Corrections

CORRECTIONS

PERSPECTIVE

The authors note that on page 582, left column, first paragraph, line 3, “10 metric tons (MT)” should instead appear as “10 million metric tons (MT).”

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BIOPHYSICS AND COMPUTATIONAL BIOLOGY

The authors note that, due to a PNAS error, the author contributions footnote appeared incorrectly. The corrected author contributions footnote appears below. The online version has been corrected.

Author contributions: H.R. and E.P. designed research; H.R. performed research; H.R. and S.M. performed fluorescence measurements; S.M.I. and B.R. contributed the computational analysis; H.R., S.M.I., S.M., B.R., and E.P. analyzed data; and H.R. and E.P. wrote the paper.

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MEDICAL SCIENCES

The authors note that the author name Klaus Schulte should instead appear as Klaus-Martin Schulte. The corrected author line appears below. The online version has been corrected.


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The authors note the following: “Recent high throughput sequencing has indicated that an upstream region of the dio3 promoter sequence in our paper was the result of a PCR fusion error. The reverse primer located –140bp upstream of the start codon was not specific to Siberian hamsters. As a result, the transcription factor binding site analysis and sodium bisulfite-treated DNA sequence analyses in the original publication were incorrect. To correct this error, we have resequenced the dio3 proximal promoter and conducted replications of the transcription factor binding site analyses and sequencing of sodium bisulfite-treated DNA, using primer sequences with confirmed specificity to Siberian hamster DNA. The corrected dio3 promoter sequence exhibited greater homology with mice and human dio3 promoter (revised Fig. S1), a greater number of CpG sites and a higher CpG frequency (revised Fig. S2) than previously reported. Analysis of sodium bisulfite-treated DNA in the acute LD-SD (Fig. 1F) and photorefractory experiments (Fig. 3E) yielded results consistent with the originally-published report: dio3 promoter DNA methylation was reduced in SD (revised Fig. 1F) and increased in SD-R (revised Fig. 3E). Revised transcription factor binding site analyses have also been performed (revised Table S1). See corrected Table S2 for primers used on sodium bisulfite treated DNA. In addition, the reverse primers for the sequencing and MSRE PCR reactions (Table S2) were originally listed in the incorrect (3′-5′) orientation; the correct orientation (5′-3′) now appears in the revised version of Table S2.

“We thank Drs. Hugues Dardente and David Hazlerigg for their assistance in identifying these errors.

As a result of this error, Figs. 1 and 3 and their legends appeared incorrectly. The figures and their corrected legends appear below. These errors do not affect the main conclusions of the article.

Fig. 1. Short photoperiods inhibit reproduction and activate hypothalamic mRNA expression via epigenetic mechanisms. Acute transfer from LD to SD photoperiods caused gonadal regression (A), increased hypothalamic dio3 mRNA expression (B), and decreased hypothalamic dnmt1 and dnmt3b mRNA expression (C) after 8 wk. (D) Immunocytochemical localization of DNMT3b (DNMT3b-ir) in the hamster mediobasal hypothalamus (MBH). DNMT3b-ir was evident throughout the MBH and in the ependymal cell (EC) layer along the third ventricle (III). (E) Transfer from LD to SD reduced DNA methylation in the dio3 proximal promoter, as measured using an MSRE assay. (F) Proportion of LD and SD hamsters in which no unmethylated DNA was detected at each of 17 CpG sites in the dio3 proximal promoter, as assessed by direct sequencing of sodium bisulfite-treated DNA. The abscissa (not to scale) depicts the 17 CpG sites from –249 to the start codon of the dio3 proximal promoter. Averaging across the entire promoter region that was sequenced, evidence of unmethylation was evident on 15% of CpG sites in LD (i.e., on 85% of CpG sites examined in LD hamsters, no detectable C-to-T bisulfite conversion occurred), whereas in SD, evidence of unmethylated DNA was present on 42% of CpG sites ($\chi^2 = 18.4, P < 0.002$). All data in panels A–E are mean ± SEM. *P < 0.05, ***P < 0.005 vs. LD value.
Fig. 3. Neuroendocrine refractoriness to SD reverses patterns of DNA methylation induced by acute SD exposure. (A) Acute (10 wk, SD) exposure to SD induced gonadal regression, whereas prolonged exposure (42 wk, SD-R) triggered neuroendocrine refractoriness and gonadal recrudescence. Refractoriness in SD-R hamsters was characterized by a complete reversal of hypothalamic dio3 and dnmt3b mRNA expression (B and C) and by remethylation of DNA in the dio3 proximal promoter (D). (E) Proportion of LD, SD, and SD-R hamsters in which no unmethylated DNA was detected in each of 17 CpG sites in the dio3 proximal promoter, as assessed by direct sequencing of sodium bisulfite DNA from the whole hypothalamus. The abscissa (not to scale) depicts the 17 CpG sites from –249 to the start codon of the dio3 proximal promoter. Averaging across the promoter region that was sequenced, evidence of unmethylation was evident on 25% of CpG sites in LD hamsters, whereas in SD hamsters, evidence of unmethylation was present on 39% of CpG sites (χ² = 5.08, P < 0.03). In SD-R hamsters, methylation patterns returned to LD-like values, and evidence of unmethylation was detected on 29% of CpG sites examined (χ² = 0.46, P > 0.40 vs. LD). Five of 17 sites (sites 16, 13, 12, 2, and 1) exhibited reversals in the pattern of methylation in SD-R hamsters. All data in panels A–D are mean ± SEM. *P < 0.05; ***P < 0.005 vs. LD value.

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Reversible DNA methylation regulates seasonal photoperiodic time measurement

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In seasonally breeding vertebrates, changes in day length induce categorically distinct behavioral and reproductive phenotypes via thyroid hormone-dependent mechanisms. Winter photoperiods inhibit reproductive neuroendocrine function but cannot sustain this inhibition beyond 6 mo, ensuring vernal reproductive recrudescence. This genomic plasticity suggests a role for epigenetics in the establishment of seasonal reproductive phenotypes. Here, we report that DNA methylation of the proximal promoter for the type III deiodinase (dio3) gene in the hamster hypothalamus is reversible and critical for photoperiodic time measurement. Short photoperiods and winter-like melatonin inhibited hypothalamic DNA methyltransferase expression and reduced dio3 promoter DNA methylation, which up-regulated dio3 expression and induced gonadal regression. Hypermethylation attenuated reproductive responses to short photoperiods. Vernal refractoriness to short photoperiods reestablished summer-like methylation of the dio3 promoter, dio3 expression, and reproductive competence, revealing a dynamic and reversible mechanism of DNA methylation in the mammalian brain that plays a central role in physiological orientation in time.

Significance

This work examined whether epigenetic mechanisms participate in the regulation of seasonal reproduction. In long-day (summer) breeding hamsters, exposure to inhibitory winter photoperiods, or winter-like patterns of melatonin, altered DNA methyltransferase expression; decreased DNA methylation in the proximal promoter region of deiodinase type III (dio3) in the hypothalamus; and, in turn, increased hypothalamic dio3 expression. Pharmacological blockade of photoperiod-driven demethylation attenuated reproductive responses to winter photoperiods. Winter demethylation was reversed in anticipation of spring: spontaneous reproductive development was accompanied by remethylation of the dio3 promoter and decreases in dio3 mRNA. Methylation dynamics in the adult brain are reversible and may constitute an important component of the mechanism by which seasonal time is represented in the nervous system.

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The results identify that DNA methylation in the adult brain is reversible, photoperiodically regulated, and plays a central role in neuroendocrine genomic plasticity.

**Results**

**Isolation and Characterization of the dio3 Proximal Promoter.** To evaluate DNA methylation in the dio3 transcriptional regulatory region, we sequenced the Siberian hamster dio3 proximal promoter (Fig. S1), defined here as the 916 bp preceding the dio3 start codon. The hamster dio3 promoter exhibited a high proportion of guanine and cytosine nucleotides (34% and 32%, respectively) and contained a total of 63 CpG sites. This CpG frequency ([Observed/Expected] × [Promoter Sequence Length] = 0.625) is considered high, and exceeded the threshold for a “CpG island” (27). Adenine and thymine comprised only 18% and 16%, respectively, of the sequenced proximal promoter. Putative transcription binding sites in the promoter region were statistically evaluated (Fig. S2 and Table S1).

**Short Photoperiods Decrease dio3 Proximal Promoter Methylation.** To examine whether changes in day length alter DNA methylation in the dio3 proximal promoter, male Siberian hamsters were exposed to SD photoperiods, which induced gonadal regression; control hamsters remained in long day (LD) photoperiods (Fig. 1A). As expected, hypothalamic dio3 mRNA expression was significantly increased in SD relative to LD (t = 2.09, P < 0.05; Fig. 1B), absent any changes in dio2 (t = 1.16, P = 0.26). The increase in dio3 mRNA is consistent with numerous reports in this species and results in marked decreases in hypothalamic T3 concentrations under SD (15).

To assess whether changes in hypothalamic dnmt expression participate in the photoperiodic stimulation of dio3 mRNA in SD, we measured expression of mRNAs for dnmt1, dnmt3a, and dnmt3b. SD treatments sufficient to up-regulate dio3 mRNA significantly decreased the expression of dnmt3b (t = 2.12, P < 0.005; Fig. 1C) and dnmt1 mRNA (t = 2.12, P < 0.05; Fig. 1C). Indeed, dnmt3b expression and dnmt1 expression were each approximately fourfold higher in LD hamsters than in SD hamsters, suggesting the potential for increases in methylation at multiple DNA residues and throughout the hypothalamus under LD photoperiods. Using immunocytochemical methods, we investigated whether DNMT3b expression was present in hypothalamic regions that also express dio3. In LD hamsters, DNMT3b immunoreactivity (DNMT3b-ir) was evident throughout the hypothalamus. Importantly, DNMT3b-ir was robust in the ependymal cell layer surrounding the third ventricle (Fig. 1D), a region of the hypothalamus in which MEL binds and dio3 mRNA is expressed in SD photoperiods (12–16). Diffuse hypothalamic expression of DNMT3b in LD suggests that increases in methylation may be occurring in multiple hypothalamic nuclei; however, photoperiodic differences in DNMT3b-ir were not quantified in the present work.

To determine if ombibus increases in dnmt3b expression in LD are associated with increased methylation of the dio3 promoter, the amount of methylated CpGs in the dio3 proximal promoter was quantified using a methylation-sensitive restriction enzyme (MSRE) assay [digestion with restriction enzymes targeting CpG sites (restriction endonucleases BstUl and HpaII)] followed by amplification with primers specific to the dio3 proximal promoter]. SD significantly decreased dio3 proximal promoter DNA methylation: methylation levels were two- to threefold higher in LD relative to SD (t = 2.14, P < 0.05; Fig. 1E). Next, to confirm the MSRE assay independently and to identify specific CpG residues that are methylated in response to changes in day length, hypothalamic DNA from LD (n = 7) and SD (n = 7) hamsters was treated with sodium bisulfite, and a region of DNA (280 bp, located within the MSRE amplicon) was amplified,
sequenced, and analyzed. Three CpG sites in the proximal promoter (sites 5, 4, and 2) exhibited fewer methylation marks in SD relative to LD, with a complete absence of methylation at sites 5 and 4 in SD hamsters (Fig. 1F). Together, these data indicate that exposure to short photoperiods is associated with reduced dnm3 mRNA expression, decreases in the number of methyl groups on specific motifs in the dio3 proximal promoter, and increases in dio3 mRNA.

**MEL Is Sufficient for Reducing dio3 Promoter DNA Methylation.** Long-duration MEL signals are necessary and sufficient for reproductive neuroendocrine responses to winter day lengths (3). To test the hypothesis that long-duration MEL signals are sufficient to decrease methylation of the dio3 promoter, LD-housed hamsters were treated with afternoon injections of MEL (50 μg administered s.c. 4 h before light offset), effectively mimicking the expansion of nocturnal MEL duration in SD by summatng with the endogenous MEL signal (28). MEL was administered for 2 consecutive days, or for 1 or 4 wk, which allowed specification of the temporal dynamics of MEL-mediated changes in hypothalamic gene expression and promoter methylation. MEL treatments for 1 or 4 wk initiated gonadal regression (P < 0.001), but 2 d of MEL did not (P > 0.05; Fig. 2A). MEL triggered short-latency decreases in dio2 expression (F = 5.55, P < 0.005; Fig. 2B), with maximal inhibition of dio2 expression evident after only 2 d (P < 0.005, all comparisons; Fig. 2B). Decreases in dio2 expression following MEL injections were not evident following ~10 wk of SD photoperiod treatments. The significance of this discrepancy is unclear and may reflect differences in time course or pharmacological effects of relatively high doses of MEL. MEL gradually up-regulated dio3 mRNA (F = 2.98, P < 0.05; Fig. 2B) over 4 wk (P < 0.005; Fig. 2B), establishing that long-duration MEL signals are sufficient to increase hypothalamic dio3 expression.

Because dnm3b is implicated in de novo acquisition of DNA methylation patterns at unmethylated CpG sites (19), we examined whether MEL signals are sufficient to inhibit dnm3b expression in a manner consistent with the pattern of disinhibited dio3 mRNA. Indeed, MEL-driven changes in hypothalamic dnm3b expression preceded changes in dio3 expression (F = 2.82, P < 0.05; Fig. 2B): Two consecutive days of MEL treatment did not alter dnm3b expression, but 1 wk of daily MEL injection reduced dnm3b expression by ~50% (P < 0.05); dnm3b inhibition was sustained by MEL thereafter (P < 0.05). Lastly, MEL also decreased DNA methylation in the dio3 proximal promoter region (F = 2.84, P < 0.05; Fig. 2C), temporally mirroring the inhibition of dnm3b expression. dio3 promoter methylation was reduced after 1 wk of MEL treatment (P < 0.05) and remained significantly lower thereafter (P < 0.05; Fig. 2C). These data establish a temporal pattern in which MEL initially inhibits dnm3b expression and dio3 promoter DNA methylation (after ~1 wk), culminating in increased dio3 mRNA (after 4 wk).

**Hypermethylation Attenuates Reproductive Responses to Inhibitory Photoperiods.** To determine if decreases in hypothalamic methylation are necessary for reproductive responses to SD, the next experiment challenged hamsters with enhanced DNA methylation [via chronic treatment with 3-aminobenzamide (3AB; 40 μg/d administered s.c.), as described in SI Materials and Methods] during exposure to LD and SD. 3AB is an inhibitor of poly(ADP ribosylation) (PARP) activity and promotes methyl binding to DNA, leading to enhanced DNA methylation (29). 3AB attenuated but did not abolish gonadal responses in SD (F = 21.53, P < 0.001; Fig. S3A). 3AB-treated hamsters had larger testes relative to SD controls after 2 wk in SD (P < 0.001). Substantial gonadal regression occurred during the next 2 wk in both SD groups, but testes of 3AB-treated hamsters remained significantly larger than those of saline-injected controls after 4 wk in SD (P < 0.05). Importantly, 3AB treatments did not directly stimulate gonadal growth in LD hamsters (F = 1.01, P = 0.37; Fig. S3A), indicating that 3AB-induced hypermethylation is not by itself gonadostimulatory. Because 3AB modifies methylation levels via its inhibition of PARP, the present data cannot exclude a contribution of reduced PARP activity in the effects of 3AB on gonadal regression. However, to establish that 3AB increased hypothalamic DNA methylation, we measured 5-methyl-2′-deoxycytidine, an indicator of methylated DNA, and confirmed that 3AB treatments were effective in globally up-regulating hypothalamic DNA methylation compared with saline-treated SD hamsters (r = 2.07, P < 0.05; Fig. S3B). The 3AB injections likely induced omnibus methylation on numerous genes, and additional convergent evidence is required to establish the sufficiency of hypermethylation to block photoperiod-induced gonadal responses. Nevertheless, the data in Fig. S3 provide a preliminary functional link between DNA methylation and the expression of seasonal phenotypic responses.

**Photorefractoriness Is Associated with Remethylation of the dio3 Proximal Promoter.** Hamster seasonal reproductive inhibition requires a direct response to SD, but restimulation of reproductive physiology in late winter/early spring is mediated by an endogenous timing mechanism that renders the hypothalamus unresponsive (refractory) to inhibitory MEL signals after ~5 mo (9, 10). Similar timing mechanisms are implicated in most vertebrate seasonal rhythms and reflect an evolutionarily conserved process for measuring seasonal time (30, 31). In light of the central role of reduced dio3 promoter methylation in the induction of the winter reproductive phenotype, we examined whether remethylation of the same region of DNA mediates...
development of neuroendocrine photorefractoriness and gonadal recrudescence. Male and female hamsters were exposed to SD for 42 wk, and age-matched controls were housed in LD or SD for 10 wk. Reproductive organ masses decreased after short-term (10 wk) exposure to SD ($P < 0.001$), but extended SD treatment caused gonadal recrudescence, indicative of neuroendocrine photorefractoriness to SD (9, 10) (Fig. 3A). Further, a morphological trait that changes seasonally and reflects pituitary lactotroph activity, confirmed the SD-induced changes in physiology and subsequent development of photorefractoriness ($\chi^2 = 8.6, P < 0.005$; not illustrated). Hypothalamic dio2 mRNA expression was comparable across all groups ($F = 0.33, P > 0.70$; Fig. 3B), but dio3 expression varied markedly across states of photorefractoriness ($F = 7.88, P < 0.01$; Fig. 3B). As expected, dio3 mRNA was elevated following acute exposure to SD ($P < 0.005$), but after 42 wk in SD, dio3 mRNA had returned to LD-like levels ($P < 0.005$ vs. SD, $P > 0.80$ vs. LD), indicating that SD-induced increases in dio3 expression are reversed in the photorefractory reproductive neuroendocrine system.

To assess whether photorefractoriness is accompanied by a reversal of the acute SD changes in dio3 methylation, we examined dnmt3b mRNA. Hypothalamic dnmt3b expression changed significantly over time in SD ($F = 6.03, P < 0.005$; Fig. 3C). Initially, SD inhibited dnmt3b expression ($P < 0.01$), but after 42 wk in SD, dnmt3b expression was dis inhibited (10 wk vs. 42 wk: $P < 0.005$) comparable to that of LD hamsters ($P > 0.90$).

Lastly, we quantified DNA methylation in the dio3 proximal promoter region of photorefractory hamsters via MSRE assay. Hypothalamic dio3 promoter methylation varied markedly over time in SD ($F = 5.41, P < 0.01$; Fig. 3D), with a clear pattern of reduced methylation on week 10, followed by a complete reversal and remethylation by week 42 in SD. Sodium bisulfite sequencing of DNA from a subset of these hamsters [LD, $n = 4$; SD, $n = 4$; SD-refractory (SD-R), $n = 6$] confirmed the MSRE data (Fig. 3E). Of the three CpG sites that exhibited clear decreases in methylation following acute exposure to SD (compare with Fig. 1F), one site (site 4) exhibited a complete reversal in DNA methylation following the development of refractoriness. Thus, refractoriness-induced inhibition of dnmt3b and inhibition of dio3 mRNA expression are accompanied by a reversal in the methylation of specific DNA sequences in the dio3 proximal promoter that could provide transcriptional control of dio3 expression.

**Discussion**

Here, we report a mechanism of photoperiod- and MEL-induced changes in methylation of the promoter region of a gene (dio3) whose expression is critical to the transduction of photoperiod information into the brain and reproductive neuroendocrine system. Exposure to SD or MEL broadly inhibited the expression of DNMTs, which regulated the methylation status of the dio3 promoter. Furthermore, a seasonally reversible cycle of changes in DNA methylation was identified in the dio3 promoter, because abundant remethylation occurred during the development of neuroendocrine refractoriness to SD. This work adds to an emerging literature on reversible methylation as a mechanism underlying physiological events in the CNS and regulating phenotypic and behavioral changes (24–26, 32, 33). Discrete regions of the dio3 promoter exhibit extremely slow changes in methylation status, occurring over the course of weeks to months; thus, the present work specifies a temporal dimension over which reversible epigenetic mechanisms operate to regulate systems-level biological processes. Methylation of the dio3 promoter likely acts as a key step for the maintenance of reproductive competence during the breeding season and permits changes in environmental photoperiod to communicate with the reproductive neuroendocrine system.

Photoperiodic plasticity in deiodinase mRNA expression is a critical step in the neuroendocrine control of reproduction in birds (13, 14, 34) and mammals (15, 16, 35); the present work identifies DNA methylation as a key mechanism by which day length and MEL exert seasonal control over dio3 expression. Long summer photoperiods drive high levels of dnmt3b expression, which maintain dio3 promoter methylation and ensure low levels of dio3 expression; collectively, this facilitates hypothalamic catabolism of the prohormone T4 into reproductively stimulatory T3 (15, 36) (Fig. S4A). Seasonal decreases in day length cause expansion of the duration of nocturnal MEL secretion, and the cumulative effect of successive long MEL signals is an acute down-regulation of dnmt3b expression; reduced methylation of the dio3 promoter increases accessibility of the DNA template to transcriptional control, and dio3 expression is markedly increased, quenching hypothalamic T3 signaling (Fig. S4B). The regulation of dio3 expression is likely complex, and methylation status presumably has an impact on the manner in which additional signals, such as “tuberalins,” thyroid-stimulating hormone $\beta$, and gonadal steroids, drive or inhibit dio3 expression.

![Fig. 3. Neuroendocrine refractoriness to SD reverses patterns of DNA methylation induced by acute SD exposure. (A) Acute (10 wk, SD) exposure to SD induced gonadal regression, whereas prolonged exposure (42 wk, SD-R) triggered neuroendocrine refractoriness and gonadal recrudescence. Refractoriness in SD-R hamsters was characterized by a complete reversal of hypothalamic dio3 and dnmt3b mRNA expression (B and C) and by remethylation of DNA in the dio3 proximal promoter (D). (E) Proportion of LD, SD, and SD-R hamsters in which methylation was present in each of 11 CpG sites in the dio3 proximal promoter, as measured by sodium bisulfite DNA sequencing. The abscissa (not to scale) depicts the 11 CpG sites in the −490 to −210-bp region of the dio3 proximal promoter. All data are mean ± SEM. *$P < 0.05$. ***$P < 0.005$ vs. LD value.](https://www.pnas.org/cgi/doi/10.1073/pnas.1310643110)
Prolonged exposure to SD eventually culminates in photorefractoriness, characterized by a complete reversal of dnmt3b expression, remethylation of the dio3 proximal promoter (specifically, site IV), and marked decreases in dio3 expression. Absent hypothalamic dio3 expression, enhanced T3 signaling triggers vernal gonadal recrudescence in refractory hamsters (Fig. S4C). How short photoperiods revert from inhibiting to stimulating, or merely disinhibiting, dnmt3b expression remains unknown, but this is likely a key step in the induction of photorefractoriness. Reduced methylation of the dio3 promoter by shorter (10 wk) intervals of SD disinhibits dio3 expression but also grants access to the promoter by transcription factors that cannot participate in the regulation of dio3 at other times of year.

Recent evidence suggests that hormone secretion can have marked effects on the methylation status of specific promoter sequences (38). In the adult brain, testosterone secretion maintains methylation of CpG sites in the promoter regions of steroid-responsive genes (22) (e.g., vasopressin, estrogen receptor α), and during early development, gonadal steroids exert enduring effects on CNS sexual differentiation, in part, via epigenetic modifications (23, 39–41); however, whether such changes are plastic or reversible in adulthood remains unknown. Here, we demonstrate that DNMT expression and downstream dio3 promoter methylation respond to the duration of elevated circular MEL in a manner that mirrors reproductive responsiveness to MEL. Importantly, emotional responses to long-term MEL lead gonadal responses by at least 1 wk, and thus are not merely consequences of changes in gonadal hormone secretion. The relatively broad hypothalamic expression of DNMT3b-ir in LD suggests that these enzymes may also be expressed in cells and nuclei that are components of CNS pathways other than those participating in the seasonal control of reproductive physiology. Epigenetic modifications are no longer viewed as being established solely during early development and maintained across the life span (42). Rather, several lines of evidence suggest that histone acetylation of specific genes is a dynamic and reversible process. For example, the circadian clock gene PERIOD1 (per1) is under transcriptional control by histone deacetylation, and repression of per1 transcription is a rhythmic and reversible daily event (24). In addition, rhythmic and reversible deacetylation of histone H4 by the histone deacetylase 3 (HDAC3)/nuclear corepressor 1 (Ncor1) complex is required for proper circadian clock function: mutations of the Ncor1 deacetylation activation domain that block its association with HDAC3 markedly shorten circadian period length and decrease rhythmic expression of circadian clock genes bmal1 and rev-erber (43). In honeybees, the reversible transition from a “nurse” to a “forager” behavioral phenotype is associated with genome-wide changes in patterns of methylation (25). The data reported here provide evidence for reversible DNA methylation of a specific gene, dio3, in the control of a reversible reproductive phenotype. By driving photoperiod- and refractoriness-induced changes in reproductive condition, the seasonal cycle in methylation described here has the potential to mediate widespread changes in physiology and behavior. This mechanism provides flexibility to regulate both autumnal inhibition and vernal recrudescence of reproductive physiology. Hypothalamic deiodinase responses to photoperiod occur in a diverse array of avian and mammalian species, suggesting that seasonal regulation of DNA methylation may be an evolutionarily ancient timing mechanism.

The present work identifies molecular mechanisms that mediate effects of day length on reproduction. SD MEL signals initially inhibit dnmt3b and reduce dio3 promoter methylation, which eventually permits dio3 expression (Fig. S4); increases in dio3 quench perihypothalamic T3 signaling. Reduced methylation in SD may also permit access by other transcription factors to the dio3 promoter. Refractoriness to SD is characterized by increases in dnmt3b expression and remethylation of the dio3 proximal promoter, suggesting that the seasonal loss of responsiveness to SD MEL signals may occur either at the level of or upstream of dnmt3b expression. Because T3 regulates the release of gonadotropin-releasing hormone (GnRH) (13, 14), epigenetic control of dio3 mRNA expression by dnmt3b likely functions as a key step in the molecular cascade of events responsible for gonadotrophin signaling from the brain to the pituitary (15). The extent to which changes in T3 signaling have an impact on hypothalamic neuromodulatory systems that converge in GnRH neurons has not been fully elaborated, but a recent report indicates that in SD-housed hamsters, exogenous T3 induces an LD phenotype in kispeptin and gonadotrophin-inhibitory hormone peptide levels in multiple hypothalamic nuclei (44), suggesting that hypothalamic T3 levels may modulate GnRH activity via multiple parallel pathways.

Taken together, the present data demonstrate that a cycle of reduced methylation and remethylation of the dio3 promoter region is driven by photoperiod- and MEL-dependent changes in dnmt3b expression. Methylation of the dio3 promoter affords transcriptional control of dio3 mRNA, and spontaneous remethylation of the dio3 promoter in late winter drives gonadal recrudescence, acting as a component of an endogenous calendar for photoperiodic time measurement.

Materials and Methods

Animals. Male and female Siberian hamsters (P. 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 1700 h Central Standard Time [CST]). Food (Teklad; Harlan Laboratories) and filtered tap water were provided ad libitum. All procedures were approved by the Animal Care and Use Committee at the University of Chicago.

Study 1: Effects of Photoperiod on dnmt and dio2/3 Expression, and dio3 Promoter Methylation. To examine the effects of acute exposure to inhibitory LD lengths (9 h light/day, lights off at 1700 h CST), male hamsters were housed in the colony LD photoperiod (n = 18) or in an SD photoperiod (n = 20) for 8 wk. Testis volumes (TVs) were determined before photoperiod manipulation (baseline), and again 4 and 8 wk later, under light isoflurane anesthesia (4% [vol/vol] induction, 2% [vol/vol] maintenance) via measurement of the length (L) and width (W) of the left testis. TV measurements were collected without knowledge of the animal’s treatment condition. TVs were calculated using the equation for a prolate spheroid (TV = 4/3 × [L/2] × W/2) (45). On week 8, hamsters were euthanized via rapid decapitation at the midpoint of the light phase, and testis weights were measured (±0.1 mg). At autopsy, the whole hypothalamus was rapidly dissected as previously described (46), frozen on dry ice, and stored at −80 °C. Following RNA and DNA extraction, mRNA expression and promoter methylation were determined, as described above. DNA from a subset of male hamsters (LD, n = 7; SD, n = 7) was used for sodium bisulfite treatment and sequencing to identify specific nucleotide residues upon which methylation occurs.

Study 2: Effects of MEL dnmt and dio2/3 Expression, and dio3 Promoter Methylation. Male hamsters were housed in an LD photoperiod and received daily injections of MEL (0.50 μg administered s.c., N-acetyl-5-methoxytryptamine; Sigma) or 0.1 mL of ethanolic saline vehicle (SAL) 4 h before lights off. This timing and concentration of MEL reliably induce gonadal regression in this species (47). Hamsters received MEL injections for 2 d (n = 12), 1 wk (n = 16), or 4 wk (n = 15); SAL injections were administered for 4 wk. All injection regimens terminated on the same calendar day, such that injection treatments for hamsters receiving MEL for 2 d and 1 wk began 26 and 21 d, respectively, after hamsters receiving MEL for 4 wk. TVs were measured on weeks 0 and 2 and 4 wk later. After the probed number of days of MEL treatment, hypothalamic tissue was extracted on the final day of the experiment as described above.

Study 3: Effects of Photorefractoriness on dnmt and dio2/3 Expression, and Promoter Methylation. On week 0, male and female hamsters were housed in an LD photoperiod (n = 12 male, n = 11 female) or SD photoperiod (n = 21 male, n = 24 female) for the next 42 wk; an age-matched cohort of hamsters was housed in an LD photoperiod for 32 wk and was transferred to an SD photoperiod for the last 10 wk of the experiment (n = 10 male, n = 11 female). TVs of male hamsters and body masses and fur scores (1 = dark “summer” fur, 4 = white “winter” fur) of both sexes were determined at multiple intervals between weeks 0 and 42 to document gonadal and somatic responsiveness and refractoriness to SD photoperiods. Testes and uteri were dissected and weighed at autopsy, and hypothalamic tissue was extracted as described above.
RNA/DNA isolation. DNA and RNA were extracted from whole hypothalamic tissue using a Qiagen RNeasy/DNeasy kit. Nucleic acid concentration and quality were determined by means of a spectrophotometer (Nanodrop; Thermo Scientific). cDNA was synthesized using SuperScript III (Invitrogen), and genomic DNA and cDNA were stored at −20 °C until quantitative PCR (qPCR) was performed.

DNA Sequencing and qPCR. Primers for the dio3 proximal promoter and dntm1, dntm2a, and dntm2b were designed based on conserved regions of mouse, rat, and human sequences using PrimerExpress software (Life Technologies) and optimized for use in Siberian hamsters (Table S2). All sequences were determined at the University of Chicago Comprehensive Cancer Center DNA sequencing facility. A standard nucleotide BLAST was used to determine sequence specificity. qPCR reactions were performed with 2 μl of cDNA using a Bio-Rad CFX384 system.

MSRE Assay. Hypothalamic DNA from all hamsters was subjected to MSRE analyses. DNA methylation at CpG nucleotides in the dio3 proximal promoter region was quantified using the MSRE assay (22).

Sodium Bisulfite Conversion and DNA Sequencing. Bisulfite conversion was conducted using Epitext Bisulfite kits (Qiagen). Ligation, transformation, growth on E. coli, and sequencing procedures are described in SI Materials and Methods.

Statistical Analyses. Tvs were analyzed using ANOVA. mRNA expression and dio3 promoter methylation were assessed using ANOVAs and unpaired t tests where appropriate. Sex differences were not evident in any measure of gene expression or methylation (P < 0.10, all comparisons), and data from both sexes were combined to increase statistical power. When violations of normality were observed, data were log-transformed. Differences were considered significant if P < 0.05.

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