**Title:**

# SCN gene expression plasticity in response to photoperiod

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**ABSTRACT**

Seasonal daylength, or circadian photoperiod, is a pervasive environmental signal that profoundly influences physiology and behavior. In mammals, the central circadian clock resides in the suprachiasmatic nuclei (SCN) of the hypothalamus and synchronizes, or entrains, physiology and behavior to the prevailing light cycle from retinal input. The process of entrainment induces considerable plasticity in the SCN, but the molecular mechanisms underlying SCN plasticity are incompletely understood. Entrainment to different photoperiods persistently alters the phase, waveform, period, and resetting properties of the SCN and its driven rhythms. To elucidate novel molecular mechanisms of photoperiod plasticity, we performed RNAseq on whole SCN dissected from mice raised in Long (LD 16:8) and Short (LD 8:16) photoperiods. Using differential rhythms analysis, we showed that fewer rhythmic genes were detected in Long photoperiod and that there was an overall phase advance of gene expression rhythms of 4-6 hours. However, a few genes showed significant phase delays, including GTP binding protein overexpressed in skeletal muscle (*Gem*), an SCN light responsive gene and light response modulator. Using differential expression analysis, we found significant expression changes in the clock-associated gene timeless circadian clock 1 (*Timeless*) and abundant changes in expression levels of SCN neural signaling genes related to light responses, neuropeptides, GABA, ion channels, and serotonin. Particularly striking were differences across photoperiods in the expression of the SCN neuropeptide signaling genes, prokineticin receptor 2 (*Prokr2*) and cholecystokinin (*Cck*), as well as convergent regulation of the expression of three SCN light response genes, dual specificity phosphatase 4 (*Dusp4*) and RAS, dexamethasone-induced 1 (*Rasd1*), and *Gem*. Transcriptional modulation of *Dusp4* and *Rasd1,* and phase regulation of *Gem,* are compelling candidate molecular mechanisms for photoperiod-induced plasticity in the SCN light response. Similarly, transcriptional modulation of *Prokr2* and *Cck* may critically support SCN neural network reconfiguration during photoperiodic entrainment. Our findings identify the SCN light response and neuropeptide signaling gene sets as rich substrates for elucidating novel mechanisms of photoperiod plasticity.

## INTRODUCTION

The mammalian brain's endogenous 24-hour timing mechanism, or circadian clock, is located within the suprachiasmatic nuclei (SCN) of the hypothalamus. SCN circadian clock neurons exhibit self-sustained circadian rhythms in gene expression, which are driven by networks of clock genes organized in transcription/translation negative feedback loops (TTFLs, for review see Hastings [1]). A key function of circadian clocks is to synchronize, or entrain, internal rhythms to local environmental time.

While much is known about the genes, neurons, and synapses that are critical for the generation of circadian rhythms, there are key gaps in knowledge regarding the molecular mechanisms of entrainment and of the pacemaker plasticity that entrainment induces. Entrainment to different seasonal day lengths (photoperiods), or even a clock reset by a single light pulse, persistently alters the phase, waveform, period, and resetting properties of the SCN circadian clock and its driven rhythms [2–10]. The long-term plasticity of circadian clocks in response to entraining light cycles is thought to be critical for stable alignment to changing seasonal photoperiods [11,12] . Misalignment of the clock to the external environment may contribute to mood disorders, SCN changes in aging, and to discordance between SCN and peripheral tissue clocks [13]. Here we describe the SCN transcriptomic response to different photoperiods to elucidate novel molecular mechanisms of SCN plasticity.

The molecular clock within SCN neurons is a nested multi-level oscillator. At its core is a two loop TTFL in which heterodimers of the transcription factors *Clock* and *Bmal1* positively drive the transcription of negative feedback period circadian regulator genes (*Per1/2*) and cryptochrome circadian regulator genes (*Cry1/2*) through E-box enhancer elements. PER and CRY proteins heterodimerize, accumulate, and enter the nucleus, inhibiting CLOCK/BMAL1-driven transcription, thus forming the core 24-hour feedback oscillator (For review see [1]). In addition, the molecular circadian clockworks comprise an extensive transcriptional network that drives circadian rhythms in the expression of clock-controlled genes in a tissue-specific and phase-specific manner, with nearly half the mouse genome thought to be under circadian control [14,15].

The timing of the circadian clock molecular oscillator loop and its clock-controlled genes can be reset by light stimuli through synaptic input to SCN neurons from melanopsin-expressing retinal ganglion cells [16–19]. The resetting transduction pathway within SCN neurons involves NMDA glutamate receptors and PAC1 PACAP receptors which depolarize the transmembrane potential and increase action potentials raising intracellular $Ca^{++}$ and cAMP levels, and ultimately stimulating MAPK and CRE transcriptional activation to induce rapid transcription of the Period genes [20,21]. Importantly, the secondary release of vasoactive intestinal polypeptide (VIP) from light-excited retinorecipient SCN neurons transmits the light input broadly throughout the SCN neural network [22,23] also through MAPK and CRE activation. Circadian entrainment theory predicts that SCN plasticity in response to light-induced clock resetting is critical to stable photoperiodic entrainment [7,11,13,14].

What then are the critical molecular mechanisms of the clock photoperiod response? GABAAR signaling and its control by the chloride co-transporters, NKCC1 and KCC2, are thought to be potential molecular elements in photoperiod plasticity in the SCN network [7,24,25]. However, these molecules alone are unlikely to fully account for the molecular bases of SCN plasticity, and the role of these transporters in the SCN has recently been called into question [26]. To reveal additional candidate molecular mechanisms of SCN photoperiod plasticity, it is important to comprehensively define what changes occur in the circadian transcriptome of the SCN following photoperiod entrainment.

Here we have used RNAseq to characterize differential gene rhythmicity and expression in response to Short winter-like photoperiods vs. Long summer-like photoperiods. To best capture the impact of photoperiod, we used a strain of mice (C3Hf+/+, [27]) with intact melatonin signaling and used perinatal exposure to photoperiods, which we have previously shown to produce robust plasticity in the SCN and robust photoperiodic responses in downstream serotonergic and dopaminergic circuits [3,28–32]. Our approach reveals specific gene expression changes in SCN neuropeptide signaling and light response genes as candidate novel molecular mechanisms for SCN light response plasticity and SCN neural network reconfiguration in photoperiodic entrainment.

## METHODS

### Animals and Housing

The C3Hf$^{+/+}$ mouse strain (a gift of Gianluca Tosini, Morehouse School of Medicine, Atlanta, GA) was used. C3Hf$^{+/+}$ mice have intact melatonin signaling and do not carry the *rd* allele that causes retinal degeneration in the C3H/HeJ parent strain [27]. Mice were paired and placed under Short (LD 8:16) or Long (LD 16:8) photoperiods (ca. 100 lux). Litters were born and maintained under these conditions. As rodent circadian rhythms develop in utero [33,34] during which maternal-fetal melatonin signaling can transmit photoperiodic information [35] and retinal melanopsin light transduction begins to function in the developing pups [36], the first litters born under the experimental photoperiods were not used in this experiment in order to avoid any potential after-effects of parental entrainment to previous photoperiod (standard institutional housing (LD 12:12)). Litters were raised with both the dam and the sire and were group housed, except for those placed in running-wheel cages for activity monitoring (see below). Mice were weaned at postnatal day 21 (P21) per standard practice and remained in the same photoperiod until postnatal day 50 (P50, late adolescence). All animal housing and procedures were approved by the Vanderbilt University Institutional Animal Care and Use Committee.

### Behavioral activity monitoring

A cohort of 10 mice (5 born and maintained under Short photoperiod and 5 under Long photoperiod) were singly housed and placed into cages with running wheels at P50. They were

kept under the same LD cycle for a minimum of 9 days before being released into constant darkness (DD), where they were allowed to freerun for at least 5 days. Wheel-running was recorded using ClockLab (version 6.1.06, ActiMetrics Software).

The phase angle of entrainment, Ψ (psi), was calculated as the timing of the activity onset in the first cycle in DD (from ClockLab) relative to the projected time of lights-off from the previous light dark cycle (projected Zeitgeber Time 12 [ZT12]). The daily duration of locomotor activity, α (alpha), was calculated separately for the LD and the DD conditions by first marking the onsets and offsets on each day in the actograms of each individual mouse using ClockLab. For each individual mouse, the average onsets and offsets (h) for each day were calculated by averaging the points. Alpha was calculated as average offset – average onset. The free-running period of the locomotor rhythms, τ (tau), was determined using Lomb-Scargle periodogram analysis in Clocklab for the days in constant darkness (DD). Statistical analysis of Ψ, α, and τ values across photoperiods were performed with GraphPad PRISM.

**SCN dissection and RNA extraction**

At postnatal day 50, the lighting regime was switched to constant darkness (DD) at the respective time of lights off (ZT 12) for each photoperiod. Mice spent a minimum of 36h in DD before sample collection began, such that gene expression in the samples likely represents the state of the endogenous clock rather than acute responses to the previously light cycle [37]. After this interval, SCN dissections took place at intervals of 4h over 2 circadian cycles [37], until a minimum n of 4 was achieved per timepoint per cycle. Short and Long photoperiod samples were collected at 36, 40, 44, 48, 52, 56, 60, 64, 68, 72, 76, and 80 hours after lights off (Fig 1). A dim red headlamp was worn by the experimenter during the first steps of dissection. Mice were euthanized by cervical dislocation and the eyes removed to prevent potential light signaling to the SCN. The subsequent steps took place under light: the optic nerve was cut and the brain was removed such that it was situated ventral side up in a petri dish. The SCN was dissected from the brain under a microscope using Vannas-Tübingen Spring Scissors (15003-08, Fine Science Tools). Each SCN sample was put in a 1.5mL sterile Dnase- Rnase-free tube, and tubes were immediately placed in powered dry ice. RNA and DNA were extracted from each SCN sample using the AllPrep DNA/RNA Micro Kit (Qiagen) according to the instructions. (DNA was stored for later use).

**RNA-sequencing**

The quality of RNA samples was assessed using an Agilent 2100 Bioanalyzer or a PerkinElmer GX TOUCH 205 Nucleic Acid Analyzer. Only those samples with an RNA integrity number (RIN) of 7.9 or above were used for sequencing. Stranded cDNA libraries were constructed using 200 ng of total RNA per sample and the NEBNext® Ultra™ II RNA Library Prep (NEB, Cat: E7765S) per manufacturer's instructions, with mRNA enriched via poly-A-selection using oligoDT beads. Paired-end sequencing produced approximately 54.6 million reads per sample (total of 11.1

billion reads), which were processed utilizing Trim Galore 0.6.7 [38] and Cutadapt 1.18 [39] to remove adapter sequences and pairs that were either shorter than 20 bp or that had Phred scores less than 20. The Spliced Transcripts Alignment to a Reference (STAR v2.7.9a) application [40] was used to perform sequence alignments to the mm39 (GRCm39) mouse genome reference and GENCODE comprehensive gene annotations (release M31). STAR's two-pass mapping approach was used to increase the detection of reads mapping to novel junctions identified during the first mapping pass. Overall, 91% of the raw sequencing reads were uniquely mapped to genomic sites. RTA (version 2.4.11; Illumina) was used for base calling and demultiplexing. All data processing was performed at the Advanced Computing Center for Research and Education (ACCRE) at Vanderbilt University.

**Data Analysis**

For downstream analysis, data from the 2 circadian cycles sampled were combined such that n ≥ 7 mice per timepoint. Differential analysis was performed by setting the Short photoperiod as the baseline in the differential gene expression pairwise comparisons. Since we used an mRNA library preparation to specifically capture protein coding genes, all analyses include and are normalized to protein-coding genes only (as determined by the gene_biotype obtained from BiomaRt).

**Differential expression analysis (DESeq2)**

To investigate genes that were significantly up- or down-regulated in Long photoperiod as compared to Short, we used DESeq2 [41]. Of all 123 samples processed, 2 samples were identified as outliers and removed from downstream analysis. The design formula was $\sim \mathrm{batch} + \mathrm{photoperiod}$ (12 sample preparations). Only genes were retained that were present in at least 3 samples with normalized counts greater than or equal to 5. Gene annotations were retrieved from BiomaRt [42] and mapped to Ensembl gene IDs. Genes without gene symbols were retained and labeled with their IDs.

**Differential rhythmic analysis (DiffCircaPipeline)**

To explore differences in rhythmic biomarkers (e.g. differential rhythmicity, phase, amplitude or MESOR) between photoperiod groups the log2CPM expression data was analyzed using the DiffCircaPipeline v0.99.9 framework [43]. Briefly, DCP uses a cosinor fit model [44] to estimate from the time sample data amplitude (A), phase (Φ), Midline Estimating Statistic Of Rhythm (MESOR, M) and the noise level (σ) of gene expression rhythms. Unlike a cosine fit model, the cosinor fit model allows for the inclusion of additional parameters such as amplitude, acrophase (timing of the peak), and MESOR (mean level). This flexibility makes it more suitable for capturing variations in the shape and characteristics of the periodic data, unlike the simpler cosine fit model. First, DCP tests whether each gene is to be considered rhythmic in each

condition (photoperiod) categorizing genes into four types of rhythmicity (TOR): arrhythmic in both groups (Arrhy), rhythmic in only group I (RhyI), rhythmic in only group II (RhyII), and rhythmic in both groups (RhyBoth) based on the cosinor fits. Then, DCP performs a differential rhythm fitness test for genes rhythmic in at least one group using the goodness of fit coefficient of determination $R^2$. For genes determined rhythmic in both conditions, the framework also calculates differences in phase, amplitude and MESOR based on the cosinor fit. To account for the cyclical nature of phase (Φ) which DCP does not account for, 24hrs were subtracted from Δ peak phase values 12hrs or greater (Δ peak-24), while 24hrs were added to values less than or equal to -12hrs (Δ peak+24), effectively normalizing phase shifts to within a -12 to 12 range. We have reported in the main text findings by DCP q-value, but also report in the Supplemental Data DCP p-value for these parameters, which is comparable to CircaCompare output. Our data was entered into the framework using median of ratios Log2 normalized data, which were corrected for batch effects using Limma [45], and then analyzed using the default settings.

**Data Availability**

Figures were generated using publicly available R-packages (The R foundation for Statistical Computing). All the code to generate the figures can be found (GitHub/Zenodo DOI available upon publication). Raw data will be deposited to NCBI's Gene Expression Omnibus and will be accessible after publication.

**RESULTS**

To confirm photoperiod entrainment and plasticity in the melatonin competent C3Hf+/+ strain, a cohort of mice were raised in Long (LD 16:8) or Short (LD 8:16) photoperiods and their locomotor activity rhythms were recorded in running wheel cages (see Methods). C3Hf+/+ mice released into constant darkness (DD) following entrainment to Long photoperiods exhibited significantly advanced activity onsets, significantly shortened activity duration, and trends toward shortened free-running period, similar to the original description of photoperiod after-effects in rodents [9] (S1). To identify candidate genes for SCN photoperiod-induced plasticity, we examined changes in the circadian transcriptome of the SCN from C3Hf+/+ mice raised in Long (LD 16:8) vs. Short (LD 8:16) photoperiods. We analyzed differential gene rhythmicity using DiffCircaPipeline (DCP) [43] and differential gene expression using DESeq2 [41] .

Analysis of the whole SCN transcriptomic datasets with DiffCircaPipeline revealed that of the 16,549 transcripts detected by DCP, 2,488 genes, or 15% of the transcriptome, were detected as rhythmic in both photoperiods. 10,501 genes (63.5%) were considered arrhythmic, and 2,864 genes (17.3%) were found to be rhythmic in Short photoperiod only, while just 697 genes (4.21%) were found to be rhythmic in Long photoperiod only (Fig 2A). Fig 2B shows heatmaps of the genes detected as rhythmic in both photoperiods by DCP. Analysis of the peak phases of rhythmic genes as determined by DCP using cosinor fits showed two lobes of gene expression peaks in both photoperiods (Fig 2C), corresponding to the well-described "day" and "night" peaks in SCN gene expression [14]. Both lobes were phase advanced in Long photoperiod by ~4-

6h relative to Short photoperiod, which also is consistent with previous smaller scale studies of SCN gene expression in different photoperiods [3,5,46]. Indeed, analysis of specific differential rhythmic parameters including peak phase, rhythmic amplitude, and the midline estimating statistic of rhythm (MESOR) (as illustrated in S2)**,** demonstrated that the majority (73.5%) of genes that were rhythmic in both photoperiods had significantly different peak phases by q-value. The vast majority (1,825) of these genes were phase advanced in the Long as compared to the Short photoperiod, while only 4 genes were phase delayed: GTP binding protein overexpressed in skeletal muscle (*Gem*), HAUS augmin-like complex subunit 3 (*Haus3*), Leucine-rich repeats and IQ motif containing 3 (*Lrriq3*), and Ras association and DIL domains (*Radil*). In contrast to peak phase, the rhythmic amplitude and the MESOR of genes were rarely found to be significantly different between photoperiods after DCP correction for false discovery rate (FDR) ($q<0.05$). However, it is worth noting here that previous analyses that employ cosinor fits to calculate the rhythmic parameters above, including the highly-cited "CircaCompare", do not use FDR correction [47,48]. Therefore, we also investigated our rhythmic results at a threshold of $p<0.05$. 155 genes showed significant changes in rhythmic amplitude (1 increased, 154 decreased), while 447 genes showed significant changes in MESOR (209 increased, 238 decreased) between photoperiods (S2, S1A Data). These DCP p-value significant changes are noted for individual rhythmic genes in the differential expression section below, and DCP output is also available parsed by the DESeq2 functional gene groups below (Circadian, Neuropeptide, Light Response, GABA, Ionic, Serotonin and Dopamine) in Supplemental Table S1B-G.

Analysis of differential gene expression using DESeq2 revealed salient differences in gene expression levels in the SCN transcriptomes of mice kept under Long versus Short photoperiods. Of the 16,856 transcripts detected by DESeq2, 1,518 were differentially expressed between the short and long photoperiods (p-adj < 0.05; Log2FC > [0.1]) (S2A Data). Of these, 972 were significantly downregulated and 546 were significantly upregulated in long photoperiod. A volcano plot of the whole SCN data shows large magnitude changes in expression in known hypothalamic photoperiod response genes downstream of the SCN, such as *Lrat* in the retinoic acid signaling pathway [49] and *Tshβ* [50], *Npvf* [51], *Dct* [52], *Dio2* [50], *Tafa3* [50], and *Slc16a2* [53] in the photoperiodic endocrine system (Fig 3), indicating that the photoperiod exposure indeed evoked a downstream photoperiodic response. Further, the direction of change agrees with multiple studies in mammals, with *Lrat*, *Npvf*, *Tshβ*, *Dct*, and *Dio2* all reported to be upregulated in Long photoperiod [49,50,52,54]. To focus on genes robustly expressed in the SCN in our dataset, we used gene expression data from recent scRNA studies of SCN neurons for exploratory analysis [55,56]. These studies have also been used to define SCN neurons as a class within the hypothalamus [57]. Photoperiod-responding genes were enriched in SCN light response genes, neuropeptide signaling genes, and synaptic signaling genes, and we therefore further analyzed these SCN functional gene groups as described below (Figures 4-7, S3 and S4).

**Circadian clock genes**

To determine whether photoperiod substantially impacted the core molecular clockworks, we analyzed differential expression and rhythmicity of 23 circadian clock genes and clock-associated genes detected by DESeq2. Overall, the expression and rhythmicity of these genes

was consistent with expectations from many previous studies [14,15] (Fig 4). Surprisingly, we found no significant differences by DESeq2 in the expression level of core clock genes between Short and Long photoperiods. However, there was a significant increase in expression level of the circadian-associated gene *Timeless* in Long photoperiod (Fig 4A, S2B Data).

In terms of differential rhythmicity, the peak phases of the clock genes found to be rhythmic in both photoperiods (*Per1, Per2, Cry1, Arntl, Rorα, Nr1d1)*, were advanced 2-5 hours in Long photoperiod (DCP, $q<0.05$), similar to the overall pattern of phase advance of gene expression (Fig 4B), and the core clock gene *Per1* showed a significant loss of rhythmic power in Long photoperiod (DR fitness ($\Delta R^2$), DCP, $q<0.05$, S3 Data). Three additional clock genes, *Per2*, *Ciart*, and *Clock* showed trend level decreases in rhythmic power in Long photoperiod as well (DR fitness ($\Delta R^2$), DCP, $p<0.05$, S3 Data). In addition, *Per1, Per2, Arntl, Nr1d1*, and *Ciart* showed significant reductions in rhythmic amplitude by DCP p-value (<0.05), however none showed significant changes in MESOR (S1B Data). *Per1* and *Ciart* also showed decreased rhythmic amplitude in Long photoperiod at DCP $q<0.10$ (S1B Data). *Cry2* and *Clock* were detected by DCP as rhythmic only in Short photoperiod (S3 Data), whereas *Rorβ* and *Timeless* were not detected as rhythmic in either photoperiod.

**Neuropeptide signaling genes**

Several neuropeptide signaling axes exist within the SCN neural network, including Arginine vasopressin (AVP), Cholecystokinin (CCK), Gastrin Releasing Peptide (GRP), Neuromedin S (NMS), Neuropeptide Y (NPY), Prokineticin 2 (PROK2), and Vasoactive Intestinal Polypeptide (VIP) [1]. A number of these peptides play important roles in SCN light responses, light resetting, and entrainment. In particular, VIP released from light-excited retinorecipient SCN neurons transmits light input broadly in the SCN neural network [22,23] and neurons expressing CCK are critical for responses to long photoperiods and may activate VIP neurons specifically under long photoperiod [58]. We investigated 26 genes detected by DESeq2 that either make up the signaling axes themselves (peptides, receptors) or are involved in their modulation. Of these, we found that prokineticin receptor 2 (*Prokr2*), neuropeptide Y receptor Y1 (*Npy1r*), and neuromedin U receptor 2 (*Nmur2*) were significantly down-regulated in Long photoperiod, while cholecystokinin (*Cck*) and dual specificity phosphatase 4 (*Dusp4*), a modulator of VIP responses [23], were significantly up-regulated (Fig 5A; S2C Data). Rhythms in *Npy1r*, *Nmur2*, and *Dusp4* expression showed significant differences in peak phase between photoperiods (DCP, $q<0.05$). *Npy1r*, *Nmur2* also showed significant decreases in MESOR, while *Dusp4* showed a significant increase in MESOR (DCP $p<0.05$), however none of these genes showed significant changes in rhythmic amplitude by DCP p-value (Fig 5B, S1C Data).

**Light responsive genes**

Persistent alterations in SCN clock period are evoked by single clock-resetting light pulses as well as by entrainment to different photoperiods [9,59] suggesting the possibility of mechanistic

overlap between these enduring responses to circadian light history. Therefore, we sought to examine whether the expression of acutely light responsive SCN genes might also be altered in our photoperiod treatments. A recent RNAseq study by Xu, et al. [60] examined which SCN genes responded acutely to light by exposing mice to pulses of light lasting 0.5-, 1-, 3-, and 6 hours. We compiled a global list of light responsive SCN genes in that study by combining the genes both up regulated and down regulated across all the pulse durations and removing those genes found in both groups. Of the 959 genes acutely regulated by light in the Xu et al., data [60], 99% of these genes were detected in our dataset, and 166 of these genes showed significant differential expression across photoperiods (S2D Data). Interestingly among the genes differentially expressed are *Dusp4* and RAS, dexamethasone-induced 1 (*Rasd1*) both of which are key modulators of the SCN light response through their actions on the critical MAPK/ERK mediation of the SCN light transduction pathway [23,61] (Fig 6A). *Rasd1* was also detected as rhythmic in both photoperiods and showed the typical advanced peak phase in Long (DCP, $q<0.05$), as well as a decrease in MESOR and no change in rhythmic amplitude by DCP p-value (Fig 6B**,** p-value $<0.05$, S1D Data). DCP results for *Dusp4* are described in the neuropeptide signaling genes results. In addition, *Gem,* which also modulates SCN phase resetting to light through feedback onto the MAPK/ERK pathway [62], was one of the very few genes that showed a significant phase delay in Long compared to Short (Fig. 6B).

**GABA signaling genes**

Virtually all neurons in the SCN communicate via the neurotransmitter GABA, and photoperiodic regulation of inhibitory vs excitatory GABA transmission in the SCN is thought to be a key mechanism underlying photoperiodic plasticity [7,24,25]. Of 36 genes involved in SCN GABA signaling detected by DESeq2, 6 were found to be differentially expressed, all down-regulated in Long photoperiod (Fig 7A, S2E Data). These included Na-K-2Cl cotransporter-1 (Nkcc1/Slc12a2), K-Cl cotransporter-1 (KCC1/Slc12a4), Betaine-GABA transporter (BGT1/Slc6a12/mGAT2), GABA transporter 2 (GAT2/Slc6a13/mGAT3), $GABA_A$ receptor subunit rho 2 (Gabrr2), and $GABA_A$ receptor subunit epsilon (Gabre) (Fig 7A)**.** None of these genes were detected as rhythmic by DCP (Fig 7B).

**Ionic signaling genes**

Previous work on SCN plasticity in response to entrainment to different cycle periods has suggested the regulation of ionic and synaptic signaling genes as a potential mechanism for mediating that form of SCN plasticity [63], therefore, we also examined calcium, potassium, sodium, and chloride voltage-gated channels genes, and related genes, in our dataset (166 genes total). Of these, 16 genes were differentially expressed between photoperiod conditions. 11 genes including *Kctd14, Kcnk5, Slc13a4, Kcnj13, Slc13a3, Kcna1, Ano1, Cacna2d4, Kcnk13, Kctd11, Kctd12* were down-regulated in Long photoperiod, while five genes including *Kcnv2, Scn4a, Clcn1, Sclt1, and Cacng7* were up-regulated in Long photoperiod (S3A; S2F Data**)**. Of these *Kctd12* was detected as rhythmic in both photoperiod with phase advanced expression in

Long (DCP, q<0.05), as well as a decrease in MESOR and no change in rhythmic amplitude by DCP p-value (S3B**,** p-value <0.05, S3B; S1F Data).

**Serotonin and dopamine signaling genes**

Serotonin and dopamine inputs to the SCN modulate SCN light responses and entrainment [64,65], and therefore we surveyed transcripts involved in serotonin and dopamine signaling in our dataset (24 genes total: 7 genes involved in dopamine signaling and 17 genes involved in serotonin signaling). The serotonin receptor 7 (Htr7) and the serotonin transporter (Slc6a4/Sert) were up-regulated, while the serotonin receptor 3B (Htr3b/5-HT3B) was down-regulated in Long photoperiod, while no dopamine signaling genes examined were found to have significant changes in expression (S4A; S2G Data). None of these genes were detected as rhythmic by DCP (S4A; S1G Data).

**DISCUSSION**

Seasonal photoperiods entrain circadian rhythms and induce enduring plasticity in the SCN clock. To elucidate potential molecular mechanisms of photoperiod plasticity we performed RNAseq on whole SCN dissected from mice raised in Long (LD 16:8) and Short (LD 8:16) photoperiods. Overall, in terms of differential rhythmicity, fewer rhythmic genes were detected and there was an overall phase advance of gene expression rhythms of a few hours in Long photoperiod. Differential expression analysis showed no significant changes in the expression levels of the core clock genes, however, there were prominent differences across photoperiods in genes involved in SCN neuropeptide signaling and light response.

The advanced phase and decreased rhythmic power of core clock genes in Long photoperiod are consistent with previously demonstrated outcomes of entrainment to long photoperiods, including altered rhythmic waveforms and decreased rhythmic amplitude, as well as increased phase dispersal of single cell SCN rhythms [3–6,8,9,46]. In contrast, the clock-associated gene *Timeless*, showed a significant increase in expression level in Long photoperiod. *Timeless* is a core clock gene in *Drosophila*, however its precise function in the mammalian SCN is unclear. Knockdown of *Timeless* abrogates SCN electrophysiological rhythms, while homozygous germ-line knockout is embryonic lethal, and heterozygotes show normal circadian rhythms [66,67]. A human mutation in *Timeless* is associated with altered sleep phase and modifies clock period and stability of PER/CRY complexes in mice [68]. Thus, the photoperiod driven changes in *Timeless* expression we describe here have the potential to affect SCN rhythmic properties.

While photoperiod-induced changes in the expression of clock genes were relatively few, there were abundant changes in expression levels of SCN neural signaling genes related to neuropeptides, GABA, ion channels, serotonin, and dopamine. In all 29 genes from these categories were found to exhibit photoperiod-dependent changes in expression level using DESeq2. Previous results have established regulation of inhibitory vs. excitatory GABA signaling

in the SCN through the expression of *Nkcc1* and *Kcc2* chloride transporter genes as a likely mechanism of photoperiodic plasticity [7,24,25]. Indeed, we found expression changes in six GABA signaling genes - Na-K-2Cl cotransporter-1 (*Nkcc1/Slc12a2*), K-Cl cotransporter-1 (*KCC1/Slc12a4*), GABA transporter 2 (*GAT2/Slc6a13/mGAT3*), GABA transporter BGT1 (*BGT1/Slc6a12/mGAT2*), $GABA_A$ receptor subunit rho 2 (*Gabrr2*), and $GABA_A$ receptor subunit epsilon (*Gabre*), all of which were reduced in expression in Long photoperiod. The expression of *Nkcc1* compared to *Kcc2* was previously found to increase in the dorsal SCN in Long days [24], however *Nkcc1* expression was reduced overall in our data, and *Kcc2* was not significantly changed. These differences may be due to the fact that photoperiod regulation of *Nkcc1/Kcc2* in the SCN is highly region-specific [7,24], and our data is taken from whole SCN, perhaps obscuring regional changes. In addition, while we found expression changes in the GABA transporters *GAT2* (*Slc6a13/mGAT3*) and *BGT1* (*Slc6a12/mGAT2*), we did not detect significant changes in the principal SCN-expressed GABA transporters of *GAT1 (Slc6a1, mGAT1)*, which is expressed in neurons and astrocytes, and *GAT3* (*Slc6a11, mGAT4),* which is expressed in astrocytes, and is critical for regulation of extracellular GABA levels and rhythms [26]. Thus, the changes in expression of GABA signaling genes we observed present the possibility of contributing to SCN plasticity, but in themselves they do not establish a very coherent picture.

Another category of SCN genes that showed a robust response to photoperiod were SCN light responsive genes – genes that change expression rapidly (within 6 hours) in response to a phase resetting light pulse [60]. Remarkably, 166 acutely light responsive genes were found to exhibit significant changes in expression after weeks-long entrainment to Long vs Short photoperiods. It is important to note that these are sustained changes in expression in our experiments, rather than acute induction by light, because photoperiod entrained mice were maintained in constant darkness for at least 36 hours prior to sampling. Intriguingly, we found significant differential expression or rhythmicity across photoperiods of three acutely light responsive genes that are all modulators of the critical MAPK/ERK pathway for light response transduction in SCN neurons, *Dusp4*, *Rasd1*, and *Gem*. This light responsive gene set appears to be a particularly rich substrate for elucidating novel mechanisms of photoperiod plasticity in the future.

What are the specific changes in SCN function following photoperiodic entrainment that beg molecular explanation? Entrainment of rodents to long photoperiods (>12hr light/cycle) has been shown to have four distinct and enduring after-effects - to advance the phase of behavioral, electrophysiological, and molecular rhythms [5,9,10]. To alter the waveform of behavioral, electrophysiological, and molecular rhythms by compressing the duration of activity ($\alpha$) and correspondingly expanding the duration of electrophysiological and *Period* gene expression [5,9,10]. To shorten the free-running period of behavioral, and molecular rhythms [5,9]. And to decrease the responsiveness of the SCN to light input and its principal synaptic mediator NMDA glutamate receptors [2,10].

How might the changes in gene expression we have observed underlie these specific functional aspects of SCN photoperiod plasticity? Focusing on genes with robust changes in expression or rhythmic parameters (DESeq2 padj<0.05, Log2FC > [0.3], [60]; DCP q<0.05) and for which there

is evidence of SCN expression and function, there are several genes in our dataset that are of potential mechanistic importance. These include *Dusp4*, *Rasd1* and *Gem* which are each key modulators of the SCN light response [23,61,62]. In addition, there were changes in the expression of *Prokr2* the widely expressed receptor for the critical PROK2 signaling network hub in the SCN [55], and in the expression of (*Cck),* which is expressed in neurons critical for light-induced phase advances and entrainment to Long photoperiods [58].

Particularly intriguing is the convergent differential expression of *Dusp4*, and *Rasd1* as well as differential rhythmic phase of *Gem* to potentially impact the SCN light response. *Dusp4* is a negative modulator of the MAPK/ERK pathway and the SCN phase shifting response to light [23], and here its expression is increased in Long photoperiod, while *Rasd1* is a positive mediator of the MAPK/ERK pathway in the SCN and the SCN phase shifting response to light [61], and here its expression is decreased in Long photoperiod. In addition, *Gem* also mediates negative feedback to MAPK/ERK transduction of light responses into gene expression changes within SCN neurons through negative modulation of Ca++ channels. *Gem* is robustly induced by light at phases at which its rhythmic expression in low, but less so when its expression is elevated near its peak [62]. Based on this pattern, the alignment of *Gem* peak phase we observe in Short photoperiod (Fig. 6B) would be predicted to decrease *Gem* light induction in the early night and therefore enhance the amplitude of response to phase delaying light pulses, compared to *Gem's* more delayed peak phase in Long. Therefore, *Dusp4*, *Rasd1,* and *Gem* are all coordinately regulated in Long photoperiod in a manner that would be expected to decrease the SCN phase-shifting light response compared to Short photoperiod. Indeed, a reduction in SCN phase resetting to acute light pulses after entrainment to long photoperiod is exactly the form of SCN photoperiodic light response plasticity that has been previously described [2,10]. Transcriptional modulation of *Dusp4* and *Rasd1* and phase modulation of *Gem* rhythms are therefore a compelling candidate molecular mechanisms for SCN light response plasticity. In addition, transcriptional modulation of *Prokr2* and *Cck* may critically support SCN neural network reconfiguration in photoperiodic entrainment [4,8] that may underlie changes in phase angle of entrainment, activity duration, or period.

In summary, using RNAseq on whole SCN dissected from mice exposed to Long (LD 16:8) and Short (LD 8:16) photoperiods we found salient differences across photoperiods in the expression of genes involved in SCN light responsive genes and neural signaling genes. We describe a remarkable convergent regulation of three SCN light response and MAPK/ERK modulating genes - *Rasd1*, *Dusp4* and *Gem* - which potentially underlie plasticity of the SCN light response, and in addition, our dataset reveals several potentially novel SCN photoperiod plasticity genes including *Prokr2*, *Cck* neuropeptide signaling genes and the clock-associated gene *Timeless*. Future experiments can test the potential novel mechanistic roles of specific genes from this dataset in SCN photoperiod entrainment and plasticity using ex vivo optogenetic entrainment [59] combined with SCN specific gene manipulation.

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## FIGURES AND LEGENDS

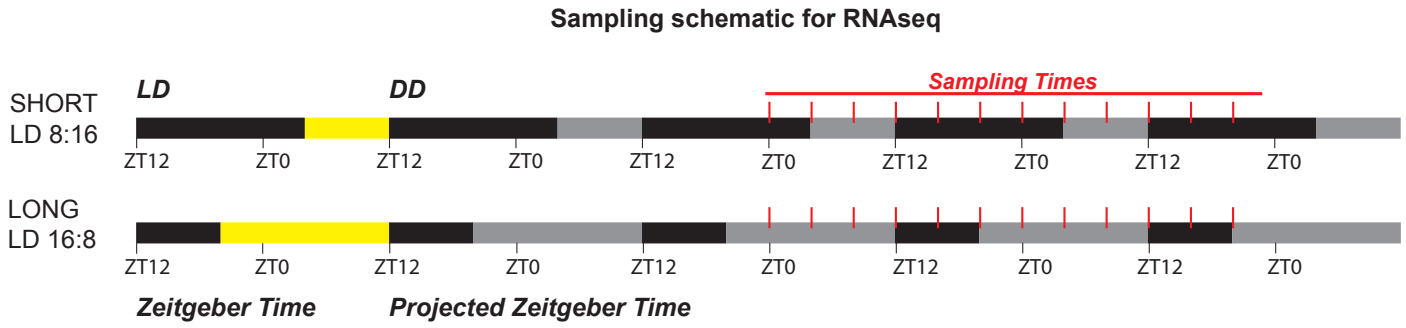


**Figure 1. Sampling schematic for RNAseq.** Last LD cycle before DD is shown at left in ZT, with yellow representing the light phase and where "lights off" is ZT12. At postnatal day 50, the lighting regime was switched to constant darkness (DD) at the respective time of lights off ZT12 as shown, (gray areas indicate the projected light phase), and spent a minimum of 36h in DD before sample collection, which occurred every 4h over 2 circadian cycles.

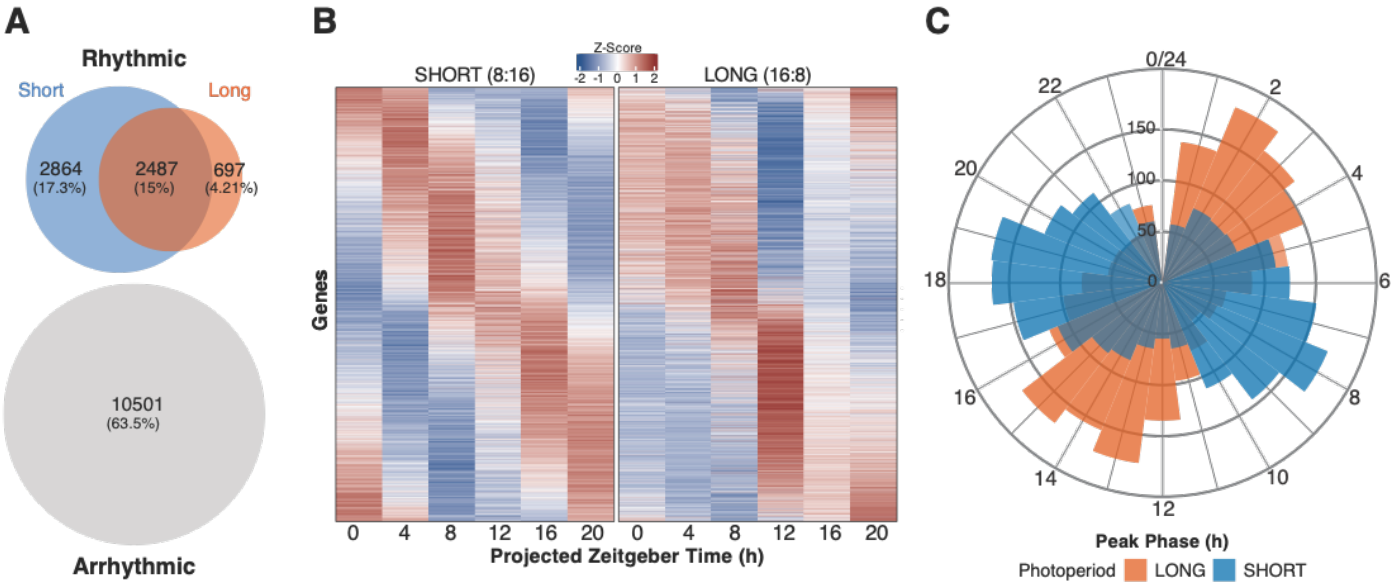


**Figure 2. DiffCircaPipeline: Analysis of rhythmicity and peak phase in the SCN transcriptome across photoperiods. A)** Venn diagram showing the number of protein coding rhythmic genes in Short, in Long, in both, and in neither photoperiod. **B)** Heatmap showing rhythmic gene expression in Short and Long photoperiods across time. **C)** Radar plot showing the peak phase for Short (blue) and Long (orange) photoperiods for genes determined to be rhythmic in both conditions.

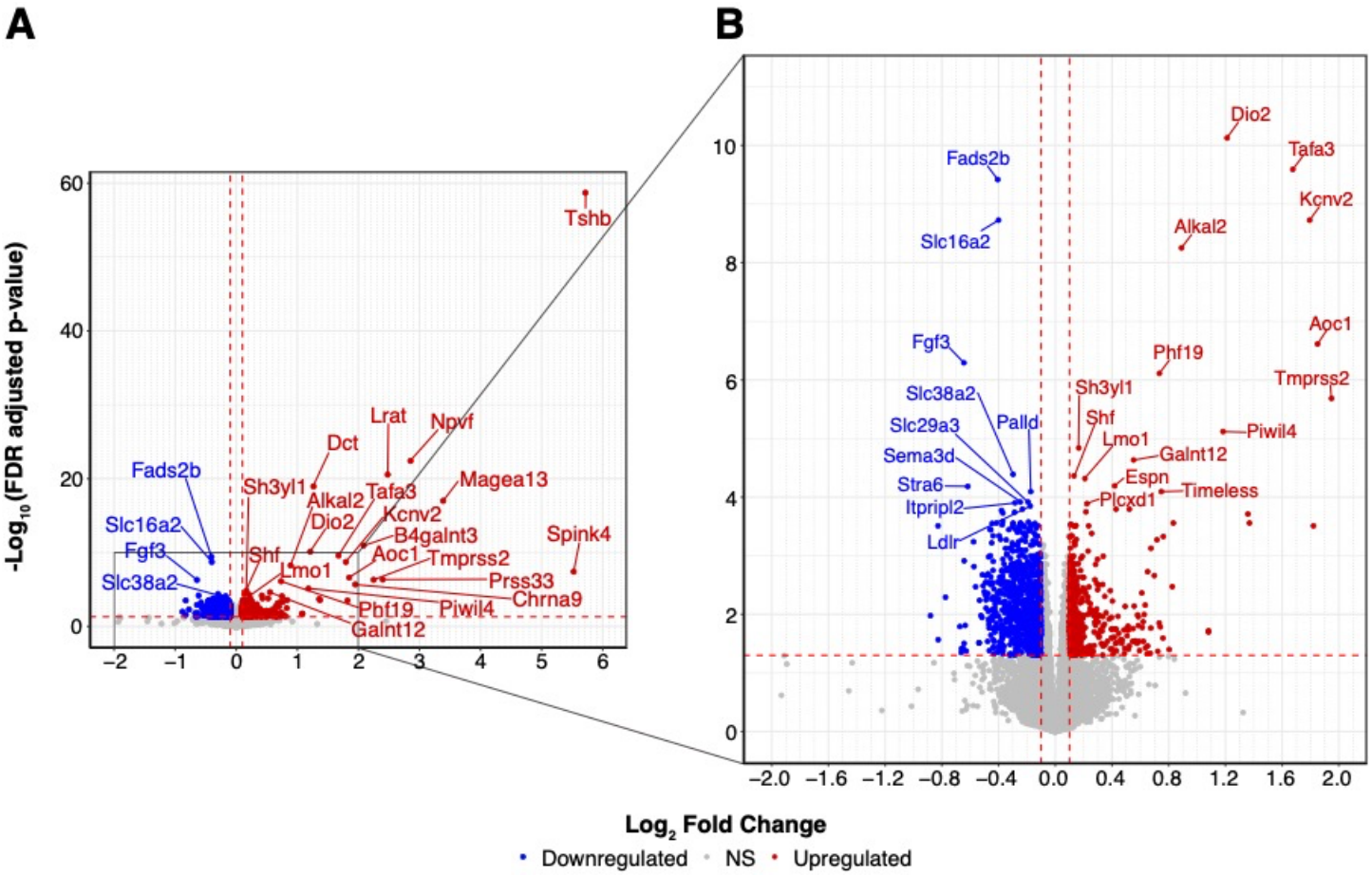


**Figure 3. DESeq2: Whole transcriptome analysis of differential expression across photoperiods. A)** Volcano plot showing changes in gene expression between Short and Long photoperiods. Directionality = from Short to Long (Long - Short). Downregulated genes are shown in blue and Upregulated genes are shown in red. Thresholds for significance were set at $\log_2 FC \geq \pm 0.1$ (red dashed vertical lines) and p-adj < 0.05 (red dashed horizontal line). The top 25 genes meeting this criteria are labeled. **B)** Zoom view of volcano plot (grey box). Threshold for significance same as panel A. The top 25 differentially expressed genes in the plot are labeled.

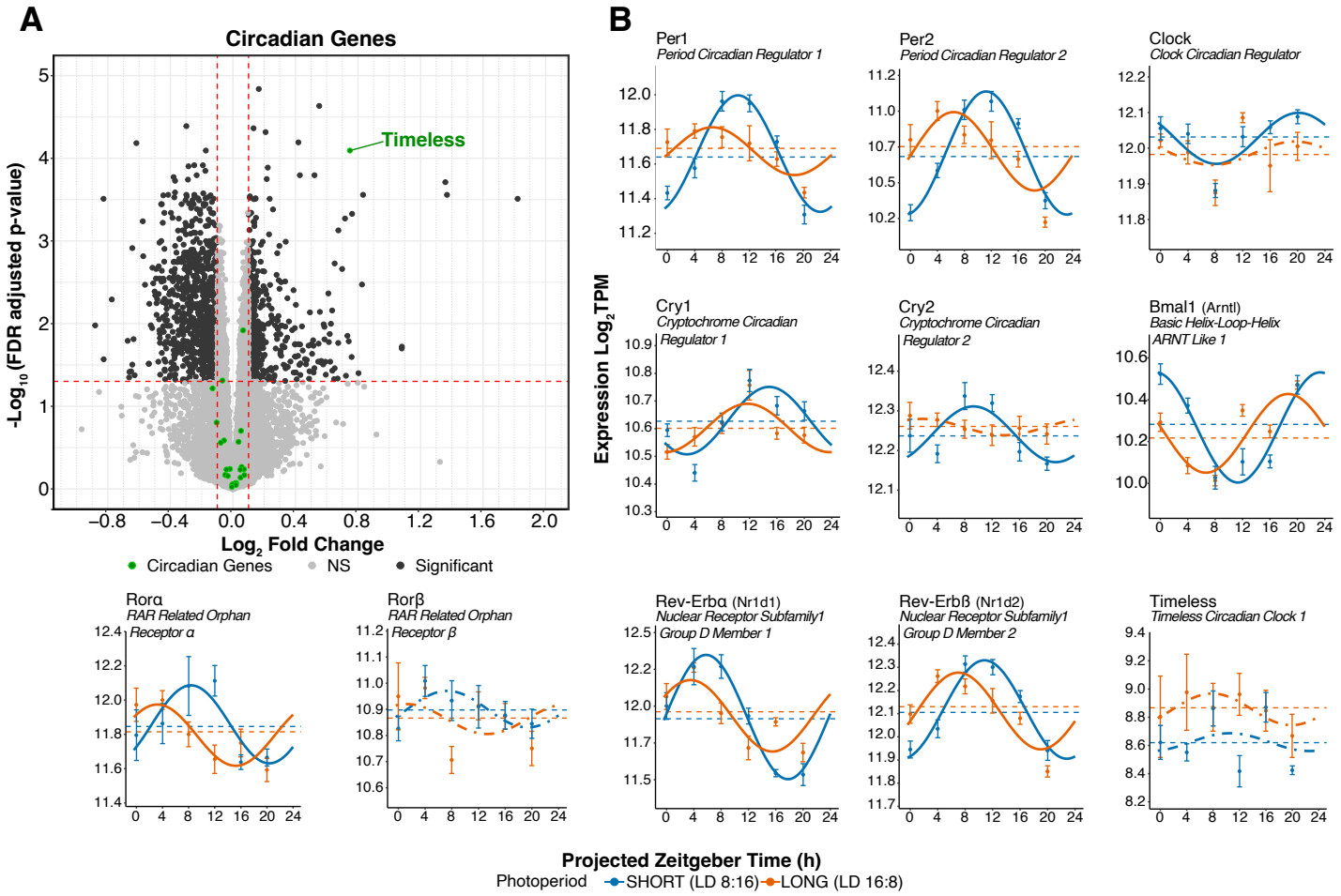


**Figure 4. Circadian clock gene differential expression & rhythmicity in response to photoperiod. A)** DESeq2 volcano plot of gene expression highlighting clock genes in green. Genes meeting the $\log_2$FC ≥ ±0.1 and p-adj < 0.05 are labeled. **B)** Cosinor plots illustrating the rhythmicity of selected clock genes. Solid lines depict significantly rhythmic cosinor fits, whereas dot-dashed traces indicate non-rhythmic fits. Dots represent mean ±SEM for samples collected at each timepoint (n ≈ 10). Short (8:16) and Long (16:8) photoperiods are represented in blue and orange, respectively.

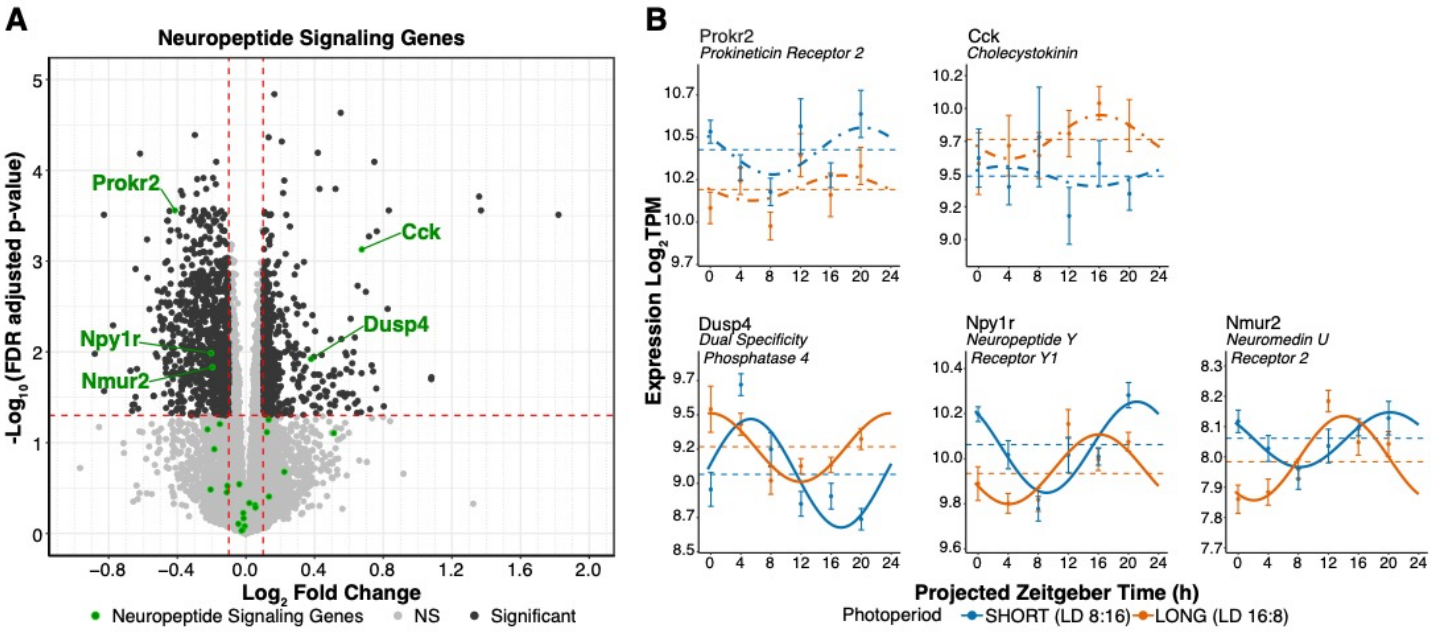


**Figure 5. Neuropeptidergic signaling gene differential expression & rhythmicity in response to photoperiod. A)** DESeq2 volcano plot of gene expression highlighting neuropeptide signaling genes in green. Genes meeting the $\log_2 FC \geq \pm 0.1$ and p-adj < 0.05 are labeled. **B)** Cosinor plots of differentially expressed genes. Solid lines depict significantly rhythmic cosinor fits, whereas dot-dashed traces indicate non-rhythmic fits. Dots represent mean ±SEM for samples collected at each timepoint (n ≈ 10). Short (8:16) and Long (16:8) photoperiods are represented in blue and orange, respectively.

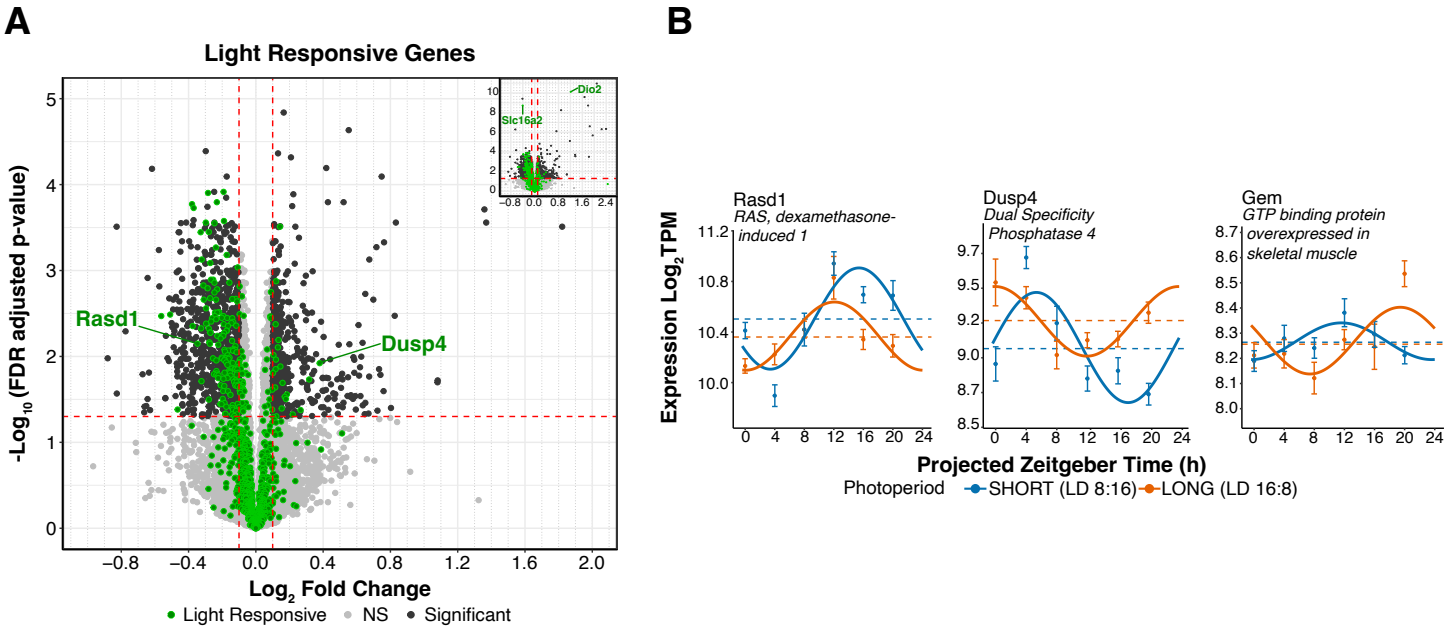


**Figure 6. Light responsive gene differential expression & rhythmicity in response to photoperiod. A)** DESeq2 volcano plot of gene expression highlighting light responsive SCN genes [60] in green at log2FC > ±0.1 and p-adj < 0.05. **B)** Cosinor plots of selected genes (described in A). Solid lines depict significantly rhythmic cosinor fits, whereas dot-dashed traces indicate non-rhythmic fits. Dots represent mean ±SEM for samples collected at each timepoint (n ≈ 10). Short (8:16) and Long (16:8) photoperiods are represented in blue and orange, respectively.

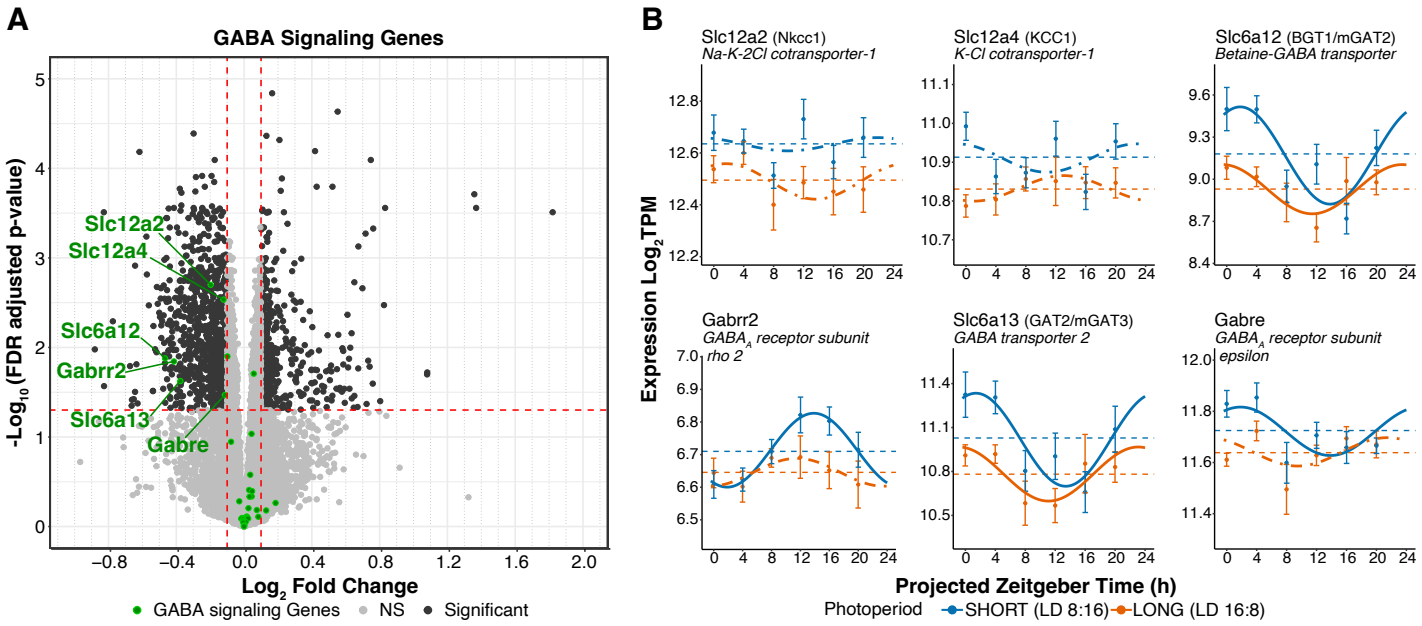


**Figure 7. GABA signaling gene differential expression & rhythmicity in response to photoperiod. A)** DESeq2 volcano plot of gene expression highlighting GABA signaling genes in green. Genes meeting the $\log_2$FC ±0.1 and p-adj < 0.05 are labeled. **B)** Cosinor plots of differentially expressed genes. Solid lines depict significantly rhythmic cosinor fits, whereas dot-dashed traces indicate non-rhythmic fits. Dots represent mean ±SEM for samples collected at each timepoint (n ≈ 10). Short (8:16) and Long (16:8) photoperiods are represented in blue and orange, respectively.