Genome sequencing and transcriptome analyses of the Siberian hamster hypothalamus identify mechanisms for seasonal energy balance

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Synthesis of triiodothyronine (T_3) in the hypothalamus induces marked seasonal neuromorphology changes across taxa. How species-specific responses to T_3 signaling in the CNS drive annual changes in body weight and energy balance remains uncharacterized. These experiments sequenced and annotated the Siberian hamster (*Phodopus sungorus*) genome, a model organism for seasonal physiology research, to facilitate the dissection of T_3-dependent molecular mechanisms that govern predictable, robust, and long-term changes in body weight. Examination of the *Phodopus* genome, in combination with transcriptome sequencing of the hamster diencephalon under winter and summer conditions, and in vivo-targeted expression analyses confirmed that proopiomelanocortin (*pomc*) is a primary genomic target for the long-term T_3-dependent regulation of body weight. Further in silico analyses of *pomc* promoter sequences revealed that thyroid hormone receptor 1β-binding motif insertions have evolved in several genera of the Cricetidae family of rodents. Finally, experimental manipulation of food availability confirmed that hypothalamic *pomc* mRNA expression is dependent on longer-term photoperiod cues and is unresponsive to acute, short-term food availability. These observations suggest that species-specific responses to hypothalamic T_3, driven in part by the receptor-binding motif insertions in some cricetid genomes, contribute critically to the long-term regulation of energy balance and the underlying physiological and behavioral adaptations associated with the seasonal organization of behavior.

triiodothyronine | proopiomelanocortin | seasonal | obesity

Rheostatic regulation of physiological processes is pervasive (1), and naturally occurring, long-term programmed seasonal reproduction and energy balance is one salient example. High-amplitude seasonal cycles in energy balance and somatic growth are common in nature and provide a unique and valuable opportunity to identify the genomic and molecular pathways involved in rheostatic control of physiology (2–5). Siberian hamsters (*Phodopus sungorus*) exhibit marked changes in energy balance as they adapt from a summer to a winter environment in nature: a decrease in day length (i.e., photoperiod) below ∼13 h light/day triggers seasonal infertility, anorexia, and a dramatic decrease in body fat (2). Consequently, *Phodopus* provide a unique and important model for neuroendocrine, physiological, and behavioral mechanisms that govern long-term seasonal regulation of body weight and reproduction (2, 5); these robust phenotypic changes in physiology and behavior can be recapitulated in the laboratory with manipulations of day length (photoperiod) alone.

Triiodothyronine (T_3)-responsive neuro-glial substrates figure prominently in the transduction of photoperiod signals into the neuroendocrine system. T_3-responsive targets in the central nervous system (CNS) constitute an evolutionarily conserved system that orchestrates morphological brain plasticity in the service of timing seasonal biology (6, 7). Enzymes that act on thyroid hormones, in particular the iodothyronine deiodinases (type 2 and 3; DIO2 and DIO3, respectively) respond to seasonal changes in photoperiod-driven melatonin secretion and govern peri-hypothalamic catabolism of the prohormone thyroxine (T_4), which limits T_3-driven changes in neuroendocrine activity. T_3 induces ligand-dependent rearrangement of the thyroid hormone receptor (TR), and T_3 drives the vast majority of TR-induced gene expression (8). Increased hypothalamic T_3 production in long summer days, driven in most amniotes by peri-hypothalamic DIO2-mediated conversion of T_4 to the biologically active hormone T_3, activates anabolic neuroendocrine pathways that maintain reproductive competence and increase body weight. Decreased T_3 signaling is afforded by peri-hypothalamic DIO3 expression, which catabolizes T_4 and T_3 into receptor-inactive amines, and is associated with adaptation to reproductively inhibitory photoperiods (9–12). In diverse taxa, DIO2

Significance

The genome and hypothalamic transcriptome of the Siberian hamster were sequenced and annotated to identify transcriptional pathways that exhibit seasonal plasticity in energy balance. Adaptation to short winter days reversed seasonal obesity and down-regulated hypothalamic proopiomelanocortin, and exogenous triiodothyronine reinstated weight gain and proopiomelanocortin expression. In silico analyses identified the evolution of thyroid hormone receptor binding motifs in the proximal promoter of the proopiomelanocortin gene of hamsters and other Cricetidae. Energetic challenges imposed by food restriction elicited orexigenic and anorexigenic neuropeptide responses in the hypothalamus, but did not affect proopiomelanocortin, which was regulated only by photoperiod. Hypothalamic proopiomelanocortin is maintained by photoperiod-driven triiodothyronine signaling and thereby affords adaptive long-term temporal organization of physiological systems that regulate energy balance.

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and DIO3 responses to environmental cues (e.g., photoperiod, or hours of light per day) have established links between tissue-specific patterns of T_3 signaling and seasonal changes in reproduction and ponderal growth/regression (2, 6). Conspicuously absent are insights into how T_3 signaling impacts hypothalamic orexigenic/anorexigenic neuropeptide systems that govern changes in energy balance mandatory to support these seasonal cycles of reproduction and life history.

Results and Discussion

Characterization of the Siberian Hamster Genome and Photoperiodic Diencephalon. Seasonal changes in day length are sufficient to induce a constellation of changes in Siberian hamster physiology that support adaptation to and survival of winter: exposure to a short-day photoperiod (SD) causes molt to a more insulative fur (Fig. 1*A*) and gonadal involution ($P < 0.001$; Fig. 1*B*), body weight is strikingly reduced in SD, white adipose tissue is depleted ($P < 0.001$; Fig. 1*C*), and brown adipose tissue thermogenic capacity is augmented (13). Nocturnal foraging persists throughout the winter months, but metabolic rate is decreased through daily torpor, which conserves energy (14). The absence of comprehensive genomic information for *Phodopus* has limited molecular insights into the physiological processes that regulate these seasonal adaptations in energy balance. To address this deficiency, we used Illumina sequencing to draft the Siberian hamster genome (*SI Appendix*, Table S1 and Fig. S1; ref. 15). To complement genome annotation and to identify molecular pathways involved in energy balance, a seasonal hypothalamic transcriptome was also sequenced and annotated. Transcriptomic analyses were performed on hypothalamic RNA from adult male hamsters following adaptation to long-day (LD) and SD photoperiods. Over 5,000 unique transcripts were identified, and 32 exhibited significant expression changes between the photoperiods [false discovery rate (FDR) < 0.05-corrected; *SI Appendix*, Table S2] with the top differentially expressed protein-coding transcripts shown (Fig. 1*D*). Multiple RNAs that have previously been implicated individually in the long-term, neuroendocrine control of reproductive physiology were identified, including thyrotropin-stimulating hormone subunit-β (*tshβ*), neuropeptide VF precursor (*npvf*), and iodothyronine deiodinase type III (*dio3*; Fig. 1*E* and *SI Appendix*, Fig. S2). Gene

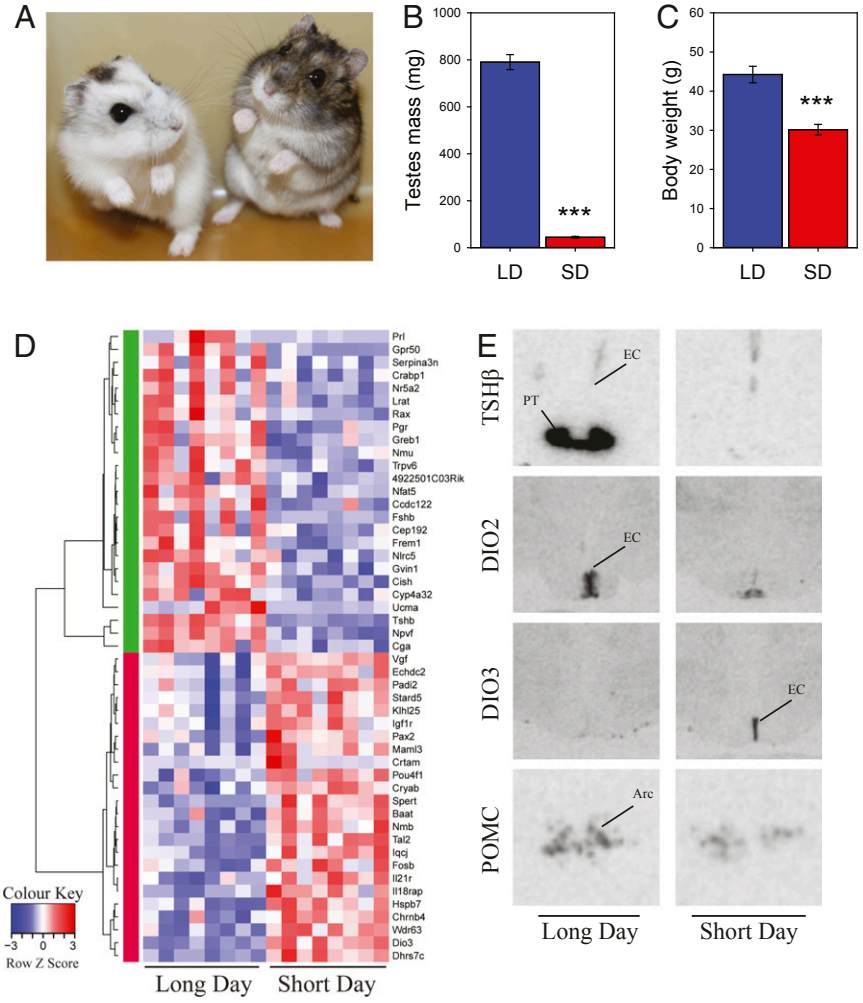

Fig. 1. Seasonal hypothalamic transcriptome in Siberian hamsters. Compared with long-day photoperiod (LD; > 13 h light/day), adaptation to short-day photoperiod (SD) induces multiple physiological adaptations including (*A*) pelage molt, from agouti (*Right*) to white (*Left*) fur, (*B*) gonadal regression, and (*C*) decreased body mass (*B* and *C* depict mean ± SEM; ***$P < 0.001$). (*D*) RNA-seq analyses revealed extensive plasticity in RNA expression for transcripts associated with neuroendocrine function. Rows indicate individual transcripts identified at a group level to be significantly (FDR < 0.05) up-regulated (red) or down-regulated (blue) by SD photoperiod. Columns indicate individual hamsters (biological replicates; LD: $n = 8$; SD: $n = 8$). (*E*) Following adaptation to LD or SD photoperiods, expression of multiple transcripts associated with energy balance, food intake, and neuroendocrine function changed in dissected hypothalamic tissue: thyrotrophin-stimulating hormone β (*tshβ*), iodothyronine deiodinase type II (*dio2*) and type III (*dio3*), and proopiomelanocortin (*pomc*). These mRNAs are anatomically localized to the pars tuberalis (PT, *tshβ*), the ependymal cells (EC, *dio2, dio3*) lining the third ventricle, or the arcuate nucleus (Arc, *pomc*).

ontology (GO) analyses identified a photoperiodic transcriptome strikingly enriched (FDR-corrected $P < 0.05$) for cellular activity related to hormone secretion and neuropeptide signaling (*SI Appendix*, Table S3 and Figs. S3 and S4). Common to many ($n = 10$) GO terms enriched by photoperiod in the hypothalamic transcriptome was proopiomelanocortin (*pomc*), a neuropeptide that is centrally implicated in energy balance, suggesting that *pomc* may be a first-order neuropeptide in the rheostatic regulation of body weight by seasonal changes in photoperiod.

Role of T_3 in Hypothalamic *pomc* Regulation. Across diverse taxa thyroid hormone signaling plays a central role in regulating seasonal physiology via actions in the brain and in the periphery (4, 5). To further examine how thyroid hormone signaling interacts with *pomc* in the regulation of seasonal energy balance, hypothalamic expression of *pomc*, along with other genes, the protein products of which participate in CNS regulation of food intake and energy balance (*npy*, *agrp*, *cart*), was quantified in LD and SD hamsters following 2 wk of daily T_3 treatment (5 μg; s.c.). Adaptation to SD decreased (Fig. 2*A* and *SI Appendix*, Fig. S5*A*), and T_3 injections increased (Fig. 2*A*) body weight. Adaptation to SD also significantly down-regulated expression of *pomc*, consistent with prior reports (16–18) Moreover in SD, T_3 increased hypothalamic *pomc* expression to a level indistinguishable from that exhibited in LD ($P < 0.05$; Fig. 2*B*). Photoperiod did not alter *npy*, *agrp* or *cart* expression ($P > 0.05$, all comparisons; *SI Appendix*, Fig. S5), and T_3 was likewise without effect on *npy* or *agrp* ($P > 0.05$, both comparisons; *SI Appendix*, Fig. S5), but did up-regulate *cart* expression in SD (*SI Appendix*, Fig. S5). Taken together, these data suggest, among hypothalamic appetitive neuropeptides, a relatively selective effect of T_3 on *pomc*.

Regulation of gene expression by T_3 occurs via binding to the TRs, which in turn bind to thyroid hormone response elements (TREs) in the DNA sequence. TREs are composed of half-sites, the spacing and orientation of which can affect their regulatory ability (19, 20). In silico analyses of the *pomc* proximal promoter sequence using PROMO (21, 22) identified two thyroid-receptor 1b (*Thrb*) half-sites: TCC-TGG-TGA and TCA-CCT-GGA (*SI Appendix*, Table S4), indicating that T_3 may be capable of directly regulating *pomc* transcription. These specific binding motifs were not identified in the proximal promoter for *npy*, *agrp*, or *cart*, consistent with the relative specificity and selectivity illustrated in Fig. 2 (*SI Appendix*, Fig. S5) and suggesting a privileged role for *pomc* among the hypothalamic targets of T_3 in signal transduction relevant to seasonal changes energy balance. Indeed, a phylogenic analysis of *pomc* proximal promoters across a range of species revealed that this specific *Thrb* motif has evolved in the Cricetidae family (Fig. 2*C*). To further evaluate responsiveness of the hamster-specific *Thrb* motif in the regulation of *pomc*, GH3 cells were transfected with a Siberian hamster or the mouse *pomc* proximal promoter sequence upstream of a luciferase reporter gene, and the ability of T_3 to drive luciferase activity was evaluated; however, this assay indicated that T_3 was no more effective than hormone-free vehicle in driving

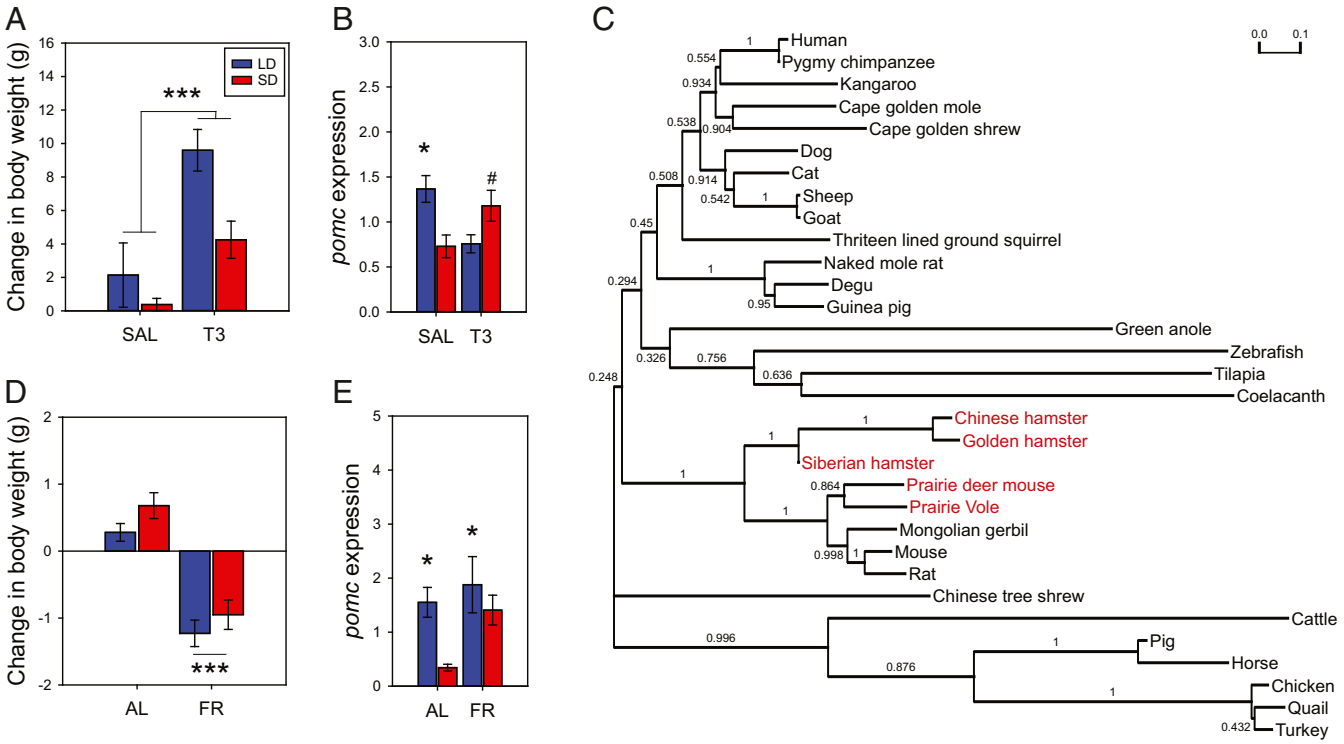

Fig. 2. Long-term photoperiodic regulation of hypothalamic *pomc* expression. Mean ± SEM (*A*) change in body mass and (*B*) hypothalamic *pomc* mRNA expression in adult male Siberian hamsters following 8 wk of exposure to LD (blue bars) or SD (red bars) and 2 wk of daily treatment with T_3 (5 μg; s.c.). (*A*) T_3 treatment increased body mass in LD and in SD (***$P < 0.001$). (*B*) SD inhibited hypothalamic *pomc* expression in saline-treated controls (*$P < 0.01$ vs. LD saline). T_3 suppressed *pomc* expression in LD-treated hamsters ($P < 0.05$) but increased *pomc* expression in SD (#$P < 0.05$). (*C*) PROMO analysis identified thyroid hormone receptor 1b (*Thrb*)-binding motifs in the Siberian hamster *pomc* proximal promoter, and phylogenetic analyses of the *pomc* proximal promoter indicated that the hamster-specific *Thrb* motif has evolved multiple times in the Cricetidae family (indicated by red font). Mean ± SEM (*D*) change in body mass and (*E*) hypothalamic *pomc* mRNA expression in adult male and female Siberian hamsters subjected to a 16-h interval of food deprivation (FR) or maintained on ad libitum feeding (AL) after 12 wk of exposure to LD (blue bars) or SD (red bars). (*D*) FR decreased body mass in both LD and SD (***$P < 0.001$ vs. AL). (*E*) SD significantly reduced hypothalamic *pomc* (*$P < 0.05$) but FR did not significantly affect hypothalamic *pomc* mRNA expression (*$P = 0.54$ within LD; $P = 0.07$ within SD).

transcriptional activity (P >0.30; *SI Appendix*, Fig. S6). The inadequacy of T_3 alone in driving luciferase activity in this assay may indicate that additional intracellular signaling pathways are required, or that functional T_3-driven transcriptional regulation at the *pomc* proximal promoter requires distal enhancer elements.

Regulation of Hypothalamic *pomc* Expression by Photoperiod vs. Food. POMC per se is functionally inert, but it is cleaved into multiple peptides, one of which, α-melanocyte–stimulating hormone, is a potent inhibitor of food intake (23). The *pomc*-knockout mice are obese (24), and acute starvation markedly down-regulates *pomc* expression, indicative of its central role in energy homeostasis (25). Therefore, we assessed the impact of acute food restriction on body weight and responses of hypothalamic neuropeptides related to energy homeostasis (*pomc*, *cart*, *npy*, and *argp*). Male and female hamsters were acutely (16 h) food-restricted (FR) after 12 wk of adaptation to LD or SD photoperiods. Again, hamsters weighed less in SD ($P < 0.001$; *SI Appendix*, Fig. S7) and acute FR further reduced body weight in both photoperiods ($P < 0.001$; Fig. 2*D*), suggesting a comparable challenge to energy homeostasis in both groups. FR also significantly up-regulated hypothalamic expression of neuropeptides that play key roles in food intake and energy balance: *npy*, *agrp*, and *cart* ($P < 0.05$, all comparisons; *SI Appendix*, Fig. S7), but failed to significantly inhibit *pomc* expression ($P > 0.05$; Fig. 2*E*), consistent with prior reports (16–18). Taken together, these data indicate that hypothalamic *pomc* is not inhibited by acute negative energy balance in Siberian hamsters but support the hypothesis that *pomc* expression is instead associated with longer-term (seasonal) states of metabolic change, specifically those linked to predictive changes in body weight and metabolism associated with seasonal/photoperiodic adjustments.

General Discussion

Here we present the sequencing and annotation of the Siberian hamster genome, a model organism for seasonal biology. The availability of well-described intergenic regions facilitated the identification of TR-binding motifs in a key neuropeptide involved in the regulation of energy balance, *pomc*. These hypothalamic transcriptomic analyses also identified several other well-described molecular markers that are regulated by photoperiodic cues: RNA-seq analyses identified changes in transcripts associated with the neuroendocrine control of reproduction, including follicle-stimulating hormone subunit-β (*fshβ*), prolactin (*prl*), and progesterone receptor (*pgr*). Moreover, we identified several previously uncharacterized transcripts involved in neuronal communication such as Glutamate metabotropic receptors (*grm*) and Gamma-aminobutyric acid type A receptor (*gabr*). Several noncoding RNA (e.g., *mir133b*) were also observed to exhibit photoperiodic variation in expression and suggest that a complex level of molecular plasticity is involved in the seasonal neuroendocrine regulation of energy balance yet to be uncovered. Mechanistic assessments confirmed that *dio3* and *pomc* were transcriptionally controlled by photoperiod in a manner that effects a down-regulation of T_3 and POMC-mediated signaling in winter. Importantly, seasonal changes in *pomc* are not merely a consequence of changes in appetite or food intake (16–18). Enhancing T_3 signaling counteracted the short-day photoperiodic inhibition of *pomc*, suggesting that interactions between T_3 and *pomc* may be in a superordinate position to drive seasonal energetic transitions.

Siberian hamsters have emerged as a key model species for investigations of biological rhythms on an annual timescale (26, 27). The hamster genome elaborated in this paper, combined with the prevalence of enrichment for POMC signaling in the RNA-seq data, directly facilitated identification of the insertions of *Thrb*-binding motifs in the *pomc* proximal promoter, which was subsequently also identified in seasonally breeding Syrian hamsters, deer mice, and prairie voles. One noteworthy pattern was increased *pomc* expression in LD hamsters, which defend a higher body weight set point and classify as obese. An observation based on common biomedical mammalian models, e.g., mice and humans, is that *pomc* has anorexigenic effects on food intake and appetite because of RNA splicing directed to the production of α-MSH as the principal neuropeptide. In Siberian hamsters, however, the actions of *pomc* may be driven by post-translational modifications such as increased carboxypeptidase E expression to drive alternative *pomc* slicing and neuropeptide synthesis (28).

The present study replicates multiple prior reports in Siberian hamsters, which exhibit decreases in body mass after ∼4 wk of exposure to SD (9–12), a winter decrease in body weight typical of other Cricetidae such as deer mice and prairie voles (28), which exhibit *Thrb* motifs in the *pomc* promoter. *Thrb* half-sites are also found in the *pomc* promoter of Syrian hamsters, which, unlike voles, deer mice, and *Phodopus*, initially exhibit modest increases in body mass and later decreases in body mass over the course of prolonged exposure to SD (30, 31). We suspect that neuroendocrine pathways other than TREs in the *pomc* promoter may explain these differences. Indeed, paralleling these species differences in body mass responses to SD are notable differences in the temporal regulation of *dio3* expression in the hypothalamus. Syrian hamsters exhibit little-to-no *dio3* expression in the hypothalamus for the first 10 wk in SD (32), an interval during which body mass responses are presumably being initiated. A delayed *dio3* response to SD would be predicted to allow elevated, LD-like, T_3 signaling to persist in the hypothalamus over the initial interval of exposure to SD and perhaps allow this species to exhibit transient weight gain. This would be consistent with the temporal expression of *dio3* and body weight change seen under natural photoperiod conditions in Siberian hamsters (33). Additionally, Syrian and Siberian hamsters may differ in the extent to which the abrupt square-wave photoperiod transitions deployed in laboratory investigations synchronize changes in body weight, compared with timing under natural photoperiodic cycles.

The results of experiment 4 provide convergent evidence in support of *pomc* as a prime candidate for long-term photoperiodic regulation of energy balance, independent of short-term energetic cues. FR elicited the expected increases in *npy* and *agrp*, but was completely ineffective in inhibiting *pomc*, confirming other reports in this species (16–18). Indeed, a nonsignificant paradoxical increase in *pomc* was observed in SD hamsters, which may reflect an additional layer of photoperiodic modulation of the response to food deprivation. Prior work indicated a relative insensitivity of hypothalamic *pomc,* body mass, luteinizing hormone, or follicle-stimulating hormone to leptin in LD hamsters (16). Collectively, these data indicate that hypothalamic *pomc* expression is regulated by longer-term predictive cues such as photoperiod (likely via T_3 signaling) independent of more proximal energetic cues such as those that reference short-term food availability (e.g., FR) or adiposity (i.e., leptin).

Finally, the availability of an annotated genome and hypothalamic transcriptome of a highly seasonal mammal may permit deeper understanding of the molecular signaling pathways that translate environmental cues into seasonal biological signals, which is relevant for understanding and mitigating the impact of seasonal disruption on health and well-being in human and nonhuman animals (34).

Methods and Materials

Additional details of experimental protocols are described in *SI Appendix*.

Animal Use and Ethics. All procedures were approved by the Animal Care and Use Committee at the University of Chicago, the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (35), or the Animal Welfare and Ethics Review Board at the University of Aberdeen, and were

conducted under the Home Office license (PPL 70/7917). All procedures were in accordance with the ARRIVE guidelines. Siberian hamsters (*P. sungorus*) were used in these studies. Hamsters were housed in polypropylene cages illuminated for 15 h or 9 h per day (LD or SD, respectively; lights off at 1700 hours CST). Food [Teklad (formerly Harlan)] and filtered tap water were provided ad libitum.

Genome Sequencing, De Novo Assembly, and Transcriptome Analyses. The data analysis workflow for genome assembly and annotation is outlined in *SI Appendix*, Fig. S1. Genomic DNA was extracted from liver tissue of two adult male hamsters using DNeasy (Qiagen; catalog #69504), and the quantity was determined using Nanodrop spectrophotometry (ThermoFisher Scientific). One paired-end library with a 250-bp insert size were prepared and sequenced on an Illumina HiSeq2000 instrument at The University of Chicago Genomic Facility. A total of 918 million 100-bp paired-end reads were generated achieving a depth of 37× (*SI Appendix*, Table S1). Hypothalamic RNA was extracted using Qiagen RNeasy (catalog #74104), and quantity was assessed using Nanodrop. Paired-end libraries were prepared and sequenced on an Illumina HiSeq 2000 instrument at The University of Chicago Genomic Facility. The transcriptome was reconstructed using a Trinity de novo assembly pipeline (v2013-02-25) (36). Transcripts differentially expressed between groups were identified at both gene and isoform level using the Bioconductor package edgeR (37), with samples of the same group as biological replicates within the group. See *SI Appendix*, Table S2 for expression fold change and FDR-corrected *P* values.

Quantification of Hypothalamic RNA Expression. The hypothalamus was dissected and expression of *tshβ*, deiodinase type II (*dio2*), and deiodinase type III (*dio3*) were measured to confirm photoperiod manipulation (*SI Appendix*). Hypothalamic RNA was extracted from tissues using TRIzol (ThermoFisher Scientific). cDNA was synthesized using SuperScript III (Invitrogen), and cDNA was stored at −20 °C. qPCRs for mRNA expression in hypothalamic tissue were performed using Bio-Rad CFX96. *SI Appendix*, Table S5, describes the qPCR parameters for each target and reference transcript. We used PCR Miner (38) to calculate reaction efficiencies and cycle thresholds, and samples were evaluated based on the Minimum Information for Publication of Quantitative Real-Time PCR guidelines (39).

In Situ Hybridization of Select Photoperiodic Genes. mRNA distribution of select photoperiodic genes (*tshβ*, *dio2*, *dio3*, *pomc*) were examined in coronal hypothalamic sections by radioactive in situ hybridization. Twenty-micron–thick sections of the hypothalamus were cut and mounted onto poly-L-lysine–coated slides. Riboprobes were generated from cloned PCR-generated fragments as previously described (33). In situ hybridization was carried out as previously described (40).

Photoperiodic and Triiodothyronine Regulation of *pomc*. Hamsters were group-housed in LD ($n = 15$) or transferred to SD (9L:15D; $n = 16$) cabinets for 8 wk (Arrowmight). LD and SD hamsters were then divided into two treatment groups that received daily saline control (LD+S and SD+S) or 5-μg T3 injections (LD+T_3 and SD+T_3). The final treatment group sample sizes were LD+S ($n = 9$), LD+T_3 ($n = 6$), SD+S ($n = 8$), and SD+T_3 ($n = 8$). These dose and injection regimens were selected based on previous work (41–43).

Impact of Food Restriction on *pomc* Expression. This experiment used adult (~6 mo of age; $n = 23$) male and female Siberian hamsters. Hamsters were housed in LD ($n = 12$) or transferred to SD for 12 wk. Body weights were measured before photoperiodic treatment (0 wk) and then at 2-wk intervals through week 12, shortly before lights off. On the last day of the experiment, a subset of LD and SD hamsters was kept on food ad libitum ($n = 10$) or had food completely removed (i.e., food restriction; FR, $n = 13$). FR started just before lights-out on the final night. Overall, there were four experimental groups: ad libitum-fed LD ($n = 5$), ad libitum-fed SD ($n = 5$), LD-FR ($n = 7$), and SD-FR ($n = 6$).

Statistical Analyses. Sigmaplot was used for all statistical analyses unless stated otherwise. For experiments 2 and 4, we conducted repeated two-way ANOVAs to examine the impact of SD treatment on body weight. We conducted two-way ANOVAs for experiment 2 (factors: photoperiod vs. T_3 injection) and experiment 4 (factors: photoperiod and food restriction) to analyze the effect of daily T_3 injections or food restriction on the change in body weight and hypothalamic gene expression. Significance was determined at $P < 0.05$.

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