Accepted Manuscript

Research paper

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Please cite this article as: Stevenson, T.J., Circannual and circadian rhythms of hypothalamic DNA methyltransferase and histone deacetylase expression in male Siberian hamsters (*Phodopus sungorus*)., *General and Comparative Endocrinology* (2016), doi: http://dx.doi.org/10.1016/j.ygcen.2016.11.011
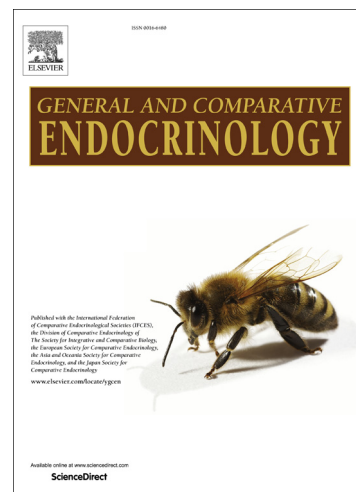
Title: Circannual and circadian rhythms of hypothalamic DNA methyltransferase and histone deacetylase expression in male Siberian hamsters (*Phodopus sungorus*).

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Running title: Rhythms in hypothalamic *dnmt* and *hdac* mRNA.

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Disclosure: The author has nothing to declare.

Manuscript length: 6223 words
Figures: 4
Supplementary Information: 3 Tables

ABSTRACT

Precise timing of gene transcription is a fundamental component of many biological rhythms.

DNA methylation and histone acetylation are two epigenetic modifications that can affect the

probability of gene transcription and RNA expression. Enzymes involved in DNA methylation (*dnmt*s)

have been shown to exhibit photoperiodic rhythms in expression in the hypothalamus, which coincide

with hypothalamic expression of deiodinase type III (*dio3*), a gene involved in the photoperiodic regulation of reproduction. It is currently unknown whether enzymes involved in histone deacetylation (*hdac*s) also vary in response to photoperiod, nor have seasonal changes in the circadian waveforms of methylation and/or acetylation enzymes been examined. The present work documents circadian and photoperiodic changes in *dnmt*s and *hdac*s in whole hypothalamic dissections obtained from male Siberian hamsters (*Phodopus sungorus*) after 5-6 weeks of exposure to SD. The data indicate that short days (SD) markedly inhibit *dnmt3a* expression, and that SD inhibition of *dnmt3a* was evident regardless of the alignment of circadian waveforms. Among *hdac*s, photoperiodic and circadian changes in expression were only observed in *hdac4* expression. Recurrent temporal waveforms in epigenetic enzyme expression may provide molecular inputs to the timing systems that reprogram RNA expression to generate daily and annual phenotypic plasticity.

Key words: epigenetic, methylation, acetylation, plasticity, seasonal

INTRODUCTION

Organisms have evolved to orient endocrine and behavioural processes across a wide range of time scales. In many temperate zone species, animals have adapted to not only circadian changes in their environment, but also annual variation. The yearly change in day length provides the predominant predictive cue that animals use to drive or entrain circannual endocrine rhythms (Goldman, 2001; Dawson et al., 2001; Paul et al., 2008; Prendergast, 2005; Stevenson & Ball, 2011; Rani & Kumar, 2014; Wood and Loudon, 2014). Siberian hamsters (*Phodopus sungorus*) are a common laboratory animal model used to investigate circadian and circannual endocrinology. In the laboratory, a change in day length from 'long days' (LD; > 14 h light/day) to 'short days' (SD; <13 h light/day) is sufficient to

initiate a constellation of neuroendocrine changes, including alter locomotor activity, gonadal involution, decreases in body mass, and moult to a winter pelage (Bartness & Wade, 1985; Heldmaier et al., 1981).

Seasonally polyphenic species exhibit marked genomic plasticity: many genes involved in the neuroendocrine regulation of reproduction and metabolism exhibit striking seasonal changes in expression (Ross et al., 2004; Mukai et al., 2008; Nakao et al., 2008; Ross et al., 2011; Stevenson et al., 2012; Stevenson & Prendergast, 2015). For example, deiodinases type II (*dio2*) and III (*dio3*) exhibit robust light-induced changes in expression in birds (Nakao et al., 2008; Perfito et al., 2012) and mammals (Ross et al., 2011; Barrett et al., 2007; Prendergast et al., 2013; Stevenson & Prendergast, 2013; Sáenz de Miera et al., 2014). Changes in these enzymes modulate localized synthesis of thyroid hormone in periventricular regions of the central nervous system (CNS; reviewed by Wood & Loudon, 2014) and evidence suggests that *dio2* and *dio3* expression play a central role in triggering seasonal changes in the activity of the hypothalamo-pituitary gonadal (HPG) axis. In Siberian hamsters, mediobasal hypothalamic (MBH) expression of *dio3* is significantly greater in SD conditions (Watanabe et al., 2007; Barrett et al., 2007). Photoperiod- and melatonin-induced decreases in DNA methylation in the *dio3* proximal promoter region have been reported, which may play a role in LD-induced decreases in *dio3* mRNA expression (Stevenson & Prendergast, 2013). Although the mechanisms by which photoperiod and melatonin disinhibit *dio3* expression are not fully characterized, the decreases in the expression of mRNAs that code for DNA methyltransferases (*dnmt*s) occur in parallel with decreases in *dio3* promoter methylation (and increases in *dio3* mRNA expression), suggesting that circannual changes in thyroid hormone catabolism in the periventricular region of the MBH may be mediated, in part, by epigenetic modifications.

Mechanistic and functional studies of epigenetic processes have largely focused on development (Waddington, 1952; Roth & Sweatt, 2010) and inheritance (Weaver et al., 2004). Epigenetic modifications commonly include two processes: DNA methylation and histone acetylation. DNA methylation involves the binding of methyl groups (CH_3) onto cytosine-guanine paired nucleotides in genomic DNA (Klose & Bird, 2006; Suzuki & Bird, 2008), and the activity of DNA methyltransferases are critical for the methylation of these residues. Increased levels of methylation are associated with reductions in RNA expression (Jones, 2012). A complimentary epigenetic process—histone acetylation— is mediated by a family of enzymes known as histone deacetylases (*hdacs*); *hdacs* participate in the removal of acetyl groups from histones (Struhl, 1998). Decreased histone acetylation leads to chromatin retraction, thereby inhibiting access to the DNA template and decreasing gene transcription (Struhl, 1998). The identification of the circadian and circannual changes in the epigenome is important for determining the functional relevance of epigenetic plasticity on rhythmic physiology and behaviour (e.g., Masri & Sassone-Corsi, 2013; Azzi et al., 2014).

Accumulating evidence indicates that some epigenetic processes are plastic, and even reversible. Circadian changes in metabolism and behaviour are strongly associated with circadian cycles in histone acetylation (Masri & Sassone-Corsi, 2010, 2013), and there exist clear circadian changes in levels of DNA methylation in a number of gene promoter regions (Azzi et al., 2014). In mice, both *dnmt3a* and *dnmt3b* expression can be induced in response to light in a circadian phase-dependent manner (Azzi et al., 2014). Exposure to SD sufficient to induce gonadal regression downregulates expression of *dnmt*s in the hamster hypothalamus; moreover, SD-induced reductions in hypothalamic expression of *dnmt*s reverts back to LD-like expression levels following the development of photorefractoriness, indicating plasticity over a circannual timescale (Stevenson & Prendergast, 2013).

Because there exist separate lines of evidence for circadian as well as seasonal changes in DNA methylation (Azzi et al., 2014), this experiment tested the hypothesis that these rhythmic processes interact, thus I examined whether seasonal time (photoperiod) affects the circadian waveforms of hypothalamic *dnmt* (*1*, *3a* and *3b*) mRNA expression. Transcript levels were analysed for three alignments: light-onset [*Zeitgeber* time], dark-onset [clock time] and mid-point of the dark/light phase). This statistical approach is not commonly used, but in this case I sought to evaluate, as critically as possible, whether photoperiod-driven differences in gene expression reflect artefacts of phase alignment or instead reflect more enduring/persistent differences between the seasonal phenotypes. If, for example, differences between photoperiod groups persist irrespective of phase alignment, this would constitute evidence in favor of the latter interpretation. Additionally, as no evidence exists in hamsters on the hypothalamic expression of *hdac* enzymes over season or time, this study also tested the hypothesis that *hdac1-4* mRNAs were regulated by these photoperiodic conditions. Relatively high-frequency sampling intervals (3 h) were used, which permitted characterization of circadian waveforms.

METHODS

Animals. Adult male Siberian hamsters (*Phodopus sungorus*) were selected from a colony maintained at the University of Chicago. Hamsters were housed in polypropylene cages illuminated for 15 h per day (LD; lights off at 17:00 h CST). Food (Harlan, Teklad) and filtered tap water were provided *ad libitum*. All procedures were approved by the Animal Care and Use Committee at the University of Chicago.

Photoperiod treatment. On week 0, hamsters were either transferred to a reproductively-inhibitory short day length (9 h light/day; lights off at 17:00 h CST; SD; n=65), or remained in their natal LD photoperiod (n=64). Hamsters remained in the LD and SD photoperiods for 40-41 days (<6 weeks)

weeks, a time interval near which MBH *dio3* expression has been reported to peak in SD in this species (Barrett et al., 2007).

Tissue collection. Hamster hypothalami (approximately n=8/time point/photoperiod) were collected at 3 hour intervals starting at 1.5 hour after light onset. Animals were anesthetized with isoflurane (3% in O_2), brains were isolated via rapid decapitation, and the whole hypothalamus was dissected and quickly frozen in powdered dry ice. In all studies, the anatomical boundaries for hypothalamus dissection were: the optic chiasm at the anterior border, the mammillary bodies at the posterior border, and laterally at the hypothalamic sulci. Extracted tissue was cut dorsally 3-4 mm from the ventral surface (cf. Prendergast et al., 2013). Shortly (1-2 h) after collection tissues were transferred from dry ice to storage at -80ºC.

RNA extraction. RNA was extracted from hypothalamic tissue using a QIAGEN AllPrep DNA/RNA mini kit. RNA concentration and quality were determined by spectrophotometer (Nanodrop, Thermo Scientific, Wilmington, DE). cDNA was synthesized using Superscript III (Invitrogen, Carlsbad, CA), and stored at -20ºC until quantitative PCR was performed.

Quantitative Polymerase Chain Reaction. Primers for *dnmts (1, 3a* and *3b*) and *hdacs (1-4*) were designed based on conserved regions of mouse, rat and human sequences using PrimerExpress software and optimized for use in Siberian hamsters (Table S1). A Standard nucleotide BLAST was used to confirm sequence specificity. qPCR reactions were performed with 2µl of cDNA using a BIORAD CFX384 system. Following initial denaturation at 95°C for 30 s, we performed 39 cycles of: i) 95°C for 10 s, ii) annealing at temperatures that varied based on the target mRNA (see Table S1) for 30 s, and iii) extension at 72°C for 30 s. Quantification of mRNA expression was accomplished with iQ Sybr Green

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Supermix (BIORAD, Hercules, USA). After the final PCR cycle, all reactions were subjected to melting curve analyses to determine the quality and specificity of each reaction. Select amplicon sequences were run in a 2.5% agarose gel to confirm PCR product size and primer specificity and were directly sequenced via dideoxynucleotide sequencing at the University of Chicago Comprehensive Cancer Center, DNA Sequencing & Genotyping Facility. Reaction efficiencies (E) and cycle thresholds (CTs) were calculated using PCR Miner (Zhao & Fernald, 2005). In accordance with MIQE guidelines (Bustin et al., 2009), samples with E values <0.8 or >1.2 were excluded from subsequent analyses. The expression of each target gene of interest was calculated for individual samples using $2^{-(delta-deltaCt)}$ calculation (relative to the reference gene, glyceraldehyde phosphate dehydrogenase [*gapdh*]).

Effects of photoperiod x time on control gene expression were analysed by factorial ANOVA followed by Bonferroni-corrected pairwise comparisons for each of the 24 pairwise comparisons. There was a photoperiod x time interaction for *gapdh* expression when aligned for lights on ($F_{7,106}=3.24$, $P<0.05$) and lights off ($F_{7,106}=4.14$, $P<0.05$) but not midpoint ($F_{7,106}=1.80$, $P>0.05$). Post-hoc analyses indicated a significantly lower CTs for *gapdh* expression in LD at two time points in the lights on alignment (1.5ZT and 19.5ZT; $P<0.005$) and in the lights off alignment (0630h and 1830h; $P<0.005$). Thus, the fold-expression values at these points have the potential underestimate mRNA expression levels in LD samples only. Due to limited tissue and RNA availability; additional reference genes could not be examined. To exclude a small number of outliers (<5%), samples with fold-changes greater than 2 standard deviations above the mean for a specific gene were removed from statistical analyses.

Statistical analyses:

mRNA expression values were log-transformed to establish normality. 2-way ANOVAs were conducted for *dnmt1,3a,3b* to examine photoperiod × circadian interactions. However, because exposure

to SD has been previously shown to inhibit hypothalamic *dnmt1, 3b* and *dio2*, and to increase *dio3* expression over intervals that range from a days (Herwig et al., 2012; Prendergast et al., 2013) to weeks (Watanabe et al., 2007; Barrett et al., 2007; Stevenson & Prendergast, 2013), *a priori* planned comparisons (*t*-tests; two-tailed, and one-tailed [where justified by expectations from the literature]) were used to assess directional changes in these mRNA values (all indicated below). For *hdac*s, no *a priori* knowledge was available, thus two-way ANOVAs were used to assess photoperiod and circadian effects, and circadian × photoperiod interactions. If warranted by a significant *F* statistic, post-hoc pairwise comparisons were conducted using Fisher's PLSD tests to limit experiment-wise α inflation.

As discussed in the Introduction, there can be no perfect matching of circadian phase in different photoperiods, thus 3 separate circadian temporal alignments were used for each analysis: (1) ZT alignment: compared values relative the onset of the light phases; (2) Dark Onset: compared values relative to the onset of darkness; (3) Midpoint (MP): compared values relative to the MP of the light phase. This afforded a less-biased evaluation of whether photoperiodic effects reflect enduring changes in physiology or may be artefacts of collection time.

CircWave (v1.4) software (www.huttlab.nl; www.euclock.org, courtesy of Dr. Roelof Hut) was used to assess mRNA rhyhtmicity (Zhou et al., 2014). One sine wave function was applied to the data; *F* and *P* values, and centres of gravity (COGs±SD) were obtained for each fitted curve. All other statistical analyses were performed using SigmaPlot 13.0; differences were considered significant if P≤0.05.

RESULTS

SD exposure reduced hypothalamic *dio2* and increased *dio3* expression

Assessment of *dio2* and *dio3* expression confirmed the anticipated SD effect on hypothalamic gene expression. One-tailed *t*-tests revealed that SD significantly reduced hypothalamic *dio2* (t_{117}=-2.218; P<0.05; Fig1a) and increased *dio3* (t_{114}=3.21; P<0.001; Fig1b) expression.

Effects of circadian time on photoperiodic changes in *dnmt* expression:

Two-way ANOVA indicated a non-significant trend for greater *dnmt1* expression in LD compared to SD ($F_{1,103}$=3.20, P=0.07; Fig2a; FigS1a). There were no significant main effects for *dnmt1* when aligned for lights on ($F_{7,103}$=1.19, P=0.19; Fig2a); lights off ($F_{7,103}$=1.52, P=0.16; Fig2d) or midpoint ($F_{7,103}$=0.62, P=0.73; Fig2g). Moreover, there was no significant photoperiod x circadian interaction for lights on ($F_{7,103}$=1.43, P=0.19) or lights off ($F_{7,103}$=1.33, P=0.24). Given the *a priori* hypothesis that *dnmt* expression is increased in LD hypothalami, *post-hoc* one-tailed t-tests were performed. When aligned for lights-on, *dnmt1* was significantly greater in LD hypothalami at ZT13.5 (P<0.01) and ZT19.5 (P<0.01; Fig2a); when aligned for lights off, *dnmt1* was greater in LD at (0330h (P<0.05) and 2130 (P<0.05);Fig2d). *dnmt1* was significantly greater in SD when aligned for lights off at 0630 h (P<0.05; Fig2d). Lastly, there was a significant photoperiod x time interaction when data were aligned by MP ($F_{7,103}$=3.20, P<0.05). Post-hoc analyses revealed *dnmt1* expression was greater in LD hypothalami at -9hr (P<0.001) and at MP (P<0.05), no additional differences were evident between LD and SD *dnmt1* when aligned for MPL

Two-way ANOVA revealed a significant increase in *dnmt3a* expression in the LD hypothalamus ($F_{1,105}$=10.15, P<0.005; Fig2b; FigS1a). Moreover, there were significant main effects for *dnmt3a* when aligned for lights on ($F_{7,105}$=5.26, P<0.001; Fig2b) and MP ($F_{7,105}$=6.46, P<0.001; Fig2h), but not when aligned for lights off ($F7_{,105}$=1.34, P=0.23; Fig2e). Significant interaction effects of photoperiod x circadian time for *dnmt3a* were identified for lights on ($F_{7,105}$=3.20, P<0.005), lights off ($F_{1,105}$=7.16,

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P<0.001) and a non-significant trend was identified for MP ($F_{7,105}$=1.96, P=0.06). *dnmt3a* expression was significantly greater in LD at ZT1.5 (P<0.001), ZT13.5 (P<0.05) and ZT19.5 (P<0.01) after lights on (Fig.2b). When aligned for lights off, *dnmt3a* expression was greater in LD at 0330h (P<0.001), 1530h (P<0.01), 1830h (P<0.01) and 2130h (P<0.05), and higher in SD at 0630h (P<0.001) after lights off. When aligned for MP, *dnmt3a* was higher LD at -9h (P<0.05), +6h (P<0.05) and +9h (P<0.05). At -3h relative to MP *dnmt3a* was significantly higher in SD hypothalami (P<0.05).

There was no significant main effect of photoperiod on *dnmt3b* expression ($F_{1,101}$=1.03, P=0.31; Fig2a; FigS1a) nor did circadian time affect expression when aligned for lights on ($F_{1,101}$=1.81, P=0.09; Fig2c); there was a non-significant trend towards an effect fo photoperiod when aligned for lights off ($F_{7,101}$=1.97, P=0.06; Fig2f), but not for MP ($F_{7,101}$=1.51, P=0.17; Fig2i). There were non-significant trends for a photoperiod x circadian time interactions when aligned for lights on ($F_{7,101}$=1.78, P=0.09) and MP ($F_{7,101}$=1.97, P=0.06), but not for lights off ($F_{7,101}$=1.60, P=0.16). One-tailed tests indicated that *dnmt3b* expression was significantly greater in LD hypothalami when aligned for lights on at ZT19.5 (P<0.005) and ZT22.5 (P<0.05; Fig2c); when aligned for lights off at 0330h (P<0.05; Fig2f) and when aligned for MPL at -9hr (P<0.005; Fig2i). *dnmt3b* was significantly greater in SD hypothalami when aligned for lights off at 0630h (P<0.05).

Effects of circadian time on photoperiodic changes in *hdac* expression:

There was no significant main effect of photoperiod on *hdacs1-3* expression (*hdac1*: $F_{1,102}$=0.09, P=0.76; *hdac2*: $F_{1,104}$=0.72, P=0.39; *hdac3*: $F_{1,103}$=0.15, P=0.69; FigS1b). Moreover, there was no significant circadian time main effect for lights on (*hdac1*: $F_{7,102}$=1.46, P=0.18; *hdac2*: $F_{7,104}$=0.87, P=0.52; *hdac3*: $F_{7,103}$=0.51, P=0.82; Fig3a), lights off (*hdac1*: $F_{7,102}$=0.68, P=0.68; *hdac2*: $F_{7,104}$=0.62, P=0.73; *hdac3*: $F_{7,103}$=0.68, P=0.69; Fig3e) or MP (*hdac1*: $F_{7,102}$=0.41, P=0.88; *hdac2*: $F_{7,104}$=0.43,

P=0.87; *hdac3*: $F_{7,103}$=0.47, P=0.85; Fig3i) alignments. In addition, *hdacs1-3* did not have exhibit significant photoperiod x time interactions: lights on (*hdac1*: $F_{7,102}$=0.47, P=0.85; *hdac2*: $F_{7,104}$=0.72, P=0.39; *hdac3*: $F_{7,103}$=0.78, P=0.60), lights off (*hdac1*: $F_{7,102}$=1.30, P=0.25; *hdac2*: $F_{7,104}$=0.67, P=0.69; *hdac3*: $F_{7,103}$=0.60, P=0.75) or MP (*hdac1*: $F_{7,102}$=1.64, P=0.13; *hdac2*: $F_{7,104}$=0.76, P=0.61; *hdac3*: $F_{7,103}$=0.83, P=0.55).

There was no significant main effect of photoperiod on *hdac4* expression ($F_{1,104}$<0.001, P=0.99; FigS1b). However, there were significant main effects of circadian time when aligned for lights on ($F_{7,104}$=3.29, P<0.005; Fig3d), and MP ($F_{7,104}$=2.80, P<0.01; Fig3l), but not for lights off ($F_{7,104}$=1.39, P=0.21; Fig3h). There were significant photoperiod x time interactions for lights on ($F_{7,104}$=2.08, P<0.05), lights off ($F_{7,104}$=3.95, P<0.001) and MP ($F_{7,104}$=2.52, P<0.05). Post-hoc analyses indicated that *hdac4* expression was significantly greater in LD hypothalami when aligned for lights on at ZT13.5 (P<0.05); when aligned for lights off, *hdac4* was greater in SD 0630h after lights off in SD (P<0.001), but greater in LD 1530h after lights off (P<0.01). When aligned by MP, *hdac4* was greater in SD at -6 h (P<0.05), but greater in LD at +6 h (P<0.05).

CircWave analyses of *dnmt3a* and *hdac4*

Table S2 (*dnmt*s) and Table S3 (*hdac*s) provide the complete CircWave analyses. In LD hamsters, longitudinal waveform analyses revealed clear circadian rhythms in *dnmt3a* (F=5.10, P<0.01) and *hdac4* (F=7.17, P=0.001) expression (Fig. 4A). No other mRNAs exhibited significant circadian rhythms in the LD hypothalamus (P>0.18, all other analyses). In SD hamsters, *dnmt3a* (F=4.49, P<0.01) exhibited a significant circadian rhythm, and there was a trend for *hdac4* expression (F=2.76, P=0.07; Fig. 4B). All other mRNAs were not significant (P>0.33, all other analyses).

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DISCUSSION

Circannual rhythms are ubiquitous in nature, and photoperiodic regulation of reproduction is one salient example. Scores of genes exhibit diurnal variation in expression levels (Duffield, 2003); however photoperiod alters both the amplitude and the waveform of RNA expression in many tissues (Johnston et al., 2005; Inagaki et al., 2007; Sosniyenko et al., 2010). In the present work, hypothalamic *dnmt3a* and *hdac4* expression exhibited robust circadian rhythms. For *dnmt3a*, and to a lesser extent *dnmt1, dnmt3b* and *hdac4*, there were multiple points in the circadian phase at which expression was significantly greater in the LD compared to SD hypothalami. Overall, these data are consistent with the hypothesis that that widespread epigenomic reprogramming occurs over circadian and circannual timescales, and possibly via multiple distinct mechanisms.

Light induced changes in DIO enzyme expression in the hypothalamus appear to be an evolutionarily-conserved mechanism that contributes to the generation of seasonal rhythms in reproductive physiology (Wood & Loudon, 2014). Here, *dio2* and *dio3* expressed were analysed to establish the neuroendocrine response to photoperiodic cues at a molecular level. In Siberian hamsters, SD increases the expression of *dio3* mRNA in the periventricular ependymal cell layer (Watanabe et al., 2007; Barrett et al., 2007; Stevenson & Prendergast, 2013); upregulation of *dio3* is associated with a decrease in T3 signaling (Barrett et al., 2007; Herwig et al., 2013). The data indicate that *dio3* mRNA is consistently greater in SD, and given the large sample size the power to detect the SD-induced decrease in *dio2* expression. Previous analyses had established that LD was associated with significantly greater DNA methylation in the *dio3* promoter (Stevenson & Prendergast, 2013). An examination of DNA methylation and/or histone acetylation of the *dio2* or *dio3* promoter were beyond the scope of the present investigation. However, if the *dio3* promoter is actively methylated in LD and demethylated in SD, then insights into temporal relations between *dnmt* and *dio* expression may prove useful in further

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understanding the mechanisms of *dio3* regulation. In LD hypothalami, *dio3* mRNA expression was lowest during an interval beginning ~16:30 h and extending until ~22:30 h; similar to those reported by Perfito and colleagues (2012) in birds. This interval was either preceded by or overlapped with increased *dnmt1* and *dnmt3a* expression. Although these alignments do not permit causal interpretations, they depict a temporal pattern of expression that is consistent with a role for DNA methylation for the transcriptional inhibition of *dio3* in LD.

In recent years, there have been substantial developments in the field of chronobiology that have identified dynamic epigenetic modifications over the course of the day and the year in the adult mammalian brain (Masri & Sassone-Corsi, 2013; Alvarado et al., 2014). This work has implicated strong circadian rhythms in *dnmt3a* and *hdac4* expression. In mice, genome-wide analyses identified changes in methylation within the suprachiasmatic nucleus that occurred over the circadian cycle (Azzi et al., 2014). The data reported here and by Azzi and colleagues (2014) indicate that one effector of the daily epigenetic modifications to the genomic template is likely the high amplitude circadian variation in *dnmt3a* expression. DNMT3a show preferential genomic regions in HEK293T cells (Choi et al., 2011), and in the SCN, the circadian clock gene *bmal* promoter is a target for circadian variation in methylation (Azzi et al., 2014). In many rodents, one of the promoters for *dnmt3a* exhibits a number of E-box elements, a key binding site for BMAL. Together, these data indicate a BMAL-DNMT3a feedback loop that could, potentially, participate in one of the many molecular outputs of the circadian clock.

In addition to circadian variation in DNA methylation, daily variations in chromatin structure mediated by histone acetylation have significant implications for timing metabolism and locomotor behavior (Aguilar-Arnal & Sassone-Corsi, 2015; Sahar & Sassone-Corsi, 2013; Masri & Sassone-Corsi, 2013). One molecular signalling event includes the interaction between sirtuin-1 and a circadian clock gene (CLOCK) and the deactylation of *bmal1* and *per2* (Nakahata et al., 2008; Asher et al., 2008). In

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association with SIRT1; this complex of epigenetic activity links metabolic state and circadian information to regulate neuronal function (Masri & Sassone-Corsi, 2010). The present report extends these insights into the epigenetic correlates of circadian rhythms that participate in histone deacetylation. Specifically, *hdac4* mRNA exhibited a complex pattern of changes in expression that differed between LD and SD. *hdac4* expression peaked during the late portion of the light phase in LD, and peaked during the late portion of the dark phase in SD. Thus, when aligned by ZT (light onset) *hdac* expression levels were greater in LD at one time point over the circadian cycle; but when aligned by dark onset or photophase midpoint, *hdac4* was higher in LD at one time point, and higher in SD at another. Absent information on which chromatin regions are being deacetylated by *hdac4*, the biological significance of *hdac4* expression is not presently clear. However, phase relations between the timing of peak *hdac4* expression and the expression of other genes in the hypothalamus warrants further examination.

In contrast to *hdac4*, no effects were detected on *hdac1, hdac2,* or *hdac3* expression in the hypothalamus. Whereas *hdacs1-3* are expressed in a wide range of tissue and cell types throughout the body, *hdac4* is predominantly expressed in the brain (Darcy et al., 2010). Omnibus changes in mediobasal hypothalamic *hdac4* expression suggest that histone deacetylation, and concomitant inhibitory effects on gene transcription, may participate in circadian photoperiodic decreases in RNA expression. A limitation of the present work is that the absence of an effect of photoperiod on expression of *hdacs1-3* in mediobasal hypothalamic tissue samples cannot exclude these enzymes as possible participants in photoperiodic control of reproduction and behavior: a given photoperiod may upregulate RNA expression in some hypothalamic nuclei and downregulate it in others, with a net effect of no difference at the level of the entire diencephalon.

Circannual and photoperiodic variations in DNA methylation are becoming better elaborated, from a comparative perspective as well. For example, in the photoperiodically regulated seasonal

diapause response in the wasp (*Nasonia vitripennis*), *dnmt3* expression was significantly increased in SD conditions and blocking DNA methylation prevented the development of diapause (Pegoraro et al., 2016). Similarly in hibernating ground squirrels (*Ictidomys tridecemlineatus*) there was a marked reduction in liver *dnmt1* and *dnmt3b* during the development of torpor (Alvarado et al., 2015). Previous work in Siberian hamsters has shown that tissue-specific *dnmt1, dnmt3a* and *dnmt3b* exhibit circannual changes in expression that are driven by photoperiod and melatonin (Stevenson and Prendergast, 2013; Stevenson et al., 2014; Lynch et al., 2016): 8 or 10 weeks of exposure to SD significantly reduced hypothalamic *dnmt1* and *dnmt3b*. Although a number of differences preclude direct comparisons between this and prior reports in this species, the present report confirms the SD reduction in hypothalamic *dnmt1* expression and the absence of photoperiodic modulation of *dnmt3a* at the midpoint of the light phase; the additional sampling points, however, revealed a robust circadian change in *dnmt3a* expression. Similar to previous work, hypothalamic *dnmt3b* expression was reduced in SD hypothalami at specific circadian time points. Experimental differences between the present and prior work may provide insights into the temporal sequence of *dnmt* expression following adaptation to SD. The present work study examined hypothalamic mRNA expression after <6 weeks of exposure to SD, as compared to the 8-10 weeks of SD exposure in a prior report (Stevenson & Prendergast, 2013). Similar to the scores of photoperiod regulated transcripts in the hamster hypothalamus (Petri et al., 2016), the present data suggest that SD inhibition of the phase and magnitude of *dnmt3a* and *dnmt3b* expression may be dependent on the length of time after exposure to SD. *dnmt3a* may be expressed at higher levels shortly after exposure to SD, whereas *dnmt3b* may require longer intervals of SD to exhibit decreased expression at more than one point in the circadian cycle, although this conjecture awaits a more detailed time course of photoperiodic changes in the circadian waveform of these transcripts. Substantial differences in experimental power at specific circadian points also exist between the two reports (n=5-8

per group in the present work vs. n=18-25 in Stevenson & Prendergast, 2013). However, the major advantage/trade-off of experimental power for the present study is the assessment of mRNA expression at multiple circadian points; this approach permits the dissection of robust daily variation in *dnmt* levels. These data indicate that *dnmt3a* exhibits robust photoperiodic changes in the hamster hypothalamus and may function to impart circannual/photoperiodic epigenomic re-programming in LD conditions. Given the robust SD-induced decrease in hypothalamic *dnmt3a* expression; and similar change recently reported in the testes and uteri of hamsters (Lynch et al., 2016), photoperiodic variation in *dnmt3a* may warrant special attention as a key enzyme involved in early and sustained photoperiod-driven epigenomic plasticity. Lastly, the present work suggests that *dnmt1* down regulation may be an early response to SD photoperiods.

The current limitations of the paper are the functional outcome and anatomical localization of annual and circadian rhythms in *dnmt3a* and *hdac4* expression. Indeed *dnmt3a* is expressed in many hypothalamic regions, such as areas involved in circadian rhythms (Azzi et al., 2014), long-term regulation of hypothalamic neuromorphology (Stevenson & Prendergast, 2013) and energy balance (Kohno et al., 2014). Similarly, medial hypothalamic *hdac4* expression has been shown to be sensitive to energy balance (Funato et al., 2011). Future directions will examine the anatomical specificity of circadian rhythms in hypothalamic *dnmt3a* and *hdac4* expression coupled with targeted genetic manipulations that permit the identification of the functional consequences of disrupted epigenetic modifications for the daily rhythms in hypothalamo-pituitary signalling.

In summary, the present data identified photoperiodic changes in the circadian waveforms of *dnmt3a* and *hdac4* expression in the hamster hypothalamus, illustrating dynamic changes in the expression of key epigenetic enzymes. SD also decreased *dnmt1* and *dnmt 3b*, but did not affect *hdacs1-3*. The vast majority of circannual breeders use the annual change in day length as a predictive cue to

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time reproduction. Scores of genes located in key neuroendocrine brain regions exhibit marked circannual changes in expression in both mammalian (Ross et al., 2011; 2004) and avian (Stevenson et al., 2012, Mukai et al., 2008, Nakao et al., 2008) models. The extent to which photoperiodic changes in methylation and histone acetylation participate in theses annual patterns of RNA expression remains largely unexplored. Prior work has established a link between DNA methylation in the *dio3* promoter; it is yet to be examined whether similar epigenetic modifications regulate *dio2* expression. It is likely that some of the annual changes in RNA expression are governed by entirely separate mechanisms from epigenetic modifications, yet it is a potentially fruitful avenue towards a deeper understanding of the molecular mechanisms underlying circannual timing.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1 – Photoperiodic regulation of relative *dio2* and *dio3* expression. Short days (SD) significantly reduced hypothalamic *dio2* expression (a) and increased *dio3* expression (b) compared to long day (LD) hamster hypothalami. $*P < 0.05$; $** P < 0.01$; $***P < 0.001$.

Figure 2 – Circadian and photoperiodic variation in relative *dnmt* expression. *dnmt1* (a, d, g) expression tended to be greater in long days (LD) compared to short days (SD) with significantly higher

levels in midpoint alignment at -9hr and midpoint (MP). *dnmt3a* (b, e, j) expression was

significantly greater in LD compared to SD regardless of circadian alignment. Several

pairwise comparisons indicate that *dnmt3a* levels were higher in LD compared to SD. *dnm3b*

expression did not show large circadian or photoperiodic differences. (Top) lights on;

(middle) lights off and (bottom) midpoint of the light phase (MP). Asterisks indicates

significance at *$P < 0.05$; ** $P < 0.01$; ***$P < 0.001$.

Figure 3 – Circadian and circannual variation in relative *hdac* expression. There were no significant

circadian nor circannual changes in *hdac1* (a, e, i); *hdac2* (b, f, j) or *hdac3* (c, g, k). *hdac4*

expression (d, h, l) revealed significant circadian rhythms with generally greater expression

occurring in LD conditions. Data are aligned by lights on (Top); lights off (middle) and

midpoint of the light phase (MP; bottom). White box depicts lights 'on' and black box

depicts lights 'off'. MP indicates midpoint. *$P < 0.05$; ** $P < 0.01$; ***$P < 0.001$.

Figure 4 – Circadian rhythms in relative *dnmt3a* and *hdac4* expression. (A) *dnmt3a* expression exhibits

a significant circadian waveform in long day (LD) and short day (SD). *hdac4* expression also

exhibits a significant circadian waveforms in LD (B); but a slight trend in the SD condition.

CircWave generated waveforms overlay *dnmt3a* and *hdac4* expression from Fig.2b and

Fig.3d, respectively. Data are double plotted and white-black boxes indicate lights 'on' and
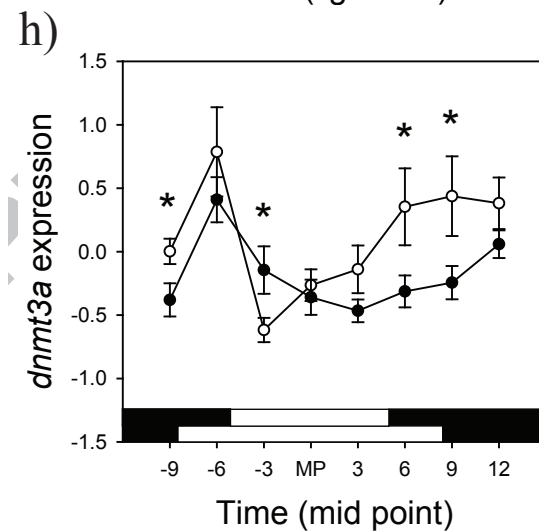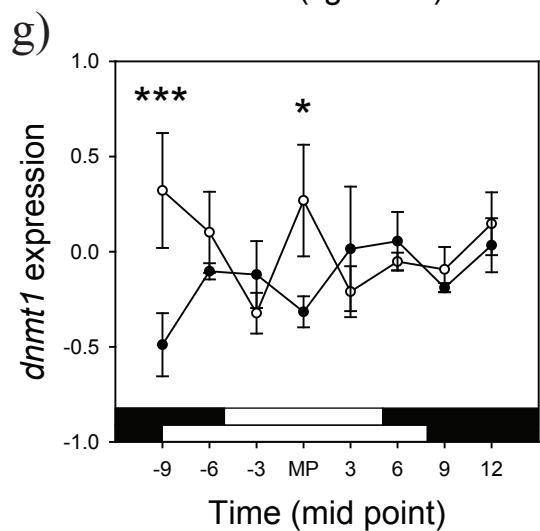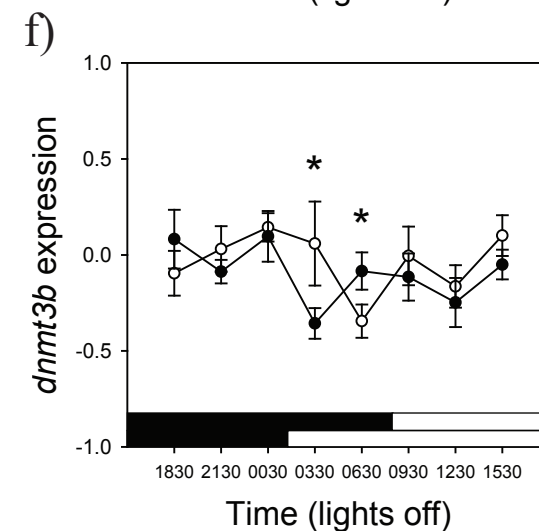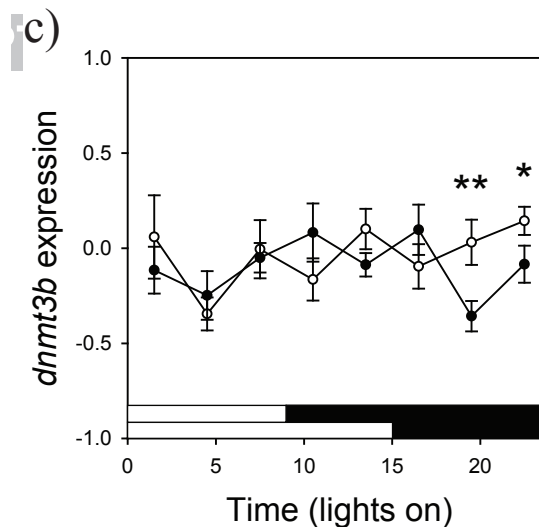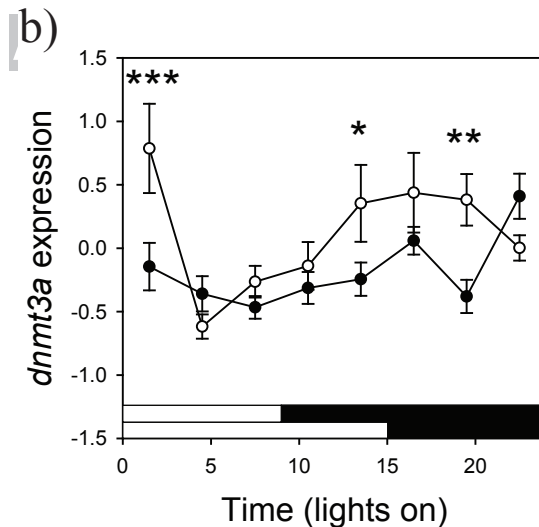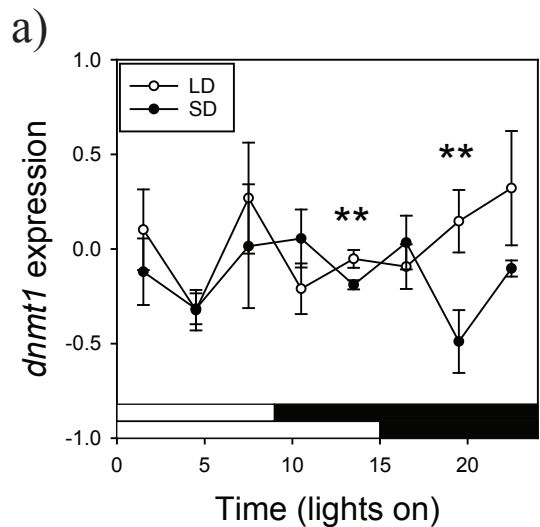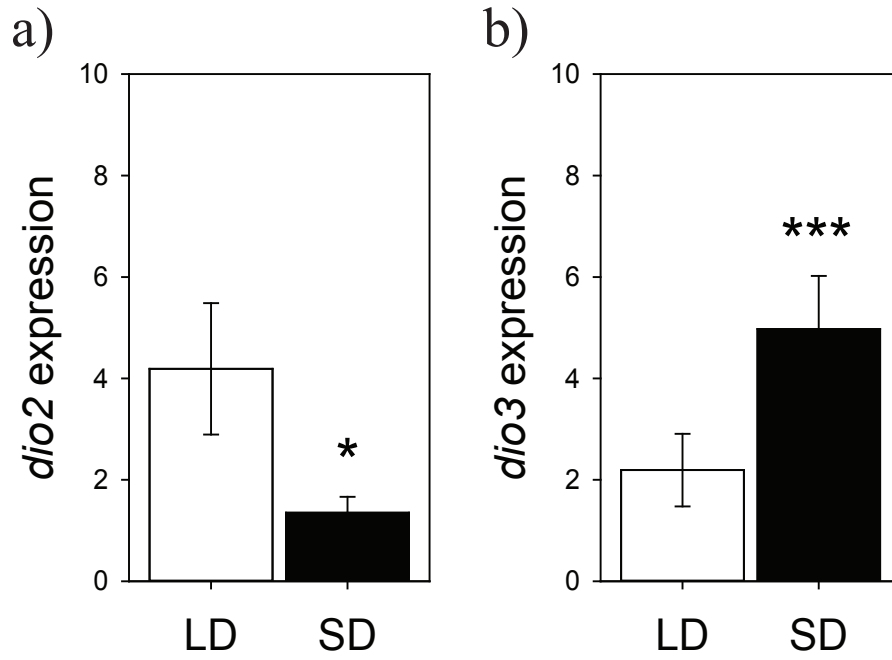
'off', respectively.

A)

B)

a)
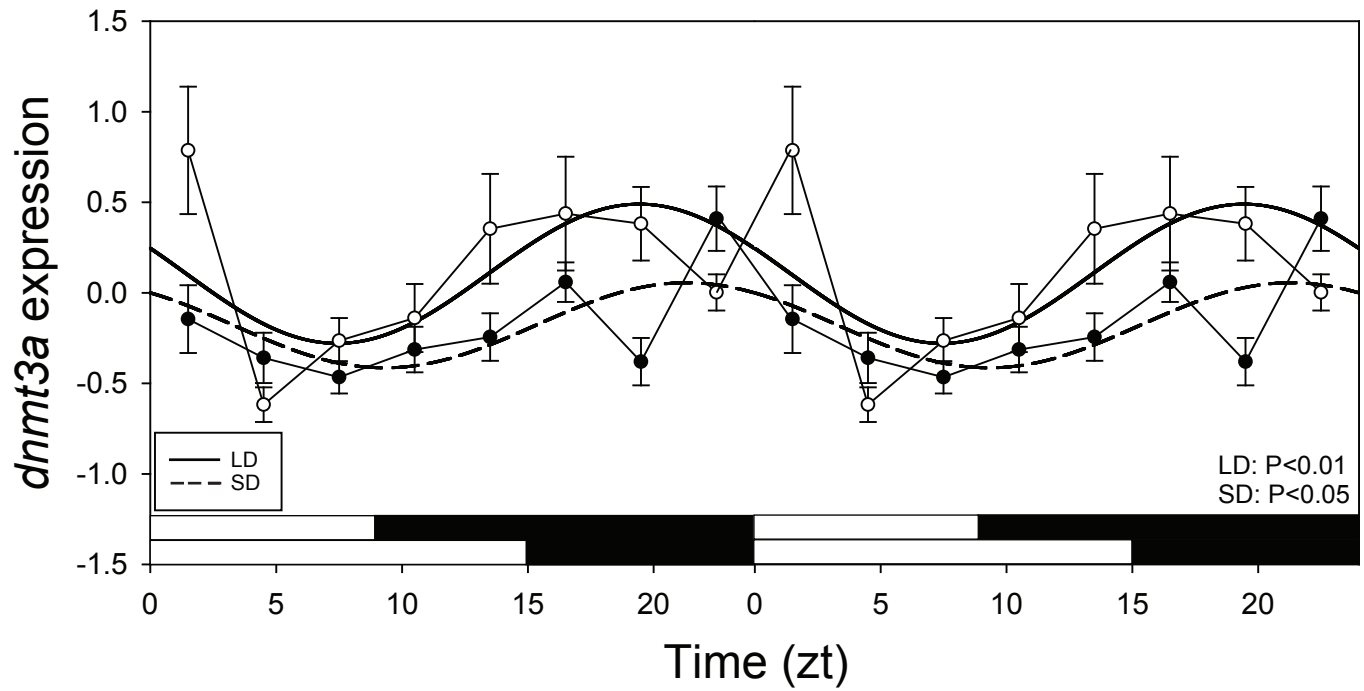
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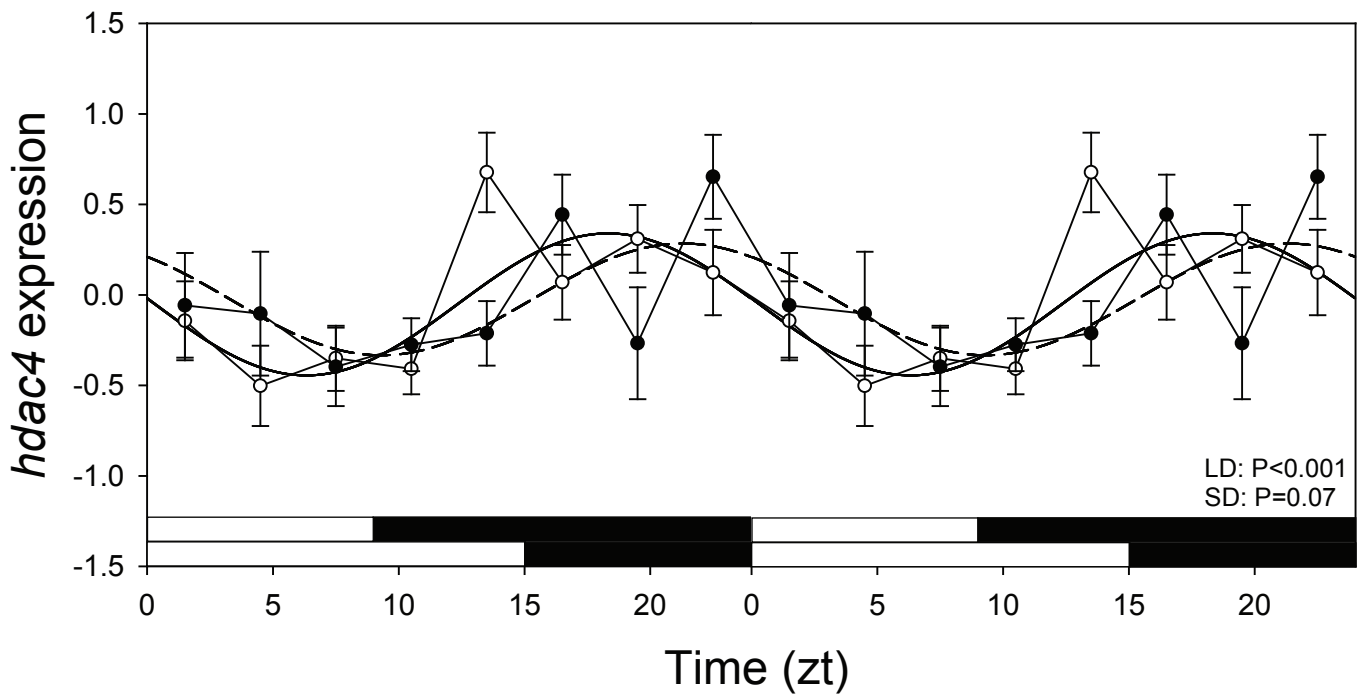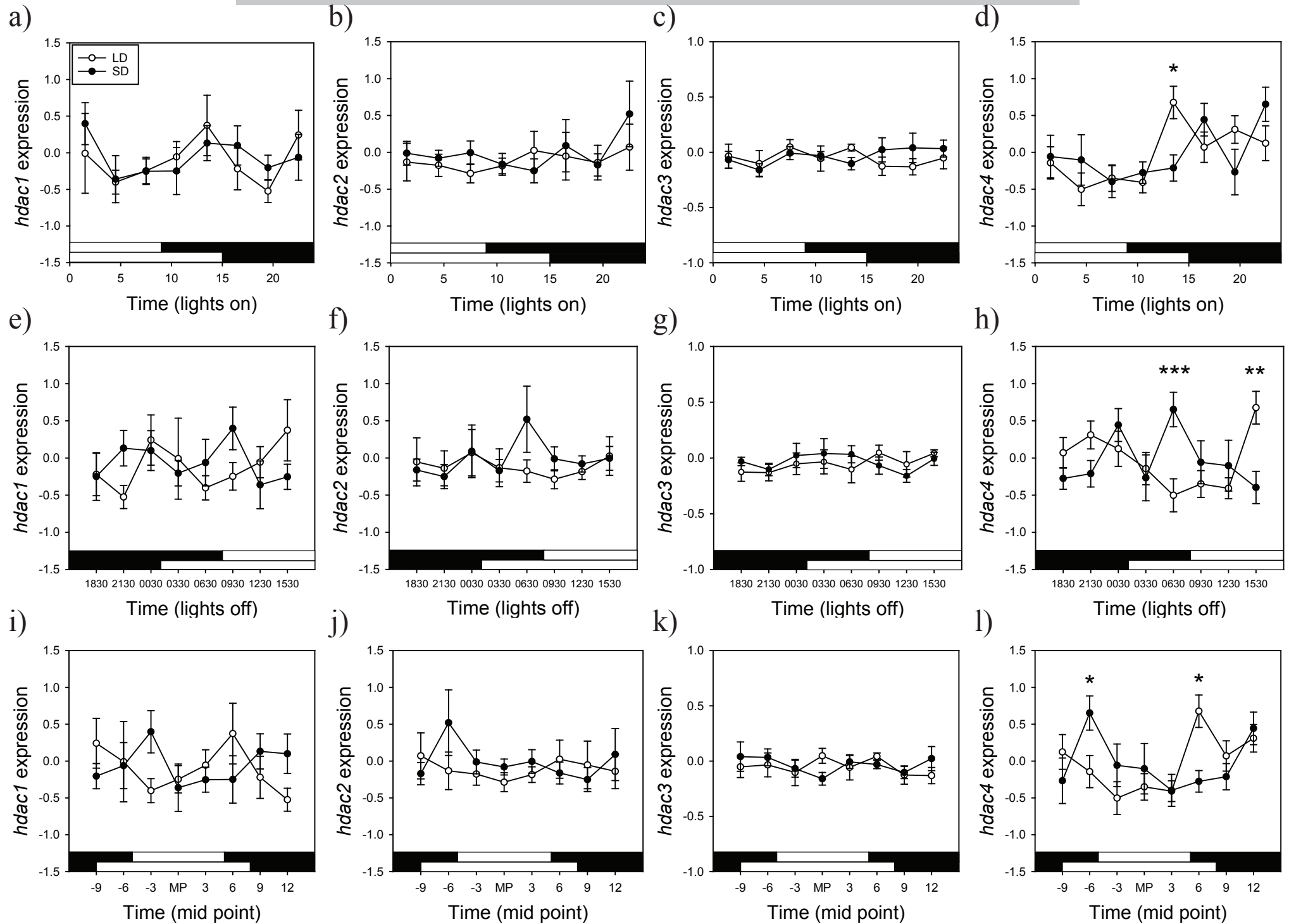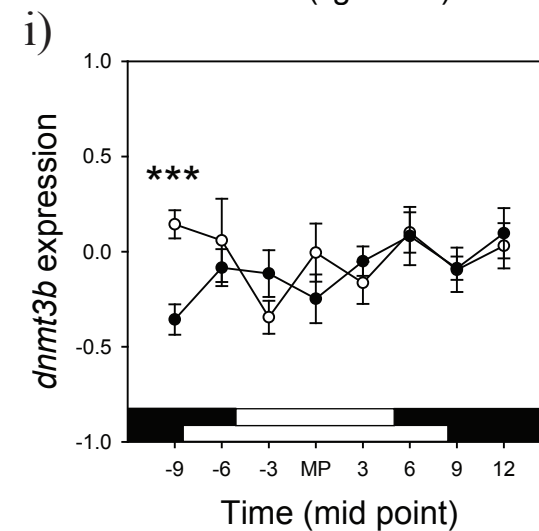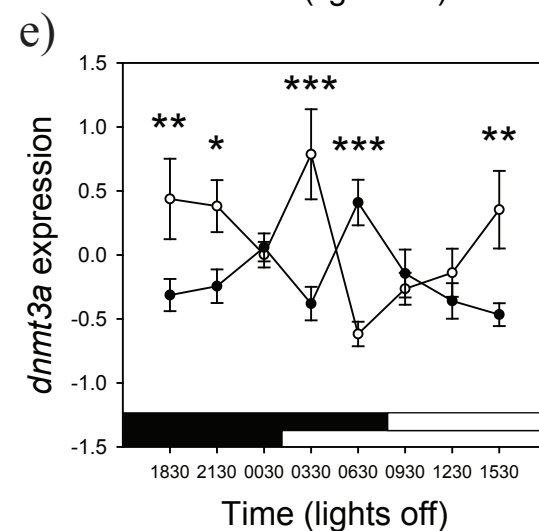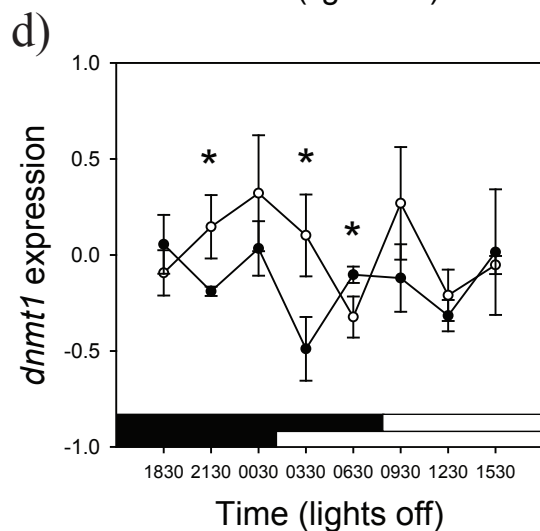
Highlights

1. Epigenetic enzyme transcript expression varies considerably over multiple timescales

2. Hypothalamic *dnmt* expression is significantly reduced short day, winter conditions

3. *dnmt3a* and *hdac4* expression are elevated during the circadian dark phase

4. Evidence indicates epigenetic regulation of timing in multiple neuroendocrine systems