Whitham, Essex, England) and water were ad libitum available during the whole baseline and experiment. Cages were cleaned and changed once a week except when animals were in experimental conditions. Before the experiment, animals were kept in a 12:12 LD cycle (lights on at 07:00 (Zeitgeber Time; ZT0) and off at 19:00 (ZT12)). Lights were gradually dimmed on and off in 1 hour (ZT0 to ZT1, and ZT 12 to ZT13, respectively).

Experimental design

Animals were exposed to different light protocol consisting of a baseline, exposure and recovery phase (see figure 1). In the baseline, all groups were in 12:12 LD of standard white light. In the exposure phase, the rats were exposed to 7 days of prolonged photoperiod (20:4 LD; lights on ZT0 to ZT20), in either standard white light or blue-enriched light. In the recovery phase, animals received 3 days of 12:12 LD in standard white light. A control group was housed in 12:12 LD, standard white light, throughout the whole experiment.

Tissue collection

Five groups of animals were used in this experiment (n=10/group). Following 7 days of prolonged light exposure in either white or blue-enriched light (WE7 and BE7 groups) or 3 days in recovery (WR3 and BR3 groups), animals were anesthetized with isoflurane, and sacrificed with decapitation (figure 1). A separate group never exposed to prolonged light condition was used as control to investigate specific protein variation. All animals were sacrificed at ZT12. The dissected tissue of prefrontal cortex was immediately stored at -80 °C for further analysis.

Light characteristics

Two light conditions were used in this experiment. Standard white light consisted of illuminance at 267 lux, irradiance 67 W/m^2 and photon flux 1.96E+14 $cm^{-2}s^{-1}$. Blue-enriched light had similar illumination as standard white light (266 lux), an irradiance of 187 W/m^2 and photon flux of 4.93E+14 $cm^{-2}s^{-1}$. The spectral distribution of both conditions is illustrated in figure 2.

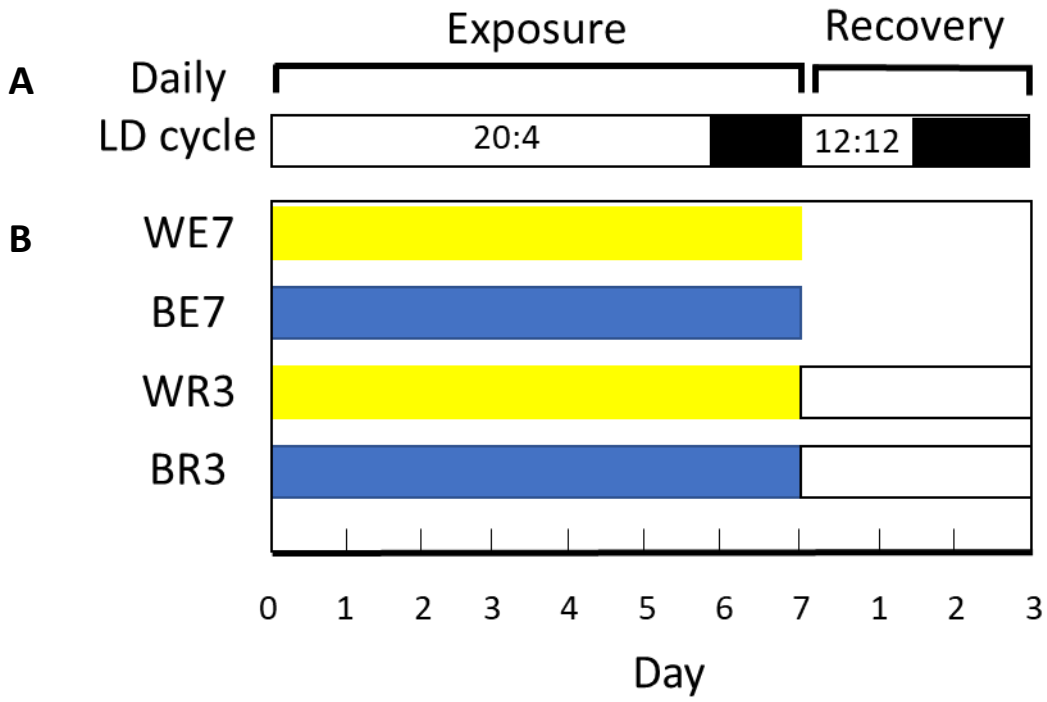

Figure 1. Experimental protocol. A. Light-dark cycle during exposure (20:4 LD, ZT0-ZT20) and recovery (12:12 LD, ZT-0-ZT12). B. Spectral qualities used for prolonged photoperiod during exposure phase. Yellow denotes the standard white light in 20:4 LD, blue the blue-enriched light during in 20:4 LD, and the white bars during recovery indicate the 12:12 LD cycle with standard white light.

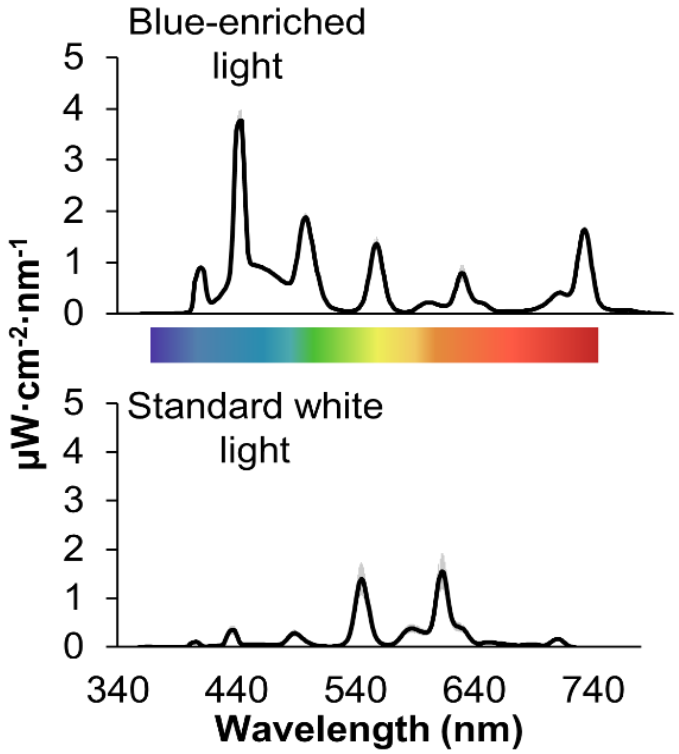

Figure 2. Light spectra of the blue-enriched light (top) and standard white light (bottom).

RNA expression analysis

PFC hemispheres (left vs right) were randomly assigned to either RNA or protein analyses. The use of left and right hemispheric tissues was balanced, to avoid measuring hemisphere-dependent effects. The RNA isolation was performed using the RNeasy Lipid Tissue Mini Kit (Qiagen). Total RNA was eluted in 100 µl RNase-free water. Genomic DNA was removed using on-column DNase digestion (Qiagen, #79254). For protocols of RNA isolation and DNA digestion, see supplementary information 1. RNA concentration and quality were determined by measuring 260 nm absorbance and 260/280 absorbance ratio using a spectrophotometer (NanoDrop 2000c, Thermo Fisher Scientific) before storage at -80 °C. Dilutions to 20ng/µl were made for all RNA samples. Subsequently, a collaborator made cDNA and performed real-time qPCR on PFC tissue using the primers listed in table 1. The fold change compared to control condition was determined by using the ΔΔCt method using ribosomal protein large (RPLP0) as an endogenous control for normalization of expression and the mean of the 12:12 LD control animals as baseline. RPLP0 was chosen as control gene for its stable expression throughout the day.

Gene	Primers	Reason
Arc	F: AGTCTTGGGCAGCATAGCTC R: GGTATGAATCACTGCTGGGGG	BDNF-induced transcriptional program, synaptic plasticity (Wibrand et al. 2006)
BDNF	F: TGGGACTCTGGAGAGCGTGAATGG R: CGGGACTTTCTCCAGGACTGTGAC	Synaptic plasticity (Wibrand et al. 2006; Bramham and Messaoudi 2005)
BMAL-1 / ARNTL	F: TTAGCCAATGTCCTGGAAGG R: GTGGGATGAGTCCTCTTTGG	Clock gene (Ko and Takahashi 2006)
CLOCK	F: TTCACTCAGGACAGACAGATA R: GTCACTAATTTGGTCACAACT	Clock gene (Ko and Takahashi 2006)
DCLK1 / Carp	F: ATAGAAGTTAATGGAACCCCTGG R: AAATCATCCGACGAGAGAGGG	BDNF-induced transcriptional program, synaptic plasticity (Wibrand et al. 2006)
Klf10 / TIEG1	F: GCCAACCATGCTTAACTTCGG R: CGCTTCCACAGCTTCAAAGTC	BDNF-induced transcriptional program, synaptic plasticity (Wibrand et al. 2006)
NPAS4	F: GGACCTAGCCCTACTGGACA R: TTTTCAGCCAACAGGCGGTA	Synaptic plasticity (Maya-Vetencourt 2013)
Nptx2 / Narp	F: GGAGAGAGGCAACAGTGCAT R: TGGTGAAGGCATACAGCTCG	BDNF-induced transcriptional program, synaptic plasticity (Wibrand et al. 2006)
Nrn1 / Neuritin	F: CGCGGTGCAAATAGCTTACC R: TGTTCGTCTTGTCGTCCAGG	BDNF-induced transcriptional program, synaptic plasticity (Wibrand et al. 2006)
RPLP0 (P0)	F: CATTGAAATCCTGAGCGATGT R: AGATGTTCAACATGTTCAGCAG	Endogenous control (Skrede et al. 2017)
Per2	F: CAGTGATGCCAAGTTTGTGG R: AGCTGTGGAACACACTGACG	Clock gene (Ko and Takahashi 2006)

Table 1. Primers used for qPCR analysis. Abbreviations: Arc; Activity-regulated cytoskeleton-associated protein, ARNTL; Aryl hydrocarbon receptor nuclear translocator-like protein 1, BDNF; Brain-Derived Neurotrophic Factor, BMAL-1; Brain and muscle Arnt-like protein-1, Carp; calcium/calmodulin-dependent protein kinase-related peptide, DCLK1; Serine/threonine-protein kinase DCLK1, Dcx1; doublecortin 1, Klf10; Krüppel-Like factor 10, Narp; Neuronal Activity-Regulated Pentaxin, NPAS4; Neuronal PAS domain-containing protein 4, Nptx2; Neuronal Pentraxin 2, Nrn1; Neuritin 1, RPLP0; ribosomal protein large P0, Per2; period 2, TIEG1; TGFβ-inducible early gene-1.

m^7 GTP (Cap) pull-down and protein analysis

The PFC tissue was homogenized by hand in 750 µl lysis buffer (RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, #89901) containing 1 mM Na_3VO_4 and 1X Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific, #78430)). To remove debris, the homogenate was centrifuged for 15 min at 13200 rpm at 4 °C . Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, # 23227).

For the cap pull down, 30 µl Immobilized γ-Aminohexyl-m^7GTP beads (Jena Bioscience #AC-141) were used per sample. Beads were washed in PBS before adding lysate containing 400 mg protein. After 2,5h of spinning at 4 °C, beads were washed in lysis buffer. 20 µl 2X XT sample buffer was added to the beads and boiled for 10 min at 70 °C.

For western blot analysis, 3 µl 4X XT sample buffer (Bio-Rad #1610791) containing 2-mercaptoethanol (1:10) was added to lysate containing 20 mg protein and boiled at 100 °C for 5. Samples were loaded on a Criterion™ TGX™ Midi Gel (Bio-rad, 4-15%, #5671084), transferred on a nitrocellulose membrane from a Trans-Blot® Turbo™ Midi Nitrocellulose Transfer Pack (Bio-rad, #1704159). 5% non-fat dry milk was used to block the membrane for 1h. Subsequently, the membrane was developed using chemiluminescence reagents and scanned in a Gel DOC XRS+ (Bio-rad). Membranes probed with antibodies recognizing phosphorylated proteins were stripped using Restore™ Western Blot Stripping Buffer (Thermo Fisher Scientific, #21059) for 10 min, washed 3x 5 min in PBS, blocked in 5% non-fat dry milk for 1h and reprobed with antibody recognizing total protein (see supplementary information 1 for extended protocol).

The optical density values were determined with ImageJ software (NIH, Bethesda, MD, USA) and normalized to tot-eIF4E and GAPDH on the same immunoblot for cap pull down and western blot respectively. In addition, the phosphorylated proteins were further normalized to the total protein on the same immunoblot.

Antibodies which were used are shown in table 2. Only eIF4G, p-BMAL1, total-BMAL1, CYFIP1, FMRP and eIF4E-BP2, p-eIF4E and eiF4E were used in both cap pull down and western blot since these proteins are associated with binding and regulation of the eIF4F complex.

Protein	**Dilution**	**Origin**
eIF4E-BP2	1:1000	Cell Signaling #2845
Arc	1:500	Santa Cruz Biotechnology #sc17839
p-BMAL1	1:500	Cell Signaling #13936
BMAL1	1:500	Santa Cruz Biotechnology #sc365645
Clock	1:1000	Abcam #ab3517
Cry2	1:1000	Thermo Fisher Scientific #PA5-13125
CYFIP1	1:1000	Upstate #07-531
p-eIF4E	1:1000	Cell Signaling #9741
eIF4E	1:1000	Cell Signaling #9742
eIF4G	1:1000	Cell Signaling #2498
FMRP	1:1000	Abcam #ab17722
GAPDH	1:5000	Santa Cruz Biotechnology #sc32233
p-S6K1	1:1000	Santa Cruz Biotechnology #sc-7984
S6K1	1:1000	Sigma #SAB4502691
Anti-Mouse Secondary Antibody	1:10000	Thermo Fisher Scientific #31430
Anti-Rabbit Secondary Antibody	1:10000	Thermo Fisher Scientific #31460

Table 2. Antibodies used for cap pull down and western blot analysis. Abbreviations: eIF4E-BP2; eIF4E-binding protein 2, Arc; activity-regulated cytoskeleton-associated protein, BMAL1; Brain and muscle Arnt-like protein-1, Clock, Cry2; Cryptochrome 2, CYFIP1; cytoplasmic fragile X mental retardation-interacting protein, eIF4E; eukaryotic initiation factor 4E, eIF4G; eukaryotic initiation factor 4G, FMRP; fragile X mental retardation protein, GAPDH; Glyceraldehyde 3-phosphate dehydrogenase, S6K1; ribosomal protein S6 kinase beta-1.

Statistics

Statistics were performed using IBM SPSS Statistics 24 and graphs were made using Prism5. Values were determined outliers and excluded from analysis when they were outside 2.5*SD. Significant effects of light conditions on RNA and protein compared to control was determined by independent samples Student's t-tests. Significant effects on RNA and protein expression on time and light condition were determined by using 2 x 2 factorial analysis of variance (ANOVA), with time (2 levels) and light condition (2 levels) as independent variables. Fisher's LSD was used as post hoc test and performed using Statistica. The alpha-level for significance was set to 0.05. Cohen's d is calculated to estimate effect size, where d of 0.2 indicates a small, 0.5 a medium and >0.8 a large effect size (Cohen 1992).

Results

RNA expression

Changes in RNA expression were measured using qPCR. All statistical results are given in supplementary tables S2A, S2B, and S2C.

Exposure to prolonged photoperiod significantly changed the RNA expression of clock genes *Per2* and *BMAL1* (figure 3). *Per2* expression was decreased at E7, compared to control ($t(15)=4.64$, $p<0.001$, $d=-1.69$). ANOVA revealed a time effect ($F(1,36)= 10.99$, $p<0.01$), where *Per2* expression increased from E7 to R3 (+51.8%, $p<0.01$, $d=1.63$). However, the *Per2* expression at R3 was still lower than control condition ($t(16)=2.40$, $p<0.05$, $d=-1.14$). There was no main effect or interaction effect with light. Blue-enriched light affected *Per2* expression similarly to white light with a reduced expression both at E7 ($t(15)=3.46$, $p<0.01$, $d=-2.29$) and at R3 ($t(15)=2.72$, $p<0.05$, $d=-1.35$) compared to control condition.

BMAL1 showed no significant change at E7 compared to control. No time effect of *BMAL1* expression was found, yet an increased expression compared to the control condition was observed at R3 ($t(16)=-4.58$, $p<0.001$, $d=2.21$). No significant light or interaction effects were observed for *BMAL1* expression. In contrast to white light, exposure to prolonged photoperiod in blue-enriched light increased *BMAL1* compared to control ($t(15)=-2.36$, $p<0.05$, $d=1.17$), while no significant change was observed at R3.

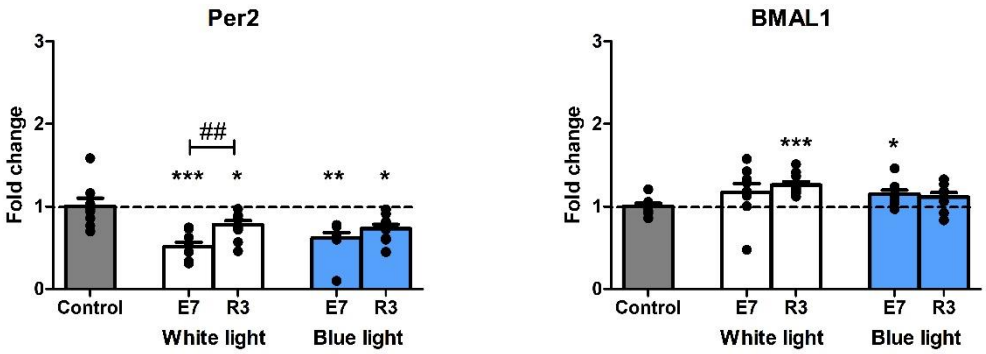

Figure 3. RNA expression of clock genes in PFC. Values are normalized to RPLP0 and expressed as fold change from 12:12 LD control. Mean fold change + SEM and individual values are shown. *** $p<0.001$, ** $p<0.01$, *$p<0.05$ compared to 12:12 LD control. ##$p<0.01$ post hoc analysis of time effect. N= 8-10 /group.

For synaptic plasticity associated genes, the experimental light conditions induced changes in expression of *Arc, BDNF,* and *NPAS4* (figure 4). *Arc* expression was reduced compared to control at E7 ($t(15)=3.74$, $p<0.01$, $d=-2.03$). In addition, *Arc* exhibited a time effect ($F(1,38)=10.45$, $p<0.01$), showing an increase from E7 to R3 (+214.3%, $p<0.01$, $d=1.45$). The observed decrease in *Arc* at E7 was normalized at R3 ($t(16)=0.14$, $p=0.89$, $d=0.07$). No light or light-time interactions effects were found. Blue-enriched light had similar effects as white light on expression of *Arc*, showing a decrease at E7 compared to control ($t(16)=4.06$, $p<0.001$, $d=-2.10$), which was largely normalized at R3 ($t(16)=1.43$, $p=0.17$, $d=-0.68$).

Expression of *BDNF* was not significantly affected compared to control at E7. *BDNF* expression also showed a time effect ($F(1,35)=5.47$, $p<0.05$), while the post hoc revealed no significant change in white light. No significant changes in *BDNF* were found at R3. Furthermore, the ANOVA revealed no significant light or interaction effects. The found time effect showed in a significant increase from E7 to R3 in the blue-enriched light condition (+42.3%, $p<0.05$, $d=1.28$). Moreover, recovery from the prolonged blue-enriched light increased expression of *BDNF* ($t(14)=-3.79$, $p<0.01$, $d=1.90$).

No significant change compared to control was found for *NPAS4* at E7. Similar to *Arc*, *NPAS4* also showed a significant time effect ($F(1,36)= 9.39$, $p<0.01$), increasing from E7 to R3 (+89.7%, $p<0.05$, $d=1.07$). Recovery from prolonged light exposure did not alter *NPAS4* expression compared to control. In contrast, prolonged exposure in blue-enriched light significantly decreased the expression of *NPAS4* at E7 ($t(15)=3.53$, $p<0.01$, $d=-1.82$), which was normalized at R3 ($t(15)=0.1$, $p=0.92$, $d=-0.05$). The increase from E7 to R3 was also significant in blue-enriched light (+125.6%, $p<0.05$, $d= 1.18$).

Expression of *Clock, Dclk1, Dcx1, Klf10, Nptx2,* and *Nrn1* was not significantly changed from control (S2A), or exhibited any main ANOVA effects of light and time or interactions, except for a light-time interaction effect in *Nptx2* ($F(1,38)=6.66$, $p<0.05$; see supplementary table and 2B).

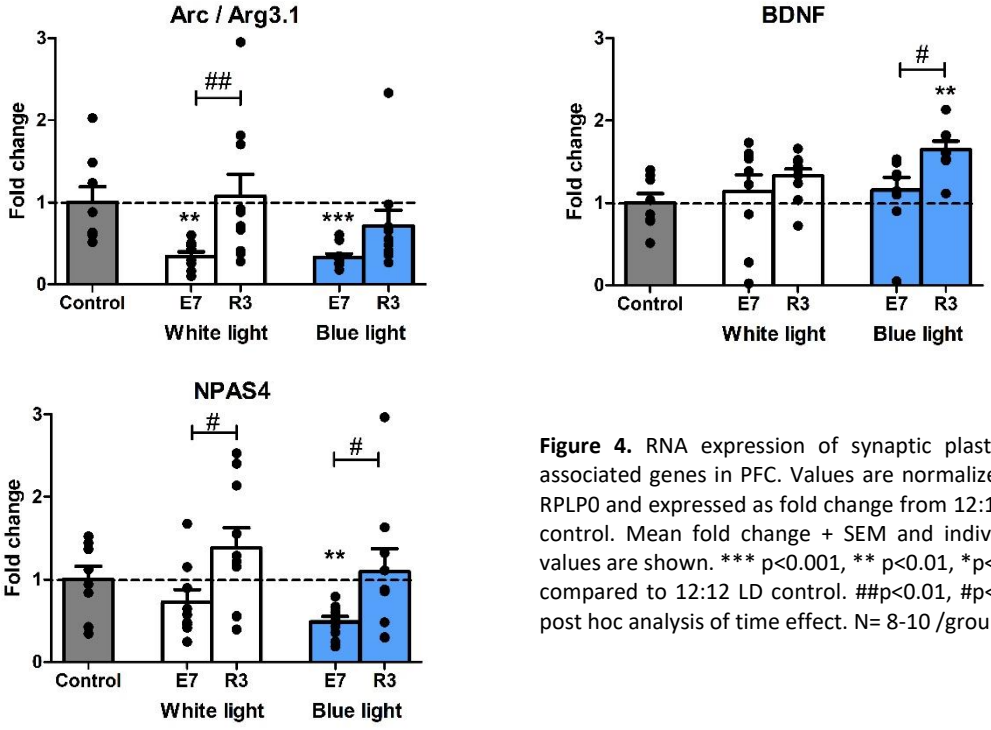

Figure 4. RNA expression of synaptic plasticity-associated genes in PFC. Values are normalized to RPLP0 and expressed as fold change from 12:12 LD control. Mean fold change + SEM and individual values are shown. *** p<0.001, ** p<0.01, *p<0.05 compared to 12:12 LD control. ##p<0.01, #p<0.05 post hoc analysis of time effect. N= 8-10 /group.

m^7 GTP (Cap) pull-down and protein expression

Expression of actively cap-bound proteins, as well as the total concentration of proteins, was determined from PFC lysates. Representative blots for m^7GTP (Cap) pull-down and western blot analysis are shown in supplementary information 3. Statistical outcomes are shown in tables S4A, S4B, S4C, S5A, S5B, and S5C. Graphs showing the total concentration of the cap-binding proteins can be found in supplementary information 6.

Protein expression of clock genes CLOCK and Cry2 was not significantly changed due to exposure to prolonged photoperiod (figure 5) (t's(15-18)<0.55, p's>0.58, *d's*<0.28). ANOVA revealed no significant time effects (F's(1,33-36)<3.47, p's>0.07). Furthermore, no significant changes at R3 were observed (t's(15-17)<1.23, p's>0.24, *d's*<0.66). Moreover, no significant light, interaction effects were found (F's(1,33-36)<3.59, p's>0.07). In addition, the blue-enriched light condition did not impact expression of clock genes CLOCK and Cry2 differently compared to control (t's(14-17)<1.57, p's>0.13, *d's*<0.74).

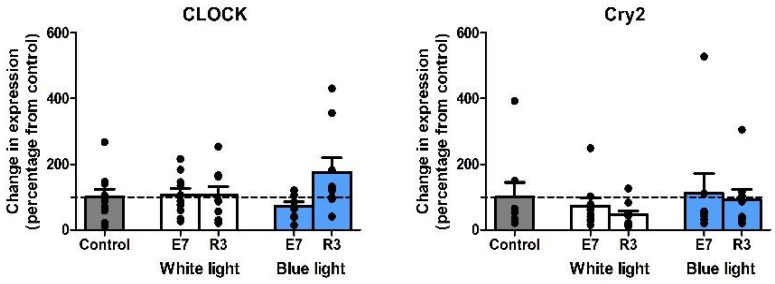

Figure 5. Photoperiodic responses in expression of clock genes analyzed by western blot of PFC lysates. Tissue was collected at lights off (ZT12) Values are expressed as percentage change relative to undisturbed controls (normalized to 100%). Graphs show mean + SEM as well as individual values. Significant differences compared to the control group: *p<0.05; **p<0.01; ***p<0.001. N=8-11/group

Of the proteins that are involved in promoting protein translation (cap-bound eIF4G, p-eIF4E, p-BMAL1, tot-BMAL1 and total protein concentration of p-S6K1), only eIF4G expression was significantly increased compared to controls after exposure to prolonged photoperiod (figure 6A and 6B, t(15)=-2.46, p<0.05, *d*=1.26, while other promoters t's(15-16)<1.54, p's>0.14, *d's*<0.73). No time effects or significant changes compared to control were found at R3 in the promoters of protein translation (F's(1,33-36)<3.15, p's>0.09 and t's(14-15)<1.04, p's>0.31, *d's*<0.52). However, eIF4G showed a light effect (F(1,35)=11.49, p<0.01), revealing a higher expression at white light compared to blue light at E7 only (+126.1%, p<0.001, *d*=1.38). Furthermore, p-BMAL1 showed a light x time interaction effect (F'(1,34)=4.31, p<0.05), yet a non-significant large effect found in post hoc analysis (p>0.26; *d*=1.37). Blue light had no further impact on the expression of individual promoters of translation compared to control (t's(13-15)<1.45, p's>0.17, *d's*<0.76). Even though results are not significant, high effect sizes indicate that photoperiod could affect promoters of protein translation (S4C and S5C). Specifically, there seems a global pattern where the expression of promoters is slightly lower in blue-enriched light compared to white light.

No significant changes from control were found in cap-bound repressors of translation initiation CYFIP1, FMRP, and eIF4E-BP2 at E7 (figure 6D, t's(9-18)<0.60, p's>0.55, *d's*<0.30). ANOVA also did not reveal any time effects of the repressors of protein translation (F's(1,21-40)<0.68, p's>0.41). At R3, no significant changes were found in CYFIP1, FMRP and eIF4E-BP2 (t's(10-18)<0.45, p's>0.65, *d's*<0.24). No significant effect of light or time-light interaction was found in the individual repressors (F's(1,21-40)<1.45, p's>0.23). Blue light did not change expression at E7 and R3 compared

to control (t's(9-18)<1.29, p's>0.21, *d's*<0.63). Similar to promoters of protein translators, medium effect size in the expression of some repressors indicate that photoperiod could modulate protein translation by affecting repressors of proteins translation (S4C). In addition, all repressors appear to show a trend where they are lower expressed in white light compared to blue-enriched light.

Prolonged light exposure did not significantly change expression of synaptic plasticity regulator Arc at E7 compared to control (figure 6C; t(18)=0.62, p=0.54, *d*=0.29). Moreover, no significant time effect was found for Arc (F(1,38)=3.53, p=0.07). Additionally, at R3, there were no significant changes in Arc (t(18)=1.59, p=0.13, *d*=0.76). No light or interaction effect or impact of blue light compared to control was found in Arc (F's(1,38)=1.82, p's=0.19 and t's<2.08, p's>0.05, *d's*<1.13). Although effects on Arc are not significant, an almost significant time effect in combination with high effect sizes suggest that Arc is increased especially in recovery (>+100.4%, *d*'s>0.76).

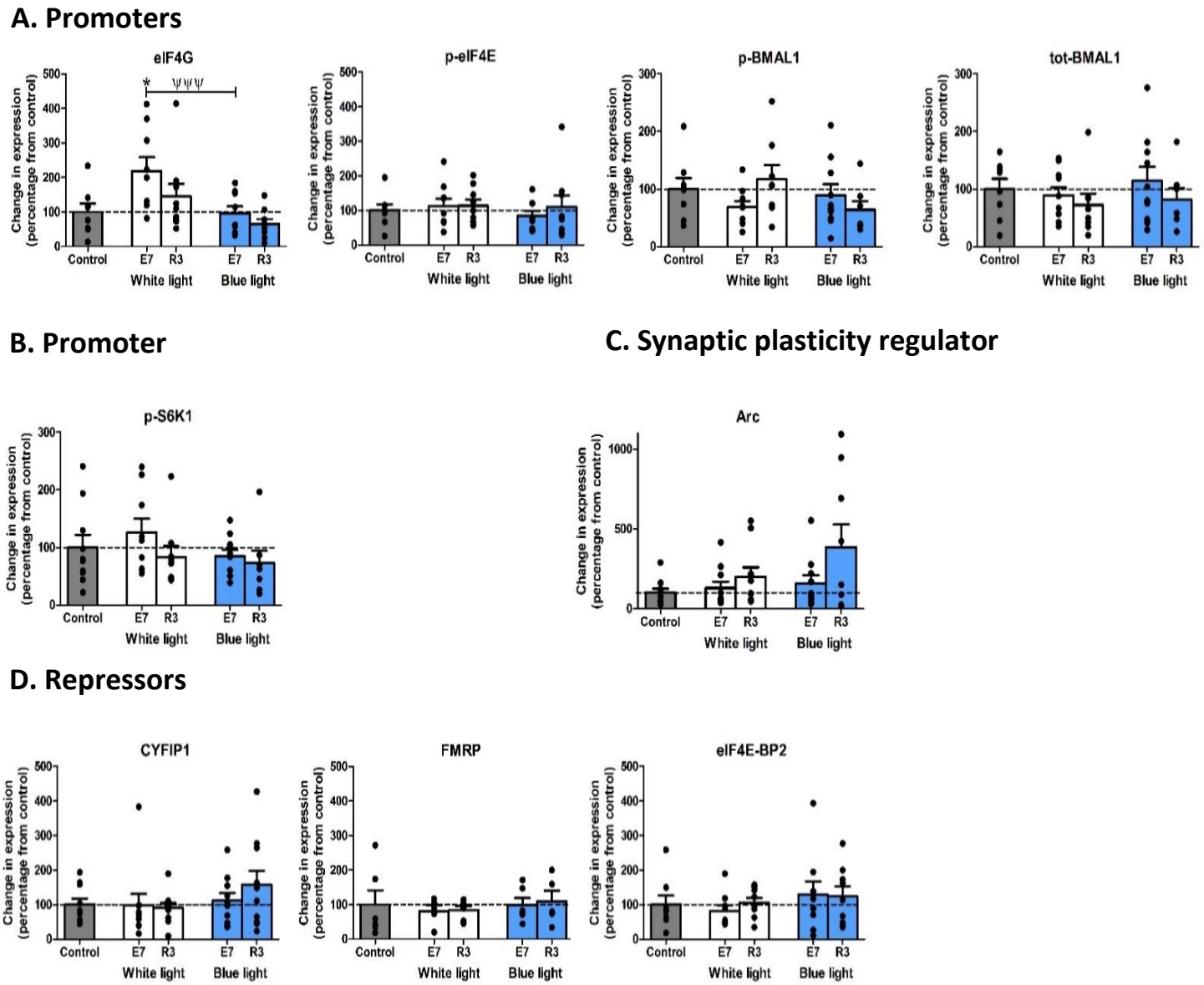

Figure 6. Photoperiodic responses in expression of promoters (A,B) and repressors (D) of translation initiation, and synaptic plasticity regulators (C). A,D). m^7GTP pull-down analysis of PFC lysates. B,C) Western blot analysis of PFC lysates. Tissue was collected at lights off (ZT12) Values are expressed as percentage change relative to undisturbed controls (normalized to 100%). Graphs show mean + SEM as well as individual values. Significance compared to control group: *p<0.05; ΨΨΨp<0.001 post hoc analysis of light effect. N=5-11/group. Note: scale differences on y-axis.

Western blot analysis of cap-binding proteins showed no significant changes, except for CYFIP1 (figure S6 and tables S5A and S5B). CYFIP1 was decreased in R3 in white light ($t(16)=2.19$, $p<0.05$, $d=-1.06$) In addition, CYFIP1 showed a time effect ($F(1,38)=4.80$, $p<0.05$), which showed a significant decrease from E7 to R3 in the blue-enriched light condition only (-27.2%, $p<0.05$, $d=-0.87$). Similar to the white light condition, CYFIP1 was decreased at R3 in the blue light condition ($t(17)=2.54$, $p<0.05$, $d=-1.20$). This indicates that, except for CYFIP1, availability for the cap-binding proteins to bind to the cap was not changed due to the experimental procedure.

Discussion

This project was aimed at investigating the effects of exposure to a prolonged photoperiod (20:4 LD) on the expression of clock genes, synaptic plasticity-associated genes and markers of protein translation in the prefrontal cortex of the rat. In addition, recovery from prolonged photoperiod was studied by changing the photoperiod from 20:4 LD to 12:12 LD. Lastly, the impact of blue light was examined by comparing the effects of exposure to and recovery from prolonged polychromatic white light and blue-enriched light. It is known that changing light exposure can have profound effects on circadian rhythmicity and PFC-dependent behavior, yet molecular changes underlying photoperiodic responses have not been investigated in the prefrontal cortex. Furthermore, ipRGC-mediated processes are known to be most sensitive to blue light, though photoperiodic responses in blue-enriched light have not been studied in the prefrontal cortex.

In summary, we found that exposure to prolonged photoperiod decreased RNA expression of *Per2* and *Arc.* A change from 20:4 LD to 12:12 LD, increased expression of *Per2, Arc,* and *NPAS4*. Expression of *Arc* and *NPAS4* was normalized at R3, while *Per2* was partially normalized. As for the impact of blue light, protein translation markers showed a global blue light-specific pattern. Moreover, *BDNF* was increased in recovery and showed an increase from E7 to R3 in the blue light conditions. Nevertheless, changes in photoperiod appeared to have stronger effects than light spectrum, especially since changes in expression largely followed the same pattern in both light conditions. These results suggest that (changes in) photoperiod have stronger effects than the spectral quality of light on the expression of clock genes and synaptic plasticity genes in the prefrontal cortex of the rat. In addition, the found changes in clock genes, synaptic plasticity markers, and protein translation markers may provide a possible mechanism for the light-induced effects on PFC-dependent behavior. Results on the expression of clock genes, synaptic plasticity markers, and protein translation initiators will be discussed subsequently in more detail.

Clock genes

The different experimental light conditions caused alterations in clock gene expression, which were only observed in changes in RNA expression. In line with the hypothesis, the important clock gene *Per2* was strongly decreased in the prefrontal cortex at ZT12 after 7 days of exposure to prolonged photoperiod. This decrease in expression could be due to the combined result of photoperiod-induced changes in circadian rhythmicity and sleep-wake dynamics. Evans et al. (2015) examined photoperiod-induced changes in circadian rhythmicity by examining *Per2* expression in non-SCN brain areas in mice exposed to 20:4 LD. Exposure to 20:4 LD caused an overall decrease in *Per2* expression and the peak of *Per2* expression stayed exclusively in the dark phase. In 20:4 LD, the output of the SCN shell, which coordinates circadian rhythmicity (e.g. *Per2* expression) in the brain, is reduced (Evans et al. 2015). Therefore, the observed reduced expression of *Per2* at E7 may be due to a prolonged photoperiod-induced attenuation in SCN shell output and the fact that the *Per2* peak is locked to the dark phase (which shifts in our protocol from ZT12-ZT24 to ZT20-ZT24 in E7). However, *Per2* expression was not determined at multiple time points, so it is not clear if the measured change was due to an overall decrease of *Per2* expression or a shift (related to the dark phase) in *Per2* rhythmicity.

In addition, *Per2* expression is related to sleep-wakefulness (low during rest phase and high during active phase) (Cirelli, Gutierrez, and Tononi 2004). Prolonged photoperiod-induced changes in the sleep-wake cycle could also contribute to the changes in *Per2*. Light has a sleep-inducing effect through ipRGC signaling in nocturnal rodents (Lupi et al. 2008). Indeed, in a separate experiment using the same prolonged light exposure protocol, an increase in total sleep time was observed during the

prolonged light exposure (unpublished results). This indicates that the rest phase of the animals might have been extended in the prolonged light exposure, which may affect the expression of *Per2* at E7. Even though the exact time point of sacrifice was the same in all groups (ZT12), the relative time point of sampling changed in the prolonged light exposure groups. In 20:4 LD, ZT12 falls in the middle of the rat's rest phase compared to the end of the rest phase for a 12:12 LD in the control and recovery groups. Measuring in the middle of the rest phase is associated with a lower expression of *Per2* (Cirelli, Gutierrez, and Tononi 2004). Thus, the rest phase-induced decrease of *Per2* could also have contributed to the observed decrease in *Per2* at E7.

Looking at the expression of clock genes in recovery, *Per2* increased from E7 to R3, yet does not completely normalize to control levels, matching our hypothesized recovery pattern. It seems that *Per2* expression indeed follows the 7-day lasting resynchronization process by the SCN (Evans et al. 2013). This timeline of *Per2* expression in the PFC fits with the fact that other circadian rhythmicity processes also take approximately 7 days to resynchronize (e.g. body temperature). Body temperature recordings in a separate set of animals undergoing the same experimental protocol as the present study showed disrupted circadian rhythmicity of body temperature upon return to 12:12 LD from 20:4 LD (unpublished results). This resynchronization of circadian body temperature took up to 7 days, which fits the timeline of resynchronization in the SCN (unpublished results, Evans et al. 2013). Thus, both the expression of *Per2* and circadian rhythmicity of body temperature seem to follow resynchronization signals from the SCN in the recovery phase. Against the hypothesis, blue-enriched light did not amplify the effects on clock gene expression since expression patterns in the blue light condition largely followed the same patterns as in the white light condition. However, the fact that the time-dependent increase in *Per2* from E7 to R3 was only significant in white light and the small spectral-specific changes in *BMAL1* expression indicates that blue light may differentially affect the expression of clock genes. To fully understand the dynamic changes in the whole transcriptional-translational feedback loop of the molecular clock, time-of-day variations in RNA and protein expression in all clock genes need to be determined (Ko and Takahashi 2006). Nevertheless, the changes in *Per2* expression observed at E7 and R3 indicates photoperiodic-induced alterations in circadian rhythmicity in the prefrontal cortex. These changes in *Per2* reflect a more rest phase character during E7 and a partial (SCN-coordinated) return towards control at R3. The observed changes in circadian rhythmicity could also impact expression in synaptic plasticity markers.

Synaptic plasticity

Photoperiodic responses were found in RNA expression of synaptic plasticity markers. Photoperiodic-induced alterations in circadian rhythmicity (especially sleep-wake dynamics) may underlie these changes in synaptic plasticity markers. Especially since the measured plasticity marker *Arc* is known to show variability in expression of mRNA throughout the day, whereas expression is higher during the active phase and lower during the rest phase (when relatively few external stimuli are processed) (Cirelli and Tononi 2000; Cirelli, Gutierrez, and Tononi 2004; Grønli, Soulé, and Bramham 2014). The observed reduction of *Arc* compared to control at E7 at ZT12 matched the hypothesis and is in line with the literature linking low expression to increase in sleep time during the prolonged photoperiod and sampling in the middle of the rest phase. In the recovery, *Arc* expression changed in line with the hypothesis: increasing from E7 to R3, thereby returning to control levels. In contrast to *Per2, Arc* completely normalized in recovery indicating a more acute effect in the PFC after a switch from 20:4 LD to 12:12 LD. These acute effects could possibly be due to a rapid adaptation in sleep and wakefulness rather than an adaptation in circadian rhythmicity, or another role in plasticity processes

in the PFC in recovery from prolonged photoperiod, but more research is needed to elucidate this. Blue light had no specific impact on the expression of *Arc* in the exposure and recovery phase, indicating no additional (ipRGC-mediated) effects of blue light. In line with the fact that the translation of Arc does not necessarily follow changes in transcription, no significant changes in protein levels were detected (Grønli et al. 2012). In fact, the found high effect sizes even suggest that Arc protein expression may be increased during recovery of white and blue-enriched light, indicating that Arc may be involved in regulating plasticity processes after the transition from a prolonged to a normal photoperiod. Thus, the observed decrease in *Arc* RNA expression at E7 and normalization at R3 could be explained by the alterations in the sleep-wake pattern, while the protein expression of Arc may point to a role in the recovery phase.

Plasticity marker *BDNF* shows similar daily variation in mRNA expression as *Arc* (Cirelli and Tononi 2000; Cirelli, Gutierrez, and Tononi 2004). Nevertheless, against the hypothesis *BDNF* did not show a reduction compared to control at E7, possibly indicating involvement of BDNF in photoperiodic plasticity independent of the sleep-wake expression patterns. However, there was an overall increase from E7 to R3 in the blue-enriched light condition, which may reflect the relative difference in rest phase of E7 (middle of rest phase) compared to R3 (end of rest phase) at ZT12. Blue light seems to have a specific impact on *BDNF* expression. The increase from E7 to R3 in *BDNF* was only significant in blue-enriched light condition and *BDNF* was increased compared to control at R3 in the blue-enriched light condition. This may indicate that *BDNF* maybe has additional plasticity functions in the PFC after exposure to and recovery from prolonged photoperiod in blue-enriched light. In the previously mentioned experiment using the same prolonged light exposure protocol, also an increase in EEG-measured beta activity was found in the prolonged blue-enriched light condition, which persisted throughout the recovery (unpublished data). Beta activity is a measure of alertness, a process which often involves activation of the PFC (Jap et al. 2009; Kane and Engle 2002). The found increase in *BDNF* expression at R3 in the blue-enriched light condition may contribute to the long-term plastic changes in the PFC, which could underlie the persistent increase in beta activity. Altogether, the spectral-specific changes in *BDNF* expression might be necessary to facilitate long-term adaptation in recovery from prolonged exposure to blue-enriched light.

NPAS4 showed, against the hypothesis, no significant reduction due to prolonged photoperiod. However, similar to *Arc*, there was a decreased expression which had a large effect size. Furthermore, the observed increase from E7 to R3 may reflect the relative difference between measuring in the middle and end of the rest phase, suggesting that *NPAS4* might follow the same sleep-wake pattern as *Arc* (Cirelli, Gutierrez, and Tononi 2004). Blue light seemed to affect *NPAS4* expression comparable to white light, yet the large decrease of *NPAS4* at E7 reached significance in the blue light condition. Similar to the white light condition, *NPAS4* increased from E7 to R3, normalizing to control levels. Combining results of white light and blue-enriched light, showed that *NPAS4* exhibits the same pattern as *Arc* and supports the fact that *NPAS4* might be affected by acute changes in sleep and wakefulness. A similar pattern of increase in the active phase and decreases in the rest phase would not be surprising on the basis of the functional similarity of *NPAS4* to *Arc* since both are involved in activity-dependent regulation of synaptic plasticity (Bloodgood et al. 2013; Bramham et al. 2010; Leal, Comprido, and Duarte 2014). Further analysis consisting of multiple time point sampling is needed to confirm time-of-day variations in *NPAS4.* Like *Arc* but in contrast with *Per2*, *NPAS4* levels returned to control levels in recovery. This may indicate differential expression dynamics during the recovery between genes involved in circadian rhythmicity and synaptic plasticity. It is possible that circadian rhythmicity follows the SCN, whereas synaptic plasticity markers follow the sleep-wakefulness rhythm, but this needs

further investigation. These differential expression dynamics of circadian rhythmicity and synaptic plasticity processes may be due to different (ipRGC) pathways, whereas some ipRGC projections go to the SCN while others directly impact non-SCN brain areas (Hattar et al. 2006; Chen, Badea, and Hattar 2011; Reifler et al. 2015). The difference in photoperiodic-responses between clock genes and synaptic plasticity genes may also be important for the treatment of mood disorders which are based on changing light conditions (e.g. light therapy or blue-blocking glasses). Both ipRGC pathways and clinical implications will be discussed below. Future studies need to examine the possible different photoperiodic responses on circadian rhythmicity and synaptic plasticity.

Protein translation regulators

Against the hypothesis, no clear changes were found in the protein expression of regulators of protein translation, which may indicate that neither prolonged photoperiod nor spectral quality has a big impact on the protein synthesis at the measured time point (ZT12). However, measuring at one time of the day may not be sufficient to fully capture the dynamic, circadian clock-mediated regulation of the initiation of protein translation (Lipton et al. 2015). A study by Marti and colleagues (2017) showed that there is a time-of-day variation of the phosphorylation of BMAL1 (translation promoting), which could be affected by circadian disruption by shift work. Nevertheless, a strong interaction effect on expression of cap-bound pBMAL1 and general patterns in promoters and repressors may indicate spectral-specific photoperiod responses on the regulation of protein translation. Even though no significant changes were found in the expression of individual promoters or repressors of translation initiation, high effect sizes support the hypothesis that there are (spectral-specific) photoperiodic responses on regulators of protein translation. However, there was not sufficient statistical power to detect them. But even combined effects of all promoters and repressors may cause significant alterations in the regulation of protein translation. To further study this hypothesis, it is needed to increase the sample size and also sampling at more time points. Moreover, the global spectral-specific pattern of decreased promotors and increased repressors in blue-enriched light compared to white light, and the role of ipRGCs in this effect, would be interesting to further investigate. These proposed studies would provide insight into the possible dynamic spectral-specific photoperiodic-induced changes in protein translation initiation, both during exposure to and recovery from 20:4 LD.

In the present study, only photoperiodic-induced changes in brain plasticity were examined on a RNA and protein level. However, to put this study further into context, there are more plasticity levels which can contribute to the brain's response to photoperiods such as epigenetic regulation and neurotransmitter switching.

First, epigenetic regulation is involved in modulating plasticity mechanisms which react to light. Epigenetic mechanisms consist of highly dynamic and reversible changes on the DNA without changing the nucleotide sequence. For example, DNA methylation or histone modifications change the accessibility of the chromatin, thereby influencing the possibility of gene transcription (Bird 2007; Strahl and Allis 2000). Contributing to the circadian clock plasticity mechanisms are the fact that in the SCN light is shown to modify chromatin, and light-induced histone modulation is important for clock gene expression (Crosio et al. 2000; Naruse et al. 2004). In addition, changes to a non-24h light dark-cycle caused changes in DNA methylation in the SCN which were reversible by returning to a 24h cycle, indicating a possible mechanism by which the SCN could regulate the transcriptional changes required for adaptation to a changing light-dark cycle (Azzi et al. 2014; Brancaccio et al. 2014). Therefore,

epigenetic mechanisms, especially DNA methylation, could impact the plasticity of the circadian and circannual system.

A second level of regulation of the light-induced plasticity mechanisms is the concept of neurotransmitter switching. For a long time, it was thought that neurons stably expressed one neurotransmitter. However, Dulcis and colleagues (2013) it has been shown that neurons can change their neurotransmitter phenotype in response to light. In response to short and long photoperiod, interneurons in the hypothalamus that control the release of corticotropin-releasing factor switched between neurotransmitters dopamine and somatostatin. By means of this neurotransmitter switching, stress-related behavior such as depressive-like behavior could be affected (Dulcis et al. 2013). Neurotransmitter switching in response to photoperiod may contribute to photoperiodic-induced changes in behavior.

In sum, the brain uses a wide variety of plasticity mechanisms to respond to light, acting on different molecular levels. Although present study only investigated this phenomenon on RNA and protein levels, it is likely that epigenetic mechanisms, neurotransmitter switching and the coupling of the SCN network are also involved. Future studies need to entangle the interactions within and between the various levels of plasticity mechanisms. It is important to distinguish between photoperiodic-induced effects on plasticity mechanisms that act through the circadian system (e.g. via the SCN-coordinated rhythmicity) and which may use more direct pathways (e.g. direct ipRGC projections).

The non-visual effects of light, including regulation of circadian rhythmicity, pupillary light response, sleep, mood, and cognition, are mainly modulated by projections of ipRGCs (Hattar et al. 2006; Legates, Fernandez, and Hattar 2014; Panda et al. 2003). ipRGC (subtypes) projections go to many different brain areas, which most likely reflect their different roles in the wide range of non-visual responses to light (Schmidt and Kofuji 2009; Zhao et al. 2014). Currently, 5 subtypes of ipRGCs are distinguished (M1-M5) based on and morphological and electrophysiological characteristics. Projections and functions of M1 ipRGCs are best characterized. M1 can be subdivided into M1 Brn3b+ (project to olivary pretectal nucleus (OPN), involved in pupillary light response) and M1 Brn3b- (project to suprachiasmatic nucleus (SCN), involved in circadian rhythmicity) (Chen, Badea, and Hattar 2011). Moreover, M4 and M5 ipRGCs have direct projections to thalamic areas involved in primitive forms of vision (Estevez et al. 2012; Stabio et al. 2018). Except for morphological and electrophysiological characterization, not much is known about M2 and M3 subtypes (Reifler et al. 2015). Light can elicit many non-visual responses by direct ipRGC projections or an indirect pathway most likely via ipRGC projections to the SCN. However, it is currently unknown how signals from ipRGCs impact the PFC. Our data suggests that circadian rhythmicity in the PFC may be regulated by SCN-coordinated rhythmicity, while other pathways cause acute changes in synaptic plasticity markers. However, further characterization of projections and functions of the different ipRGC subtypes is needed to untangle the complex retinal and neuronal basis of non-visual responses to light.

One of these non-visual effects which need further characterization is the effect of light on mood. Mood and mood disorders are strongly linked to light (McClung 2007; Lazzerini Ospri, Prusky, and Hattar 2017; Bedrosian and Nelson 2017). Light-induced changes in plasticity processes are most likely the basis of the non-visual effects on cognition and mood. Increasing the understanding of these processes is crucial in optimizing light intervention therapies for mood disorders (Cajochen et al. 2005; Legates, Fernandez, and Hattar 2014; Vandewalle, Maquet, and Dijk 2009). The current study shows that by altering the daily light exposure, synaptic plasticity markers can be changed. By increasing daily light exposure, the rest phase of the nocturnal rat was extended. Since synaptic plasticity markers are

decreasing during the rest phase, a longer rest phase may decrease overall daily expression of synaptic plasticity markers (Cirelli, Gutierrez, and Tononi 2004). Low plasticity markers are thought to be part of the etiology of mood disorders (Duman and Monteggia 2006). The fact that in humans short photoperiods (short active phase) are associated with Seasonal Affective Disorder (SAD), depressive episodes in bipolar mania and light exposure therapy improves depressive symptoms may, therefore, be linked to the light-induced changes in expression of synaptic plasticity markers (McClung 2007; Wang and Chen 2013). Likewise, alteration of plasticity processes may underlie the acute improvement of mood in patients with depression after a night of sleep deprivation (artificially extending the active phase and light exposure), since sleep deprivation is also known to increase synaptic plasticity markers (Grønli, Soulé, and Bramham 2014; Wirz-Justice and Van den Hoofdakker 1999). The acute character of the mood improvement after sleep deprivation suggests that these effects are most likely not via the (relatively slow) SCN-coordinated adaptations in circadian rhythmicity, but rather through faster acting pathways directly impacting plasticity mechanisms. It might be relevant for the treatment of mood disorders to look earlier in the recovery phase of the used experimental protocol to examine more acute effects of light-induced changes in plasticity mechanisms. Interestingly, blocking exposure to blue light, and thereby extending the night (rest phase), is effective as an add-on treatment for bipolar mania (Henriksen et al. 2016, 2014). Extending the rest phase would normally decrease overall plasticity markers, a phenomenon normally associated with worsening of mood disorders (Duman and Monteggia 2006). These mood stabilizing effects of blue-blocking glasses in bipolar patients may therefore be through changes in (SCN-coordinated) circadian rhythmicity or other plasticity mechanisms currently not understood. Nevertheless, it is interesting that the effects of light on mood may be dependent on spectral qualities (e.g. blocking blue light), although this might be mood disorder-specific (Hopkins et al. 2017; Ekström and Beaven 2014). For example, treatment of depression with light therapy did not show differences between white and blue-enriched light (Meesters et al. 2011; Gordijn, 'T Mannetje, and Meesters 2012). Elucidating (spectral-specific) photoperiodic responses on circadian rhythmicity and plasticity processes can be used to optimize light-based treatments in mood disorders, although these responses may differ between mood disorders.

Conclusion and Future Directions

Photoperiodic responses were found in RNA and (cap-bound) protein expression of clock genes, synaptic plasticity markers and protein translation initiators in the PFC. Changes in RNA expression of clock genes and synaptic plasticity genes were mostly influenced by photoperiod-induced changes in sleep-wakefulness. The changes in expression generally followed the same pattern in white and blue-enriched light, so it appears that photoperiodic responses on clock genes and synaptic plasticity markers are largely independent of the spectral quality of the light. Only *BDNF* expression was increased in recovery from blue-enriched light, indicating that BDNF may be specifically involved in adaptation to blue-enriched light. Photoperiodic responses on protein translation initiation regulators were not significant but had high effect sizes. In addition, a general spectral-specific pattern in the expression of promoters and repressors of protein translation was observed. However, further research is needed to characterize photoperiodic responses on protein translation regulators. In sum, the found photoperiod-induced changes in clock genes, synaptic plasticity markers, and protein translation markers may be a possible pathway for the light-induced effects of PFC-dependent behavior and mood.

To further investigate the photoperiodic responses in RNA and (cap-bound) protein expression, more time-of-day measurements are needed to map the possible dynamic changes in expression across the day, most importantly differentiating between active and rest phase. In addition, the recovery phase should be further studied to examine the effects of changes in photoperiod, while taking the different dynamics of circadian rhythmicity and plasticity processes into account. Furthermore, it would be interesting to determine if the photoperiodic-induced changes in clock genes and plasticity markers are affected through different (ipRGC) pathways and if synaptic plasticity markers contribute to the resynchronization in the PFC in the recovery of prolonged photoperiods. Moreover, it would be important to see if the photoperiodic-induced changes in the measured markers affect PFC-dependent behavior and whether this is affected by 1) length of prolonged photoperiod, 2) time-of-day of testing, 3) switches in photoperiod, and 4) spectral quality. The knowledge about light-induced effects on circadian rhythmicity and synaptic plasticity can be used to develop mood-specific light-based therapies.

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Supplementary information

1. Protocols

RNA isolation protocol RNeasy Lipid Tissue Mini Kit

RNeasy® Lipid Tissue Mini Kit

The RNeasy Lipid Tissue Mini Kit (cat. no. 74804) can be stored at room temperature (15–25°C) for at least 9 months. QIAzol® Lysis Reagent can be stored at room temperature, or at 2–8°C.

For more information, further and more detailed protocols, and safety information, please refer to the *RNeasy Lipid Tissue Handbook*, which can be found at www.qiagen.com/handbooks.

For technical assistance, please call toll-free 00800-22-44-6000, or find regional phone numbers at www.qiagen.com/contact.

RNA purification using the RNeasy Lipid Tissue Mini Kit

Notes before starting

- Add 4 volumes of ethanol (96–100%) to Buffer RPE for a working solution.

1. Disrupt and homogenize ≤100 mg fatty tissue (≤50 mg other tissue) in 1 ml QIAzol Lysis Reagent using the TissueRuptor®, TissueLyser LT, or TissueLyser II (see Table 1).
2. Incubate the homogenate at room temperature (15–25°C) for 5 min.
3. Add 200 µl chloroform, and shake vigorously for 15 s.
4. Incubate sample at room temperature for 2–3 min.
5. Centrifuge at 12,000 x g for 15 min at 4°C.
6. Transfer upper, aqueous phase to a new tube. Be careful to avoid the interphase. Add 1 volume of 70% ethanol, and vortex. Do not centrifuge. Proceed at once to step 7.
7. Transfer up to 700 µl of the sample to RNeasy Mini spin column in 2 ml collection tube (supplied). Close the lid, centrifuge at room temperature for 15 s at ≥8000 x g, and discard flow-through.

January 2011

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Sample & Assay Technologies

8. Using the same collection tube, repeat step 7 using the remainder of the sample. Discard the flow-through.

   Optional DNase digest: Follow steps in "Optional on-column DNase digestion with the RNase-Free DNase Set" in Appendix C of the *RNeasy Lipid Tissue Handbook*.

9. Add 700 µl Buffer RW1 to RNeasy column. Close lid, centrifuge for 15 s at ≥8000 x *g*, and discard flow-through. (Skip this step if performing optional DNase digestion.)

10. Add 500 µl Buffer RPE to RNeasy column. Close lid, centrifuge for 15 s at ≥8000 x *g*, and discard flow-through.

11. Add 500 µl Buffer RPE to RNeasy column. Close lid and centrifuge for 2 min at ≥8000 x *g*.

    Optional: To further dry membrane, place RNeasy column in new 2 ml tube, close lid, and centrifuge at full speed for 1 min.)

12. Place RNeasy column in a new 1.5 ml tube. Add 30–50 µl RNase-free water, close lid, and centrifuge for 1 min at ≥8000 x *g*.

Table 1. Recommended methods for sample homogenization

Sample	Amount	Dish	Disruption and homogenization
Animal cells	<5 x 10^6	<6 cm	Add Buffer RLT, vortex (≤1 x 10^5 cells); or use QIAshredder, TissueRuptor, or needle and syringe
	≤1 x 10^7	6–10 cm	
Animal tissues	<20 mg	–	TissueLyser LT; TissueLyser II; TissueRuptor, or mortar and pestle followed by QIAshredder or needle and syringe
	≤30 mg	–	

DNase digestion protocol

Procedure

Prepare and load samples onto the RNeasy spin column as indicated in steps 1–10 of the protocol on page 14 or 19. Instead of performing step 11, follow steps C1–C4 below.

C1. Add ▲ 350 µl or ● 2 ml Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for ▲ 15 s at ≥8000 x *g* (≥10,000 rpm) or ● 5 min at 3000–5000 x *g* to wash the membrane. Discard the flow-through.*

Reuse the collection tube in step C4.

C2. Add ▲ 10 µl or ● 20 µl DNase I stock solution (see above) to ▲ 70 µl or ● 140 µl Buffer RDD. Mix by gently inverting the tube, and centrifuge briefly to collect residual liquid from the sides of the tube.

Buffer RDD is supplied with the RNase-Free DNase Set.

Note: DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube. Do not vortex.

C3. Add the DNase I incubation mix (▲ 80 µl or ● 160 µl) directly to the RNeasy spin column membrane, and place on the benchtop (20–30°C) for 15 min.

Note: Be sure to add the DNase I incubation mix directly to the RNeasy spin column membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the spin column.

C4. Add ▲ 350 µl or ● 2 ml Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for ▲ 15 s at ≥8000 x *g* (≥10,000 rpm) or ● 5 min at 3000–5000 x *g*. Discard the flow-through.* Continue with step 12 of the protocol on page 14 or 19.

* Flow-through contains Buffer RW1 and is therefore not compatible with bleach. See page 6 for safety information.

Internal mRNA isolation protocol

February, 2018

Written by Andrea Rørvik Marti and Jorrit Waslander

1. Preparations
    - Cool down centrifuge to 4 °C (all centrifuge steps are done at 4 °C)
    - Make sure the flow cabinet is cleaned with RNaseZAP
2. Move tissue into 2 mL hard tubes
    - Add 1 **metal ball**
    - Add 1 mL **QIAzol lysis reagent** (brown bottle, green cap)
3. Bring samples and frozen homogenizing plates to the 7th floor
    - Place in frozen homogenizing plates – make sure to balance the distribution of samples on plates and check if the plates are properly fixated in the machine
    - Set the TissueRuptor machine to 300 rpm, 2 minutes and press start
    - If the tissue is not properly homogenized, repeat previous steps of point 3
4. Bring samples back to 9th floor and incubate for 5 minutes
    - Don't forget to RNAseZap/clean your gloves (every time)
5. Add 200 µl **chloroform** to each tube and vortex for 15 sec
6. Incubate (wait) for 2-3 minutes at room temperature
7. Centrifuge samples 12000 g for 15 minutes
    - Write new tubes (1.5 ml) for step 9
    - Prepare DNase mix for step 14
        - Per sample: add 10 µl DNase stock solution to 70 µl Buffer RDD and mix gently by inverting the tube (do no vortex, make ~10% extra for safety)
    - If taken a coffee break, bring timer to keep track of time
8. Take all samples carefully out of the centrifuge
9. Pipet aqueous phase of sample into a new tube (~550 µl) – it is more important get **only** aqueous phase than to get **all** aqueous substance
10. Add 1 volume (~550 µl) **70% ethanol** to each tube and vortex
11. Label the RNeasy Mini spin columns with 2ml collection tube
12. Transfer 700 µl **of each sample** on RNeasy Mini spin column and centrifuge 15 sec at 8000 g
    - Repeat step 12 with remainder of the sample (note: balancing of the centrifuge)
13. Add 350 µl **Buffer RW1** to each column and centrifuge 15 sec at 8000 g
    - Throw away fluid in collection tube and put column back in for next step
14. Add 80 µl of the **DNase mix** to each sample (to make sure the fluid is on the membrane of the column, tick tube and column on table) and wait 15 minutes at room temperature
    - Write new tubes (1.5 ml) for step 19
    - If taken a coffee break, bring timer to keep track of time
15. Add 350 µl **Buffer RW1** to each column and centrifuge 15 sec at 8000 g
    - Throw away fluid in collection tube and put column back in for next step
16. Add 500 µl **Buffer RPE** to each column and centrifuge 15 sec at 8000 g
    - Throw away fluid in collection tube and put column back in for next step
17. Add 500 µl **Buffer RPE** to each column and centrifuge 2 min at 8000 g
    - Throw away fluid in collection tube
18. Put column in new collection tube and centrifuge 1 min at full speed
19. Put column in new (1.5 ml) tube

20. Add 30-50 µl **RNase-free water** to column and centrifuge for 1 min at 8000 g
    - Depending on the expected concentration of RNA, repeat step 20
21. Measure concentration of RNA with Nanodrop on 7th floor
    - Bring samples and USB-stick to 7th floor
    - Open computer programme Nanodrop, select Nucleic acid, set to RNA (right top)
    - Blank with 1 µl **RNase-free water**
    - Wipe Nanodrop machine with paper
    - Measure 1 µl **sample**
    - Wipe Nanodrop machine with paper
    - Make sure you measure each sample twice (measuring in duplo)
    - Save file as 'yyyymmdd tissue' and copy table of results to excel sheet on USB-stick
22. Store labeled samples at -80 °C

Internal protein and cap pull down protocol

Homogenizing samples

- Do everything on ice
- Make lysis buffer (RIPA, add 100 ul protease inhibitor and 100 ul Na_3VO_4 per 10ml)
    - You need approximately 1ml/sample for the whole homogenization
- Put some buffer in bouncer with the tissue
- Put the rest of the buffer in
- Push plunger in carefully, twist and crush
- 10-12 pushes
- Avoid bubbles
- Move homogenized tissue back in tube
- Wash bouncer between each tissue, with water, alcohol and water
- Centrifuge on 4°c, 10,000 rpm, 10 minutes
- Dispose of debris, move liquid over to new labelled tube with glass Pasteur pipet

Protein determination

- Make standards (dilute in lysis buffer)
    - Protein (diluted albumin BSA, 2mg/mmL)
    - Diluted to: 2000ug/ml, 1000ug/ml, 500ug/ml, 250ug/ml, 125ug/ml, 62.5ug/ml.
    - Also a blank standard (lysis buffer)
- Make working reagent
    - 50 parts BCA reagent A + 1 part BCA reagent B
- Wells
    - 190ul working reagent
    - 10ul standard, blank or sample

	1	2	3	4	5	6	7
A	Blank	Standard1	Standard2	Standard3	Standard4	Standard5	Standard6
B	X	Standard1	Standard2	Standard3	Standard4	Standard5	Standard6
C	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7
D	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7
E	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7

- Etc…
- Leave in 37° for 30 minutes

Preparing samples for Cap Pull Down

1. Do everything on ice
2. Make lysis buffer if needed
3. Prepare Lysis buffer (see 'Homogenizing samples')
    a. You need 1,5 ml per sample for step 8 and 12.
4. For each sample use 30 ul Immobilized γ-Aminohexyl-m7GTP beads (from Jena bioscience)! Also include a bead control, which is treated the same.

    Remember to cut off the end of the pipette tip with scissors, to ensure beads do not get stuck!

    Take ~50ul per sample from beads stock because they are stored in some fluid. Spin down at 2000 rmp for 1 min to check if you have enough beads
5. Wash the total bead mix and control (x3): in 500ul PBS. Flick and Spin down at 2000 rpm for 1 min (beads are delicate so too fast and they will get damaged).
6. Equally distribute the beads over the tubes. Spin down 1 min at 2000 rpm to check. If not then collect all the beads and re-distribute. E.g. for 10 samples: 200 - 300ul of beads and add lysis buffer to make total volume 1000ul and then distribute 100ul of solution in each tube with mixing every time. – distribute in 1.5ml tubes. Remember to cut the tip.
7. To the bead mixture add protein lysate: 250ug of protein should be okay; 300ug is good; 400ug to be safe (i.e. if you are looking at weakly binding proteins).

    To the bead control, add a little protein lysate from all samples
8. Add lysis buffer and make up to 500ul.
9. Put on rotating mixer and leave at 4^{o}C for **1.5 - 4 hours**.
10. Turn heat block on (70 ^{o}C)
11. Spin beads down at 2000 rpm, 1 min before washing
12. Wash the total bead mix (x3): Add 400ul lysis buffer (or washing buffer) to each tube. Spin down at 2000 rpm, 1 min.
13. On the last wash aspirate as much of the lysis buffer as possible. If you are confident you won't remove any beads, use a manual pipette to get the last bit off so the beads are dry.
14. Add 20 ul of 2X sample buffer to each sample tube under the hood, pipet the sample buffer in the beads to mix. Change tips between samples.

15. Boil and shake at 70 °C and 300 rpm for 10min if it is agarose beads.

    Samples can be stored at -20°C or loaded onto a gel straight away.

Preparing samples for western blot

- Turn heat block on (100 °C).
- Make sample buffer if necessary
    - Use 2X sample buffer. To make 2X from 4X: mix 1ml H_2O with 1ml 4X and add 2ul DTT
- Add amount corresponding to 20mg of protein from each sample in a tube
    - Consult protein determination output for amount
    - Switch pipette tips between samples
- 3 ul 4X sample buffer in each tube under the hood
- Mix well and spin down (little centrifuge)
- Boil on shaker for 5 minutes at 100 °C and 300 rpm
- **Samples can be stored at -20°C or loaded onto a gel straight away.**

Loading samples and running gel

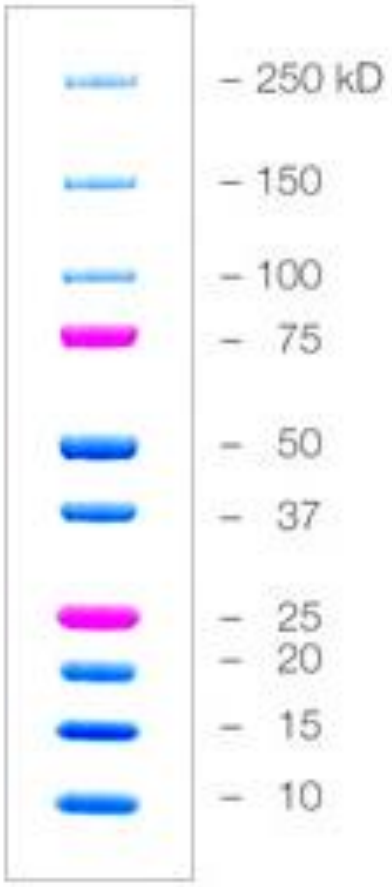
1. Take gel holder, Powerpac and Turbotransfer
2. Take gels from fridge and place in tank
3. Fill tank with 1X TGS
4. Spin the samples down (small centrifuge)
5. Load marker in correct wells. Load samples (all volume in CPD/WB sample, ~25 for WB and ~45 for CPD)
6. Put on lid. Red-red, black-black
7. Attach to PowerPac1000
8. Set voltage to 80 V
    a. Exit → manual → set V → 80 → running man
9. Wait 25 minutes (set timer)
    a. See that samples have lined up below stacking gel
10. Change voltage to 100V
    a. Hand → exit → manual → set V → 130 → running man
11. Wait for samples to run all the way through (the blue has to run off the gel)
    a. 1h-1h30, check after 45 min
12. Turn off, disconnect

Transfer

1. Take the transfer pack from the fridge
2. Take one of the cassettes from the Trans-Blot Turbo and open it
3. Place the top part of the transfer pack (including the membrane) on the drawer
    a. Use roller tool to remove air bubbles
4. Break the gel holder open with the green plastic tool
5. Take the gel and place on the membrane
    a. Use roller tool to avoid air bubbles
6. Cover the gel with the remaining part of the transfer pack
    a. Use roller tool to avoid air bubbles
7. Close the drawer and place back in Trans-Blot Turbo
8. Select programme (30 min)
    a. List → Bio-Rad → Midi gel → Run → Select right drawer

After transfer

- Mix 5% dry non-fat milk
    - 2.5g dry milk powder with 50ml TBST (use large tube or bottle and mix well)
    - Put membrane in a box with 5% dry non-fat milk (cut the membrane on a plastic board to fit in the box)
    - Put on shaker for 1h (room temperature)
    - Rinse briefly in TBST after (till all milk is gone)
- Cut membrane according to molecular weight (see ladder)
- Place in individual containers
    - Label container with your name, protein and type of secondary antibody needed
- Pour on primary antibody
    - To make new antibody in 5% BSA
        - Add 5 ml 10% BSA
        - Add 5 ml TBST
        - Add 20ul antibody (for 1:500) or 10ul antibody (for 1:1000)
- Place in cold room on shaker overnight
- Primary antibody containers stored in freezer



Protein	Molecular Weight (kD)
4G	220
CYFIP	140
PER2	136
CLOCK	100
BMAL1	75 - 86
S6K1	70
CRY2	68
FMRP	60 – 72
Melanopsin	54
ARC	50
MNK1	50
GAPDH	37
BDNF	30
4E	25
BP2	15 - 20

Next day

- Pour primary antibodies back in tubes in freezer for reuse
    - Mark one use on antibody tube
- Wash in 1x TBST 3x10 mins (TBST can go in the sink)
    - During the 3rd wash, prepare secondary antibody
    - Secondary antibody diluted in 1x TBST 1:10,000 (e.g. 20ml TBST, 2ul antibody)
        - Vortex secondary antibody before adding it to TBST
        - Antibody was 1:1 diluted in glycerin for storage, so per 10 ml TBST 2 ul antibody
- Pour on secondary antibody, shake for 1h
    - Secondary antibody goes in the sink
- Wash in 1x TBST 3x10 mins (TBST can go in the sink)
- You can leave the box (including membrane & TBST) in room temperature after 3rd wash or immediately develop

Developing

- Mix detection reagent 1&2, 1:1 to make ECL
- Lift membrane, drain off TBST by gently touching a piece of paper with the membrane
- Place membranes correctly ordered on plastic plate
- Pour on ECL, cover membrane fully
- Place plate inside molecular imager (Gel-Doc)
- Program: ImageLab
    - Protocol → new
    - Application → blots → Chemi Hi Resolution
    - Gel type → BioRad MiniProtean
    - Setup → 1st image 30, last image 300, 10
    - Position membrane within screen
        - No filter
    - Run protocol
    - Label image (sample number, antibodies) – use annotation tools
    - Merge with bands
        - Protocol → select → colorimetric
        - Gel type → BioRad miniPROTEAN
        - Run protocol
        - Image tools → merge
    - Save image
        - Yyyymmdd [Experiment] [Tissue] [Antibodies visible] [WB/CPD]

Stripping

- Use ready-made stripping buffer
- Place all membranes in one box
- Add ~10ml stripping buffer (all membranes must be covered)
- Incubate in room temperature for 10 minutes on shaker
- Wash 3x 5 min with PBS
- Return to “after transfer” step

2. T-test and factorial ANOVA RNA expression analysis

	WE7			WR3			BE7			BR3		
PFC Gene	t	df	Sig. (2-tailed)	t	df	Sig. (2-tailed)	t	df	Sig. (2-tailed)	t	df	Sig. (2-tailed)
Arc	3,74	15	0,002**	0,14	16	0,890	4,06	16	0,001**	1,43	16	0,172
BDNF	-0,35	15	0,731	-1,96	16	0,068	-0,53	15	0,603	-3,79	14	0,002**
BMAL1	-1,4	15	0,169	-4,6	16	0,0003***	-2,4	15	0,032*	-1,7	15	0,119
Clock	-0,4	15	0,700	-0,9	16	0,393	0,39	16	0,702	-0,1	16	0,943
Dclk1	0,23	15	0,821	0,28	16	0,785	1,8	16	0,091	0,63	16	0,537
Dcx1	0,02	15	0,981	-0,5	16	0,646	-0,2	15	0,836	-0,4	15	0,674
KLF10	1,67	15	0,115	0,12	16	0,905	2	16	0,063	1,12	16	0,281
NPAS4	1,73	15	0,104	-0,8	16	0,438	3,53	15	0,003**	0,1	15	0,924
Nptx2	-0,9	15	0,370	0,2	16	0,844	0,7	16	0,493	-0,9	16	0,406
Nrn1	0,06	15	0,952	0,07	16	0,948	0,71	16	0,488	-0	16	0,972
PER2	4,64	15	0,0003***	2,4	16	0,029*	3,46	15	0,004**	2,72	15	0,016*

Supplementary Table 2A. T-test of RNA expression. *p < 0.05; **p < 0.01; ***p < 0.001

	Light			Time point			Light x Time point		
	F	df	p	F	df	p	F	df	p
Arc	1,164	1,38	0,288	10,447	1,38	0,003**	1,021	1,38	0,319
BDNF	1,342	1,35	0,255	5,465	1,35	0,026*	1,047	1,35	0,314
BMAL1	1,701	1,36	0,201	0,154	1,36	0,697	0,906	1,36	0,348
Clock	1,253	1,38	0,271	0,406	1,38	0,528	0,010	1,38	0,922
Dclk1	2,409	1,38	0,130	0,564	1,38	0,458	0,549	1,38	0,464
Dcx1	0,036	1,36	0,850	0,092	1,36	0,764	0,034	1,36	0,856
Klf10	1,093	1,38	0,303	2,054	1,38	0,161	0,183	1,38	0,672
NPAS4	1,647	1,36	0,208	9,389	1,36	0,004**	0,011	1,36	0,917
Nptx2	0,387	1,38	0,538	0,154	1,38	0,697	6,662	1,38	0,014*
Nrn1	0,248	1,38	0,621	0,445	1,38	0,509	0,439	1,38	0,512
Per2	0,258	1,36	0,615	10,989	1,36	0,002**	1,767	1,36	0,193

Supplementary Table 2B. Factorial ANOVA of RNA expression. *p < 0.05; **p < 0.01; ***p < 0.001

	WE7	**WR3**	**BE7**	**BR3**
	Cohen's d	Cohen's d	Cohen's d	Cohen's d
Arc	-2.027	-0.069	-2.141	-0.678
BDNF	0.181	0.921	0.265	1.900
BMAL1	0.806	2.211	1.172	0.830
Clock	0.190	0.416	-0.184	0.035
Dclk1	-0.112	-0.132	-0.848	-0.306
Dcx1	-0.012	0.226	0.113	0.213
KLF10	-0.813	-0.059	-0.948	-0.537
NPAS4	-0.839	0.395	-1.827	-0.049
Nptx2	0.448	-0.100	-0.331	0.402
Nrn1	-0.030	-0.031	-0.335	0.017
Per2	-2.292	-1.144	-1.685	-1.348

Supplementary Table 4C. Cohen's d compared to control of RNA expression. 0.2 indicates a small, 0.5 a medium and >0.8 a large effect size.

3. Representative blots of western blot and cap pull down

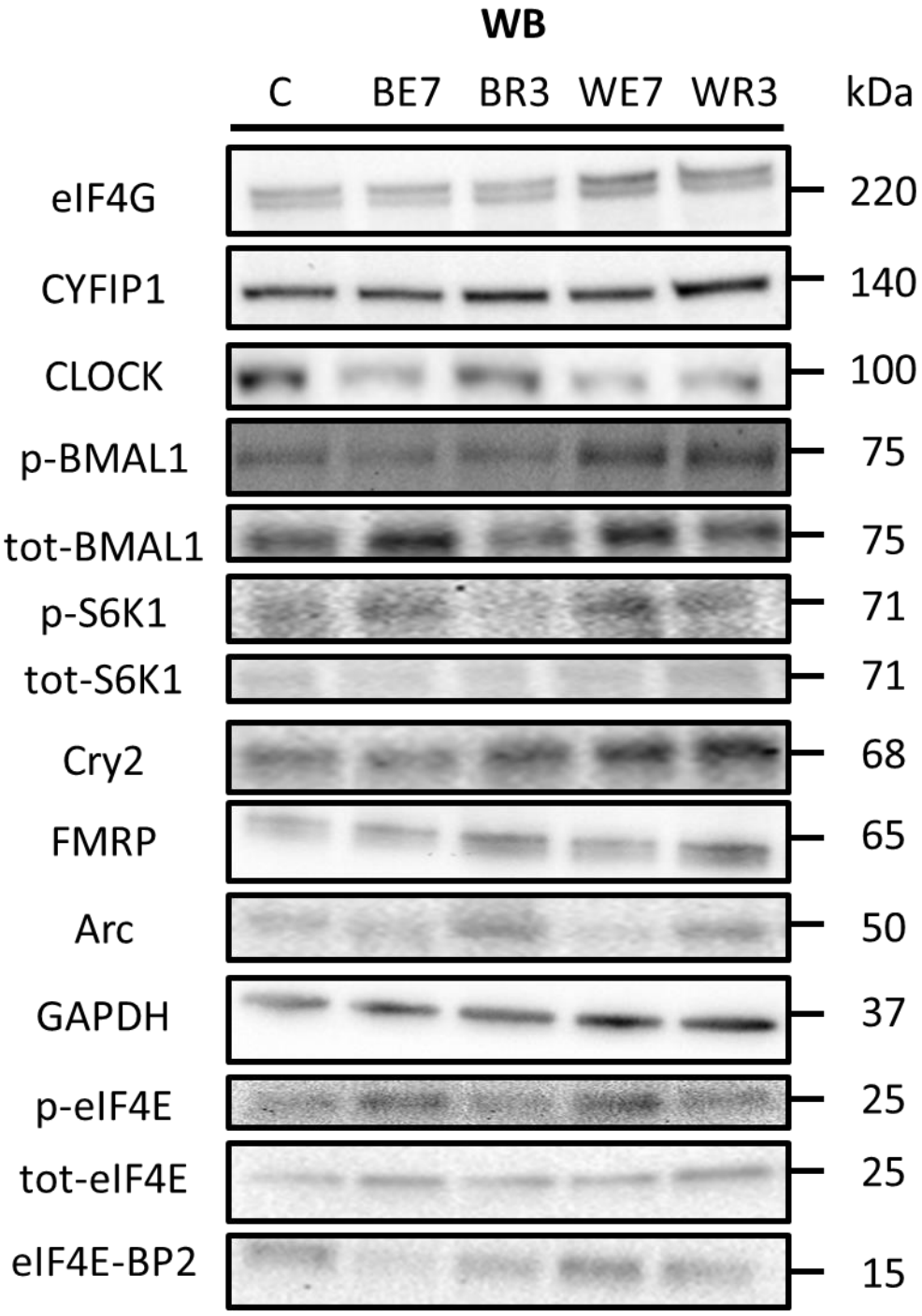


Figure S3A. Representative blots for Western Blot analysis.

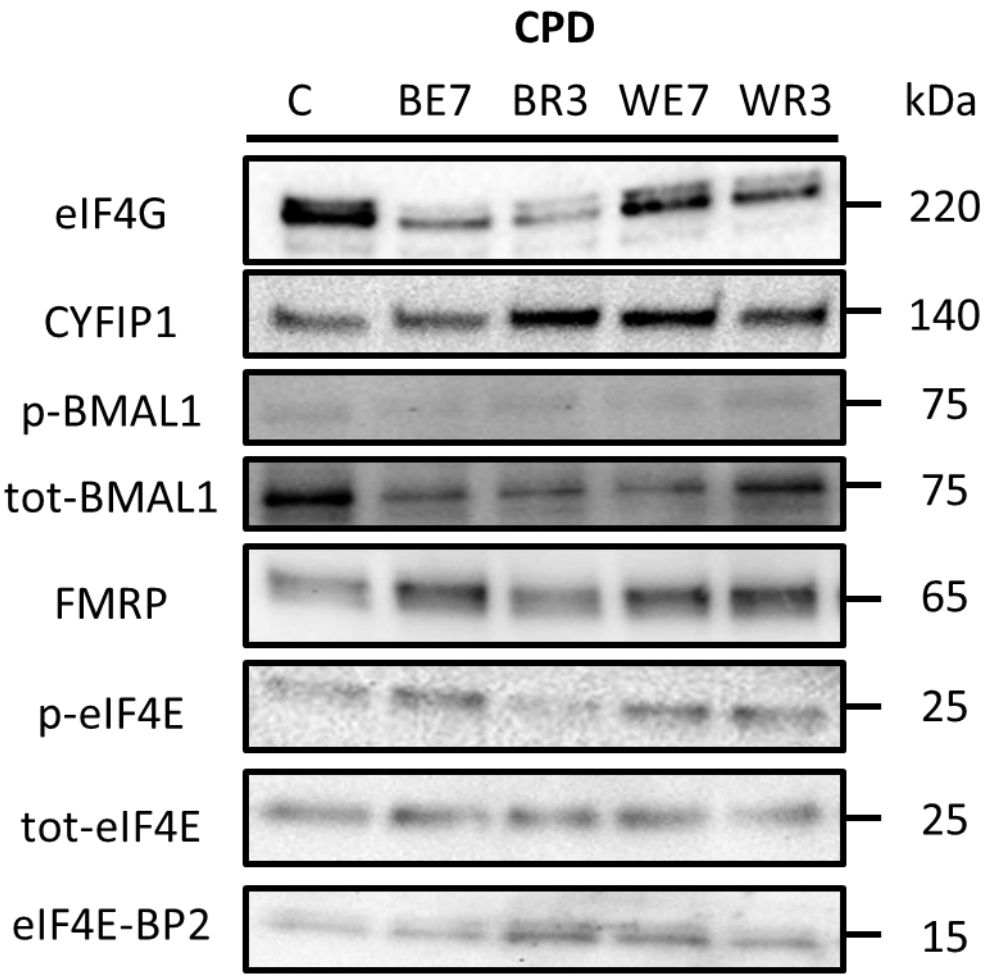


Figure S3B. Representative blots for m^7 GTP (Cap) pull-down analysis

4. T-test and factorial ANOVA for m^7 GTP (Cap) pull-down analysis

	WE7			WR3			BE7			BR3		
CPD Gene	t	df	Sig. (2-tailed)	t	df	Sig. (2-tailed)	t	df	Sig. (2-tailed)	t	df	Sig. (2-tailed)
CYFIP1	0,062	18	0,951	0,451	18	0,658	-0,037	19	0,971	-1,291	18	0,213
eIF4E-BP2	0,609	15	0,552	-0,197	15	0,847	-0,604	15	0,555	-0,611	15	0,550
eIF4G	-2,463	15	0,026*	-0,979	15	0,343	0,108	15	0,915	1,271	15	0,223
FMRP	0,394	9	0,703	0,373	10	0,717	0,033	10	0,974	-0,177	9	0,864
p-BMAL1	1,539	16	0,143	-0,552	14	0,590	0,400	16	0,694	1,445	13	0,172
p-eIF4E	-0,445	15	0,663	-0,535	15	0,601	0,716	15	0,485	-0,257	15	0,801
tot-BMAL1	0,492	16	0,629	1,040	14	0,316	-0,449	16	0,659	0,700	13	0,496

Supplementary Table 4A. T-test of m7 GTP (Cap) pull-down analysis. *p < 0.05; **p < 0.01; ***p < 0.001

	Light			Time point			Light x Time point		
	F	df	p	F	df	p	F	df	p
CYFIP1	1,458	1,40	0,235	0,683	1,40	0,414	1,194	1,40	0,282
eIF4E-BP2	1,566	1,35	0,220	0,134	1,35	0,717	0,307	1,35	0,583
eIF4G	11,485	1,35	0,002**	3,148	1,35	0,086	0,518	1,35	0,477
FMRP	1,109	1,21	0,306	0,118	1,21	0,736	0,038	1,21	0,847
p-BMAL1	0,845	1,34	0,365	0,417	1,34	0,523	4,309	1,34	0,046*
p-eIF4E	0,485	1,35	0,491	0,338	1,35	0,565	0,299	1,35	0,588
tot-BMAL1	0,705	1,34	0,407	1,491	1,34	0,231	0,159	1,34	0,693

Supplementary Table 4B. Factorial ANOVA of m7 GTP (Cap) pull-down analysis. *p < 0.05; **p < 0.01; ***p < 0.001

	WE7	WR3	BE7	BR3
	Cohens d	Cohens d	Cohens d	Cohens d
CYFIP1	-0.029	-0.202	0.016	0.625
eIF4E-BP2	-0.298	0.098	0.304	0.299
eIF4G	1.261	0.496	-0.052	-0.626
FMRP	-0.273	-0.247	-0.020	0.111
p-BMAL1	-0.730	0.278	-0.191	-0.762
p-eIF4E	0.220	0.261	-0.347	0.134
tot-BMAL1	-0.232	-0.521	0.223	-0.362

Supplementary Table 4C. Cohen's d compared to control of m7 GTP (Cap) pull-down analysis. 0.2 indicates a small, 0.5 a medium and >0.8 a large effect size.

5. T-test and factorial ANOVA for WB

	WE7			WR3			BE7			BR3		
WB Gene	t	df	Sig. (2-tailed)	t	df	Sig. (2-tailed)	t	df	Sig. (2-tailed)	t	df	Sig. (2-tailed)
Arc	-0,619	18	0,544	-1,586	18	0,130	-1,037	18	0,314	-2,083	17	0,053
CLOCK	-0,223	18	0,826	-0,173	17	0,865	0,997	17	0,333	-1,575	17	0,134
Cry2	0,554	15	0,587	1,233	15	0,237	-0,160	14	0,875	0,169	14	0,868
CYFIP1	1,564	16	0,137	2,188	16	0,044*	0,628	18	0,538	2,543	17	0,021*
eIF4E-BP2	0,097	14	0,924	-0,070	14	0,945	0,069	14	0,946	-0,036	13	0,972
eIF4G	0,451	17	0,658	0,497	18	0,626	1,635	18	0,119	1,181	18	0,253
FMRP	-0,693	15	0,499	-0,249	15	0,807	-0,177	16	0,862	0,125	15	0,902
p-BMAL1	1,532	16	0,145	1,006	17	0,328	0,614	17	0,547	0,882	17	0,390
p-eIF4E	0,011	6	0,992	1,382	6	0,216	0,618	6	0,559	1,545	7	0,166
p-S6K1	-0,797	17	0,437	0,575	17	0,573	0,614	18	0,547	0,851	15	0,408
tot-BMAL1	-1,583	18	0,131	-0,259	18	0,798	-0,129	19	0,899	-0,904	18	0,378
tot-eIF4E	-0,322	15	0,752	0,579	14	0,572	1,288	15	0,217	0,796	15	0,438
tot-S6K1	-0,838	18	0,413	-1,718	18	0,103	-0,591	18	0,562	0,702	16	0,493

Supplementary Table 5A. T-test western blot analysis. *p < 0.05; **p < 0.01; ***p < 0.001

	Light			Time point			Light x Time point		
	F	df	p	F	df	p	F	df	p
Arc	1,824	1,38	0,186	3,525	1,38	0,069	0,959	1,38	0,334
CLOCK	0,421	1,36	0,521	3,465	1,36	0,072	3,590	1,36	0,067
Cry2	1,393	1,33	0,247	0,465	1,33	0,501	0,006	1,33	0,938
CYFIP1	0,212	1,38	0,648	4,799	1,38	0,035*	0,699	1,38	0,409
eIF4E-BP2	0,000	1,30	0,992	0,021	1,30	0,887	0,000	1,30	0,982
eIF4G	1,596	1,38	0,215	0,027	1,38	0,870	0,203	1,38	0,655
FMRP	0,307	1,32	0,584	0,327	1,32	0,572	0,032	1,32	0,860
p-BMAL1	1,611	1,38	0,213	0,080	1,38	0,780	1,158	1,38	0,289
p-eIF4E	0,324	1,16	0,579	2,434	1,16	0,143	0,351	1,16	0,564
p-S6K1	1,783	1,34	0,191	2,039	1,34	0,163	0,614	1,34	0,439
tot-BMAL	0,294	1,40	0,591	0,168	1,40	0,684	3,073	1,40	0,088
tot-eIF4E	2,153	1,34	0,152	0,239	1,34	0,628	1,462	1,34	0,236
tot-S6K1	2,789	1,37	0,104	0,085	1,37	0,773	1,811	1,37	0,187

Supplementary Table 5B. Factorial ANOVA of western blot analysis. *p < 0.05; **p < 0.01; ***p < 0.001

	WE7	WR3	BE7	BR3
	Cohen's d	Cohen's d	Cohen's d	Cohen's d
Arc	0.285	0.764	0.490	1.129
CLOCK	0.100	0.079	-0.490	0.740
Cry2	-0.275	-0.661	0.081	-0.085
CYFIP1	-0.811	-1.064	-0.282	-1.196
eIF4E-BP2	-0.050	0.037	-0.037	0.020
eIF4G	-0.219	-0.222	-0.795	-0.557
FMRP	0.339	0.127	0.085	-0.061
p-BMAL1	-0.805	-0.488	-0.301	-0.415
p-eIF4E	-0.008	-1.166	-0.442	-1.150
p-S6K1	0.366	-0.267	-0.289	-0.427
tot-BMAL1	0.712	0.121	0.056	0.409
tot-eIF4E	0.158	-0.307	-0.677	-0.411
tot-S6K1	0.391	0.847	0.271	-0.339

Supplementary Table 5C. Cohen's d compared to control of western blot analysis. 0.2 indicates a small, 0.5 a medium and >0.8 a large effect size.

6. Western blot analysis of m^7 GTP (Cap) bound proteins

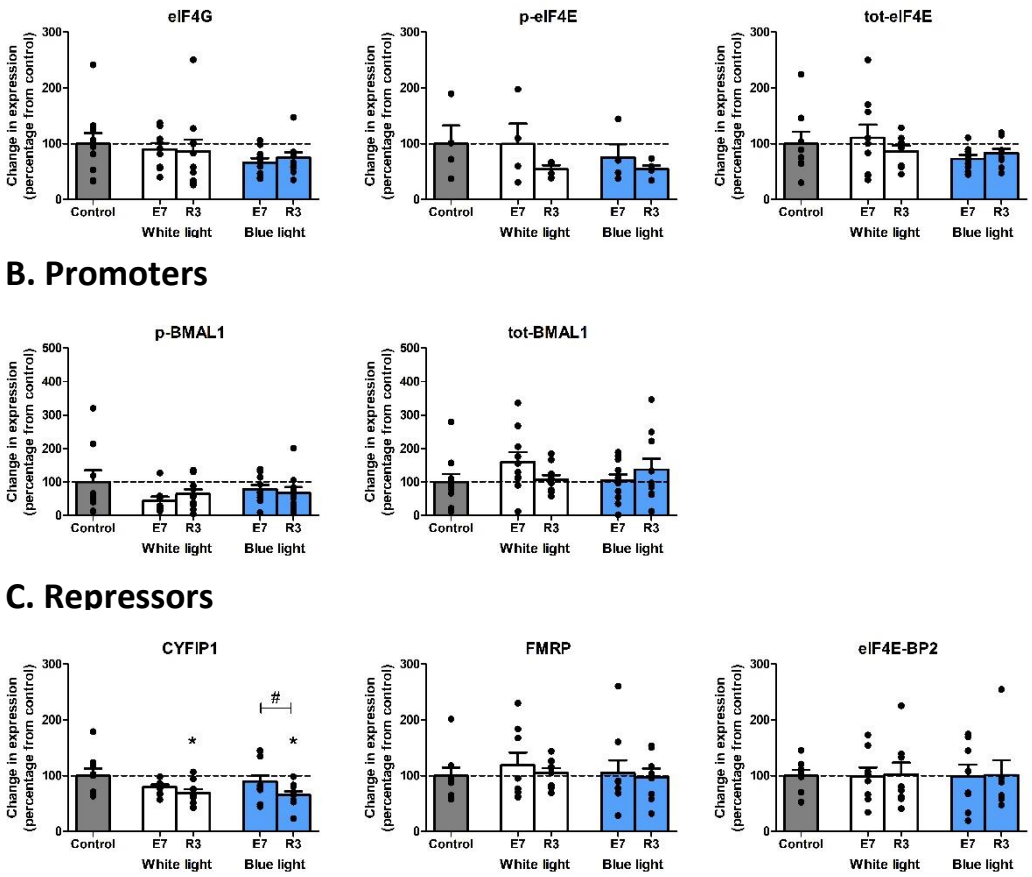


Figure S6. Photoperiodic responses in total protein expression of promoters (A,B) and repressors (C) of cap-dependent translation initiation. A,B,C) Western blot analysis of PFC lysates. Tissue was collected at lights off (ZT12) Values are expressed as percentage change relative to undisturbed controls (normalized to 100%). Graphs show mean + SEM as well as individual values. Significant differences compared to the control group: *p<0.05; **p<0.01; ***p<0.001. #p<0.05 post hoc analysis of time effect. Note: differences on y-axis scale. N=5-11/group.