

RESEARCH ARTICLE

The transcriptomes of hypothalamic micropunches reveal sex differences in regulatory processes across hibernation in the Arctic ground squirrel

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Abstract

Seasonal life-history events, such as migration, hibernation, and reproduction, depend on coordinated physiological changes. In vertebrates, a conserved thyroid hormone-signaling pathway in the hypothalamus is known to trigger many of these seasonal transitions. However, the broader processes and regulators modulating seasonal physiology are poorly defined. Recent research in Arctic ground squirrels (AGS, *Urocitellus parryii*) revealed that hypothalamic thyroid hormone signaling is activated, and markers of tancytic remodeling are expressed in late hibernation in anticipation of springtime reproduction. We conducted RNA-sequencing on hypothalamic micropunches encompassing the arcuate nucleus, median eminence, pars tuberalis, and third ventricle in male and female AGS at early and late hibernation. We found substantial sex differences in the hypothalamic transcriptome across hibernation. Functional enrichment analysis of gene expression data revealed an upregulation of processes and pathways related to hormone transport and neurogenesis in females, whereas this was less apparent in males. Transcription factor binding site analysis of differentially expressed genes identified upstream regulators involved in glial cell differentiation, neuronal development, survival, and plasticity. Notably, many of the intersecting genes from these analyses were localized to specialized glial cells (tanycytes) lining the floor and walls of the third ventricle. Our findings support a model in which annual changes in gene expression rely on a progressive remodeling of tanycytes across hibernation. This remodeling may contribute to seasonal changes in neuronal plasticity and function of the hypothalamus, priming the brain in anticipation of shifting physiological demands upon hibernation termination.

NEW & NOTEWORTHY We examine how the transcriptome of hypothalamic micropunches changes across the hibernation season. Our analyses uncover sex-specific changes to regulatory processes associated with hormone transport and neurogenesis. Genes linked to these processes and regulators are strongly localized to third ventricle tanycytes, consistent with the key role these cells play in regulating seasonal physiological changes. Our study supports that using sex as a biological variable is essential for understanding the mechanisms underlying seasonal life-history transitions.

hibernation; hypothalamus; physiology; tanycytes; transcriptomics

INTRODUCTION

Seasonal changes in temperature, food availability, and other biotic/abiotic variables act as important selection pressures that have led to the evolution of sensory and regulatory mechanisms, allowing vertebrates to anticipate predictable environmental changes (i.e., seasonality) (1, 2). Seasonality also involves critical molecular, physiological, and behavioral adaptations that allow for the precise timing and synchronization of life-history events, including reproduction, migration, and hibernation (3). These seasonal events rely on the brain receiving and translating cues to neuronal networks responsible for the output of appropriate behaviors. A changing day length is the primary cue by which organisms predict forthcoming life-history transitions, as it provides a stable year-to-year message that can be used to track time (4). The hypothalamus is a key brain region that integrates

signals and generates seasonal behaviors; physiological processes such as seasonal fattening, metabolic suppression, and reproduction rely on an intact hypothalamus (5–7). Despite the critical role the hypothalamus plays in seasonally modulating physiology, many of the molecular mechanisms that underlie anticipatory life-cycle transitions remain unknown.

One of the most extreme examples of seasonal reprogramming of physiology occurs in small mammalian hibernators. Hibernating mammals can double their body mass in just 2–3 wk in the fall, only to completely cease feeding and subsist on internal energy stores for 8 mo during hibernation (8). After a prolonged fast, metabolic suppression (i.e., hibernation) is terminated in early spring, and animals must rapidly transition to a mildly hyperphagic state as they regain body mass lost during winter dormancy. The capacity of hibernators to reprogram metabolism, a phenomenon known as energy rheostasis, is an adaptive response to living in highly



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seasonal environments (9). The mechanisms governing seasonal rheostatic events, such as the rapid resumption of appetite in the spring, are not well defined. We hypothesize that life history transitions requiring shifts in the energy rheostat, such as the termination of hibernation, are preceded by a progressive remodeling of the hypothalamus that includes changes to regulatory processes and signaling pathways.

In seasonal mammals, hypothalamic remodeling is initiated by a retrograde signaling pathway that tracks changes in daylength to gauge when to activate the reproductive axis and trigger shifts in energy rheostasis (10). Here, the brain measures changes in the duration of nocturnally secreted melatonin and alters the availability of thyroid hormone to the hypothalamus accordingly. For instance, long (summer) daylengths drive a short-duration melatonin signal that increases hypothalamic triiodothyronine (T3) levels, resulting in cellular remodeling and activation of neuroendocrine circuits involved in reproduction in species that breed in spring (11). In species that breed in the fall (e.g., sheep and goats), activation of hypothalamic T3 signaling by long days is conserved, but the effects on the reproductive axis appear to be reversed downstream of this pathway, as reproduction coincides with reduced hypothalamic T3 signaling (12, 13). Hibernating mammals that breed in the spring progressively activate this retrograde pathway when sequestered in constant darkness in their hibernacula, such that increased hypothalamic T3 remodels neuroendocrine pathways and initiates activation of the reproductive axis before hibernation is terminated (14, 15). Increases in hypothalamic T3 are proposed to underlie rheostatic shifts in energy metabolism (16) and are likely to drive the springtime resurgence in appetite and activation of the reproductive axis. Recent research implicates specialized ependymogial cells (tanyocytes) lining the floors and walls of the hypothalamus's third ventricle (3V) as playing a key role in controlling hypothalamic remodeling for processes related to energy metabolism, suggesting that they are likely critical to seasonal rheostatic processes (16–18). Given this, we hypothesize that changes in gene expression should occur across hibernation at tanyocyte cells, as animals prepare to upregulate their metabolism and appetite after the long winter fast.

Tanyocytes are versatile integrators of energy metabolism. They send long radial glial processes to metabolic nuclei in the hypothalamus (e.g., the arcuate nucleus; hereafter the ARC), affecting seasonal neuronal function (17). Their cell bodies are situated along the 3V in an organized fashion, such that specific tanyocyte subtypes have distinct functions. For example, tanyocytes along the floor of the 3V control the secretion of neuropeptides into the portal circulation but also act as a bridge to shuttle peripheral signals to the cerebrospinal fluid (CSF) (19). Along the walls of the 3V, tanyocytes integrate signals from the CSF and relay this information to specific hypothalamic nuclei via radial processes (19). Tanyocytes are integral components of the retrograde hypothalamic thyroid hormone signaling pathway, serving as transporters for PT-derived signals and acting as metabolic sensors to regulate the functioning of the reproductive axis (18, 20). Finally, tanyocytes along the base and walls of the 3V form a neurogenic niche and differentiate into various neuronal phenotypes

(21). Changing daylength affects hypothalamic cell proliferation in seasonal mammals (22), and the seasonal proliferation of neurons from tanyocyte cells in the hypothalamus has been theorized to control cycles of body weight and reproduction in seasonal mammals (23). Thus, since tanyocytes act as seasonal metabolic sensors and as a source of neural stem cells, they may dictate and impact the functioning of surrounding hypothalamic nuclei, ultimately controlling the transition between seasonal phenotypes.

Although thyroid hormone signaling is implicated in priming and remodeling the brain in preparation for life-history events (e.g., reproduction and hibernation), we lack an understanding of other important processes (i.e., functional pathways) and regulators (e.g., transcription factors) involved. In addition, how these processes and regulators vary between males and females remains elusive. We address these questions using an extreme seasonal mammalian model, the Arctic ground squirrel (AGS, *Urocitellus parryii*). AGS are the northernmost hibernators, capable of suppressing metabolism to less than 5% of basal levels for extended bouts of torpor, only to periodically arouse and become euthermic every 2–4 wk in a repeatable cycle lasting for 6–8 mo (8, 24). Males terminate hibernation in the spring before females and remain sequestered in their hibernacula for 2–4 wk, during which they consume autumnal cached food and initiate testicular recrudescence and spermatogenesis (25–27). Females, in contrast, emerge from hibernation later than males, do not subsist on cached food, and become reproductively competent within days of terminating hibernation (27). It has been hypothesized that sex differences in the vernal termination of hibernation are due to increased circulating testosterone levels in males (28, 29). However, whether seasonal increases in sex steroids feedback to the hypothalamus to control sex differences in hypothalamic remodeling is unclear. If so, this may be a regulatory mechanism that underlies observed differences in the timing of life-history transitions between males and females.

We hypothesized that the preparation of physiological processes leading to the springtime termination of hibernation occurs via hypothalamic remodeling. To test this, we conducted RNA-sequencing in hibernating male and female AGS of a micropunched hypothalamic region that included 3V cell bodies, the ARC, median eminence, and pars tuberalis. We used discovery-based analyses to test our prediction that hypothalamic remodeling should involve the enrichment of processes and pathways related to hormone signaling and transport, as well as neurogenesis (14). Specifically, we predicted that genes aligned with these processes and pathways should be associated with tanyocytes of the 3V, supporting their purported role in rheostatic shifts. Overall, we provide evidence for hypothalamic remodeling and activation of signaling pathways across hibernation in a sex-dependent manner.

METHODS

Animal Housing and Sampling

Free-living male and female juvenile AGS were trapped in the Brooks Range of Alaska from late June to early July 2020. Animals were held at the University of Alaska

Fairbanks and fed 10 pellets (~50 g) of Mazuri rodent chow daily and given water ad libitum. During the active season, AGS were kept in a warm room (16–20°C) on a 16 L:8 D light cycle that transitioned to 4 L:20 D from early July to late September. During the active season, animals were implanted with abdominal transmitters (Model TA-F40, Data Sciences International, New Beighton, MN) to allow monitoring of internal body temperature throughout hibernation. Animals were kept in the warm room until they recovered from surgery. In late September, animals were moved into the hibernaculum (2°C, 4 L:20 D). Once animals entered hibernation, males and females were sampled during natural arousal events across the hibernation cycle: 2 wk after hibernation commenced (early hibernation, EH; $n = 3$ males, $n = 3$ females) or ~20 wk after hibernation commenced (late hibernation, LH; $n = 4$ males, $n = 4$ females) (representative temperature trace: Fig. 1A). Animals were sampled during interbout arousal when body temperature exceeded 32°C. Euthermic animals were deeply anesthetized using isoflurane mixed with medical-grade oxygen, and blood was drawn via cardiac puncture. Plasma was collected into heparinized tubes and stored at –80°C. Animals were then rapidly decapitated, brains sampled and frozen using hexane chilled with dry ice and stored at –80°C. Work with animals was approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee (IACUC) under Protocol No. 1428175. Trapping was permitted by the Alaska Department of Fish and Game under Permit No. 20-137.

Brain Sectioning and Punching

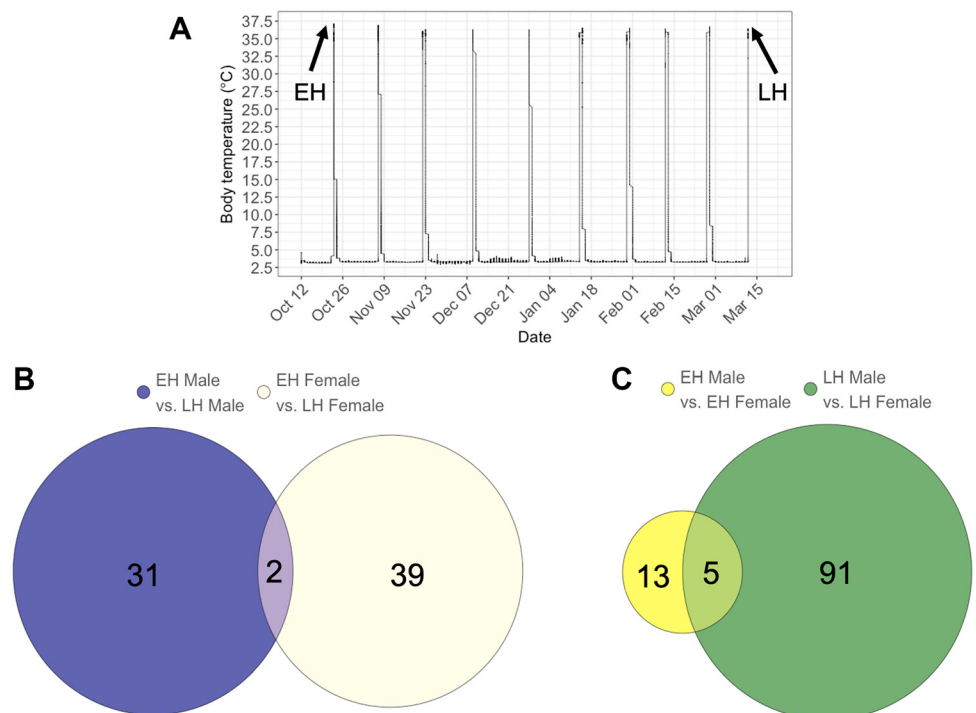
Brains were sectioned on a cryostat (Leica CM1950) using the Paxinos and Watson Rat Brain Atlas (4th edition) as a guide. Sections encompassed the rostrocaudal extent of the ARC (approximately: 2.12 to –3.60 mm, caudal to bregma).

Consecutive coronal sections (20 μ m) across this rostrocaudal region were periodically interrupted by a 100- μ m thick section used for hypothalamic punches—five 100- μ m sections were captured across the rostrocaudal extent of the ARC. All sections were mounted on SuperFrost Plus slides (VWR, Cat. No. 48311703). Furthermore, 100- μ m sections were mounted on slides, instantly placed on a dry-ice-cooled stainless steel plate, and a circular tissue punch (1,000 μ m; Electron Microscopy Sciences Cat. No. 57491) encompassing the ARC, 3V (including walls and floor), pars tuberalis, and median eminence were taken. Punches were transferred to RNase-free 1.5-mL microcentrifuge tubes on dry ice and frozen at –80°C until RNA was extracted. All 20 μ m sections were air-dried and stored at –80°C until downstream immunohistochemistry was performed.

RNA Extraction, Library Preparation, and Sequencing

RNA was extracted from punched microdissections using an RNeasy Micro Kit (Qiagen, Germantown, MD, Cat. No. 74004) according to the manufacturer's instructions, using a DNase digestion step to remove genomic DNA. All sample concentrations were measured using a Qubit 4.0 High-Sensitivity RNA Assay Kit (Invitrogen, ThermoFisher Scientific, Waltham, MA), using 1 μ L of purified RNA as input. The integrity of RNA was measured using a High-Sensitivity RNA ScreenTape Assay on a TapeStation 4150 (Agilent Technologies, Santa Clara, CA). All RNA integrity number (RIN) values were between 8.6 and 9.0. Preparations of libraries for RNA sequencing were done using a NEBNext Ultra II Directional Kit (Cat. No. E7760S), utilizing the polyA mRNA workflow. Each sample was multiplexed with unique dual index primers using the NEBNext Multiplex Oligos workflow (Cat. No. E7600S). Adapter ligated cDNAs were enriched following 15 PCR cycles on an Eppendorf Nexus Gradient Thermocycler.

Figure 1. A: representative temperature trace of a late hibernation group male Arctic ground squirrel ($n = 1$). Arrows point to early hibernation (EH) or late hibernation (LH) sampling points; animals were sampled during periodic arousals when body temperature returned to euthermia. Venn diagrams indicating the overlap in differentially expressed genes (absolute log₂-fold change of 0.5 filtered genes) between LH vs. EH in males (blue) and females (light yellow) (B), and the overlap in DE genes between sexes at EH (yellow) or LH (green) (C).



Libraries were checked for quality with an Agilent TapeStation High-Sensitivity DNA ScreenTape Assay and quantified on a Qubit 4.0 High-Sensitivity Double-Stranded DNA Assay Kit. Multiplexed libraries were pooled and sequenced on one lane of the NovaSeq X Plus platform with 150 base-pair paired-end reads (Novogene Corporation Inc., Sacramento, CA). An average of ~83 million reads per sample was obtained.

Bioinformatics

Raw fastq files were trimmed of polyG tails using *fastp* (v0.22.0) (30), with flags to disable length and quality filtering, along with adapter trimming. Illumina adapters were removed with *bbduk* (v39.01; sourceforge.net/projects/bbmap/) using default settings. Quality trimming and NEB adapter removal were done in *trimmomatic* (v0.39; RRID:SCR_011848) (31), using default trimming options (ILLUMINACLIP:NEB_adapters.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36). Trimmed files were aligned to the AGS genome (ASM342692v1) using *hisat2* (v2.2.1; RRID:SCR_015530) (32), producing an average mapping rate of 82%. Aligned reads were counted using the *featureCounts* function as part of the subRead package (v2.0.6; RRID:SCR_009803) (33), enabling flags to account for overlap and multimapping. This resulted in an average of ~64 million reads assigned per sample. Raw reads are deposited into the National Center for Biotechnology Information (NCBI) (SRA BioProject Accession No. PRJNA1134074). All downstream analysis was performed in R (v4.2.2).

Differential Expression Analysis

To address our hypothesis that annual changes in gene expression involve processes related to signal transport and neurogenesis, we performed a differential expression analysis on our generated RNA-sequencing dataset from hypothalamic micropunches. Differential expression analysis was conducted using *dream* in variancePartition (v1.28.9; RRID:SCR_019204) (34). Reads were filtered for lowly expressed genes, such that only genes greater than 0.5 counts per million (cpm) in 50% of the samples were kept, leaving us with 17,133 genes for our downstream analyses. We grouped time point and sex as a single predictor (i.e., EH♂, EH♀, LH♂, and LH♀), with sampling date modeled as a random effect. From this, we performed four planned contrasts in *dream*: EH♂ versus EH♀, LH♀ versus EH♀, LH♂ versus LH♀, and LH♂ versus LH♀. Genes were considered differentially expressed if *P*-adjusted < 0.05 (using the Benjamin–Hochberg false discovery rate [FDR]). For uncharacterized genes (e.g., “LOC”), if available, single-copy mouse orthologs were obtained from the online OrthoDB database (v11; RRID:SCR_011980). For uncharacterized differentially expressed genes of interest, Arctic ground squirrel nucleotide sequences were also compared with the corresponding *Mus musculus* nucleotide sequence using BLAST.

Differentially expressed genes from *dream* were classified as upregulated or downregulated based on an absolute log₂-fold change of 0.5. These subset genes were analyzed for functional enrichment of gene ontology (GO) terms using the *gost* function in *gprofiler2* (v0.2.1; RRID:SCR_006809)

(35); genes were ranked in decreasing order based on log₂-fold change. All filtered genes were used as a background set. GO terms were considered significant if the FDR-adjusted *P* values < 0.05, and term sizes were greater than 5. For pathway enrichment analysis, up- and downregulated genes (and background genes) were converted to EntrezID's using *bitr* in *clusterProfiler* (v4.6.2; RRID:SCR_016884) (36). Genes were analyzed using the function *enrichPathway* in the *ReactomePA* package (v1.42.0; RRID:SCR_019316) to assess enriched pathways. Enriched pathways were only considered significant if FDR-adjusted *P* values < 0.05. Differentially expressed genes were visualized using the packages *eulerr* (v7.0.0) and *ggplot2* (v3.4.0).

Validation of Genes via Droplet Digital Polymerase Chain Reaction

To validate the expression of key genes from RNA-sequencing data, RNA from hypothalamic punches was reverse transcribed to cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Cat. No. 4368814) and the following program 25°C for 10 min (annealing), 37°C for 120 min (reverse transcription), and 85°C for 5 min (inactivation). The cDNA product was stored at –80°C until droplet digital PCR was performed.

Droplet digital PCR was subsequently conducted according to the BioRad Laboratories QX200 ddPCR EvaGreen Supermix Protocol, using 10 ng of input cDNA. AGS primers [solute carrier family 16 member 2 (*Slc16a2*), *Slc24a4*, sortilin-related receptor-A 1 (*Sorll*), and *Kdm6a*] were designed in NCBI based on coding regions for the gene of interest and synthesized by ThermoFisher. Primer design ensured the spanning of an exon-exon junction, a 20-nucleotide length, and a product size of 60–180 base pairs. Annealing temperatures were calculated based on a final primer concentration in the PCR reaction (125 nM of forward and reverse primers); all primers had annealing temperatures ~60°C. No template (NT) controls were run for each gene using 5 µL of PCR-grade water instead of cDNA.

Inference of Regulatory Transcription Factors

Although functional enrichment analyses give biological insight into the potential patterns and processes for differentially expressed genes, it does not provide information on the regulatory control of global changes in gene expression. We hypothesized that functional enrichment analyses would reveal changes in gene expression related to hormone transport and neurogenesis. We also predicted that the differentially expressed gene set would be enriched for binding sites of upstream regulators (i.e., transcription factors) of these processes (relative to a background gene set). Using CiiiDER (37), we performed a transcription factor binding site enrichment analysis for upregulated and downregulated genes. We first used CiiiDER to identify the enriched transcription factor binding sites in differentially expressed genes across hibernation in both sexes to determine if males and females differ in their enriched regulatory factors. Second, we performed another enrichment analysis to identify the enriched transcription factor binding sites unique to each sex. In each sex, we identified enriched transcription factor binding sites in differentially expressed genes at late hibernation

versus early hibernation (e.g., LH♀ vs. EH♀) and determined whether these binding sites were also enriched in differentially expressed between the sexes at late hibernation (e.g., LH♀ vs. LH♂). This allowed us to identify putative transcription factors that might be responsible for programmed changes in gene expression that occur in one or both sexes across hibernation. Furthermore, using CiiiDER, we determined which differentially expressed genes were associated with the enriched transcription factor binding sites.

For CiiiDER analysis, all background gene sets were specified as filtered genes with a log₂-fold change between -0.05 and 0.05 (see Ref. 37). All differentially expressed genes (and background genes) were converted to ENSEMBL IDs using *biomaRt* (v2.54.1) (38) for input into CiiiDER. Transcription factor binding site scans in CiiiDER were performed using default settings (scans performed $-1,500$ bp upstream and 500 bp downstream of the transcription start site; deficit threshold of 0.15). Downstream analysis of enrichment results was done in R (v4.2.2). Transcription factor binding sites were considered overrepresented if P values < 0.05 and log₂ enrichment scores > 1 , indicating they were enriched in the differentially expressed gene set relative to the background set. Transcription factor binding sites were considered underrepresented if P values < 0.05 and log₂ enrichment scores < 1 , indicating they were enriched in our background gene set relative to our differentially expressed genes.

Sex Steroid Assays

Steroid hormones were extracted from $500 \mu\text{L}$ of plasma by solid phase extraction (39), using 200 mg Hypersep C18 columns (Thermo Scientific No. 60108303) on a vacuum manifold (Supelco Visiprep SPE Vacuum Manifold, MilliporeSigma, Burlington, MA). Hormones were eluted in 5 mL of 90% methanol and dried at 38°C using a steady stream of nitrogen gas (Multivap Nitrogen Evaporation System, Model 11848, Organomation Associates, Berlin, MA); samples were stored at -80°C . Before running assays, dried samples were dissolved in 100% ethanol (10% of the final volume), then fully resuspended in $450 \mu\text{L}$ (for testosterone assays) or $900 \mu\text{L}$ (for estradiol assays) of assay buffer. Samples were shaken ($1,000$ rpm) for 2 h at room temp, stored overnight at 4°C , and then shaken ($1,000$ rpm) for 1 h before running hormone assays.

Commercial enzyme-linked immunosorbent assay (ELISA) kits (Arbor Assays, Ann Arbor, MI) were used to quantify plasma testosterone (Kit No. K032) in males and estradiol (Kit No. K030) in females. All kits were validated for parallelism between the standard curve and serially diluted plasma samples, and dilution linearity with spiked samples. Extracted plasma samples were run in duplicate on a single plate, using a full standard curve. The intra-assay percent coefficient of variations for duplicates (means \pm SD) were $4.42 \pm 1.94\%$ (testosterone) and $1.41 \pm 0.84\%$ (estradiol). Data were analyzed using a one-way ANOVA. Model residuals were checked to ensure a normal distribution.

Immunohistochemistry

Coronal $20 \mu\text{m}$ sections from male and female AGS were removed from -80°C on the day immunohistochemistry was performed. Sections were air-dried at room temp, then

fixed and permeabilized in ice-cold methanol and acetone ($1:1$ v/v) for 1 min at -20°C . Sections were blocked in 5% normal goat serum (in 0.1% Triton X-100 PBS; PBST) for 60 min at room temperature. Sections were incubated with a SORL1 ($1:1,000$, rabbit monoclonal SORL1, Abcam No. 302508) primary antibody for 60 min at room temperature, then at 4°C in a humidity chamber for 24 h. Sections were washed three times with PBST for 5 min. Sections were incubated with secondary antibody ($1:500$, Alexa Fluor 647 goat anti-rabbit, Invitrogen No. A21244) for 60 min at room temp. Sections were washed three times with PBST for 5 min. Sections were then incubated with a fluorescein (FITC) conjugated vimentin (VIM) primary antibody ($1:1,000$, mouse monoclonal VIM V9, Invitrogen, Cat. No 11989780) for 120 min at room temp. Sections were washed like previous steps, and incubated with DAPI ($1:50,000$) for 5 min, followed by a 5 -min wash in PBS. Sections were mounted on slides using Fluoromount-G (SouthernBiotech, Cat. No.010001). Sections were imaged on an Olympus APX100 at $\times 10$ and $\times 40$ using cellSens software (SORL1 exposure time: 654 ms; VIM-FITC: 300 ms). Negative controls (secondary antibody only) showed no nonspecific fluorescent binding of SORL1 (Supplemental Fig. S1).

RESULTS

Differential Expression, Functional Enrichment, and Pathway Analysis of the Global Gene Set

First, we addressed our hypothesis that annual changes in gene expression alter processes related to hormone transport and signaling, as well as neurogenic-related processes. Using an unbiased analysis of the global gene set from our RNA-sequencing approach for micropunched brain sections, we found a similar number of differentially expressed genes between the sexes at late hibernation relative to early hibernation; 33 genes were differentially expressed in LH♂ versus EH♂ and 41 genes were differentially expressed in LH♀ versus EH♀ (Fig. 1B; Fig. 2, A and B). However, only two of the genes identified as differentially expressed between late and early hibernation were shared between the sexes (*Ccdc177* and *Vtn*). Most of the differentially expressed genes in males were upregulated in late hibernation (25 out of 33 genes), whereas females had a similar number of up- and downregulated genes (17 upregulated in late hibernation vs. 24 downregulated).

Given our prior work indicating gonadal development is initiated at late hibernation (14) and the known regulatory role the hypothalamus plays in puberty onset (40, 41), we next compared differential gene expression between the sexes at early hibernation and late hibernation. We observed more differentially expressed genes between LH♂ versus LH♀ (96 genes) compared with EH♂ versus EH♀ (18 genes). Only five genes were consistently differentially expressed between the sexes at both early hibernation and late hibernation: *Vtn*, *Zrsr2*, *Usp9x*, *Uba1y*, and *Zfx* (Fig. 1C). Most of the standout upregulated genes in LH♂ versus LH♀ and EH♂ versus EH♀ were predicted to be linked to the X chromosome (*LOC113176020/Eif1a*, *LOC113175911/Zrsr2*, *LOC113175909/Usp9x*, *LOC113176017/Zfx*, *LOC113175910/Kdm6a*, and *LOC113175926/Kdm5c*), based upon their single-copy mouse orthologues and nucleotide BLAST sequence

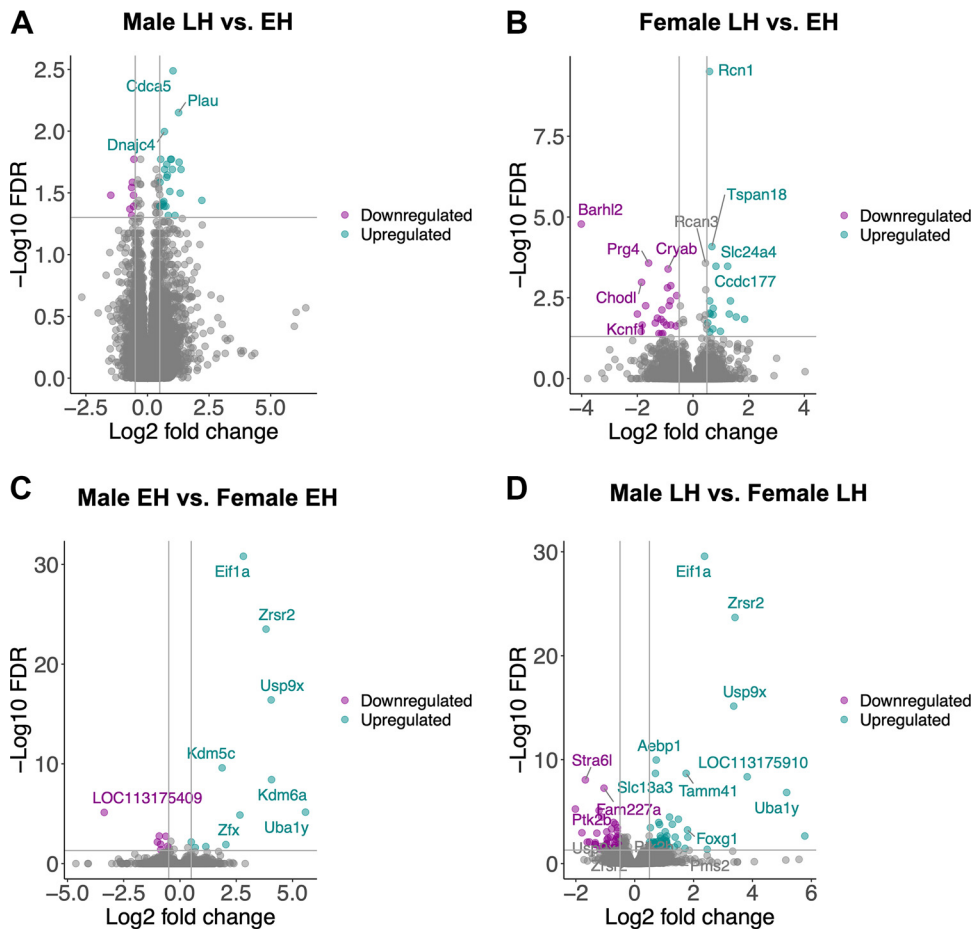


Figure 2. Volcano plots of gene expression changes for all pairwise comparisons in this study at early hibernation (EH) and/or late hibernation (LH), within or between the sexes (A–D). Horizontal lines indicate the negative logarithmic false discovery rate cutoff used in this study ($FDR < 0.05$), with the vertical lines indicating the log fold change cutoff (absolute fold change > 0.5). Dots colored green indicate the genes that are upregulated, with magenta dots indicating a downregulation.

similarities to *Mus musculus* (Fig. 2, C and D). We validated the expression of *Kdm6a* using ddPCR (Supplemental Fig. S2A). Comprehensive gene lists for all pairwise comparisons are given in Supplemental File S1.

We subsequently analyzed the functional importance of the observed changes in gene expression to determine if they were associated with processes we hypothesized to be induced late in hibernation (e.g., related to hormone signaling and neurogenesis). In females, we found that genes upregulated in late hibernation versus early hibernation were enriched for processes related to hypothalamic remodeling and signal transport (GO terms: “neural cell proliferation,” “negative regulation of neurogenesis,” “import across plasma membrane,” and “export from cell”). Genes intersecting with these terms, e.g., *Slc16a2*, *Slc24a4*, *Vax1*, and *Sor11*, were upregulated in LH_♀ versus EH_♀ but not in LH_♂ versus EH_♂. Cross-referencing these genes with the Allen Brain in situ hybridization (ISH) atlas revealed that *Slc16a2* and *Sor11* have localized mRNA expression along the base and walls of the 3V of the hypothalamus, with *Slc24a4* and *Vax1* being expressed within the ARC (Fig. 3, A–D). We validated this upregulation of *Slc16a2*, *Slc24a4*, and *Sor11* using ddPCR (Supplemental Fig. S2, B–D). Another important neurogenic regulator, BarH-like homeobox 2 (*Barhl2*), was differentially expressed in LH_♀ versus EH_♀ (Fig. 2B); *Barhl2* is implicated in cell

differentiation and specification and is expressed in 3V ependymal cells (42, 43).

To assess differences between the sexes, we then compared the hypothalamic transcriptome between the sexes at both early and late hibernation time points (i.e., EH_♀ vs. EH_♂ and LH_♀ vs. LH_♂). Relative to EH_♂, upregulated genes in EH_♀ were enriched for terms related to the complement system (“complement activation”); the complement system has been suggested to be important in the development of the central nervous system (44). Relative to LH_♂, upregulated genes in LH_♀ were enriched for processes related to cell proliferation and neurodevelopment (e.g., “negative regulation of apoptotic processes,” “system development,” “homeostatic processes”). Although GO analysis did not reveal enrichment of any biological processes in EH_♂ versus EH_♀ or LH_♂ versus LH_♀, there was evidence of enrichment for molecular functions related to “transcription regulatory region nucleic acid binding” in EH_♂ versus EH_♀, and “molecular function regulator activity” in LH_♂ versus LH_♀. All GO enrichment results are provided in Supplemental File S2.

We next used ReactomePA enrichment analysis to examine pathways of upregulated differentially expressed genes in LH_♀ versus EH_♀. ReactomePA analysis supported these prior GO term findings in females, where genes upregulated in late hibernation versus early hibernation were enriched for

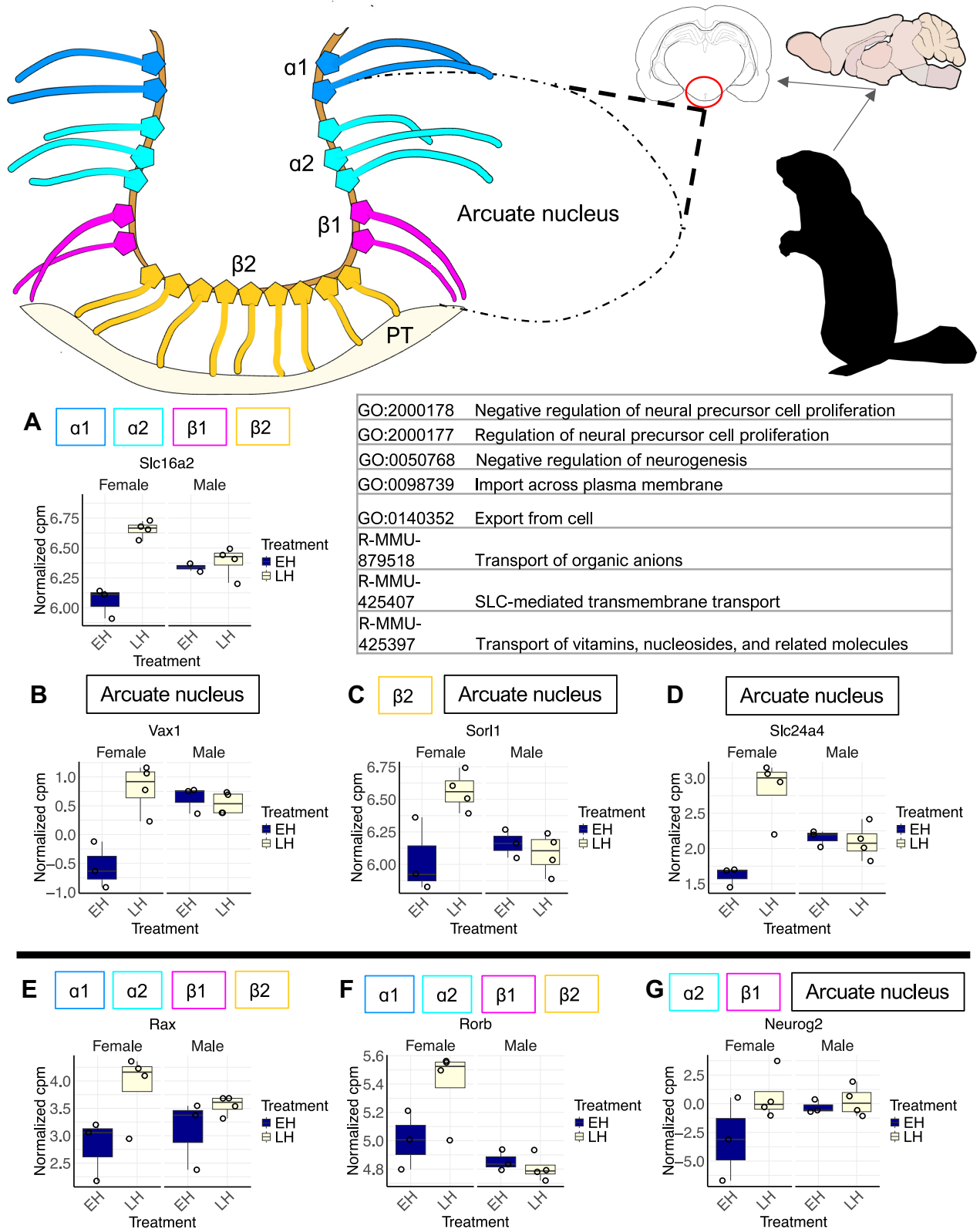


Figure 3. Differentially expressed genes from female AGS, their intersecting gene ontology (GO) and ReactomePA terms, and localized expression in various locations of the mediobasal hypothalamus, as determined by the Allen in situ hybridization Brain Atlas. Tanycyte subtype localization for each gene is denoted by both color and symbol ($\alpha 1$, $\alpha 2$ $\beta 1$, $\beta 2$) along the floor and walls of the third ventricle (3V). Differentially expressed genes intersect with GO or ReactomePA terms (A–D) or are the predicted target genes for enriched transcription factors determined by CiiDER analysis (E–G). AGS, Arctic ground squirrel.

pathways related to the “transport of organic anions,” “transport of vitamins and nucleosides,” and “SLC-mediated transport.” Consistent with our GO analysis, *Slc16a2* and *Slc24a4* intersected with these pathways. When comparing the sexes using ReactomePA analysis, enriched pathways for upregulated genes of EH♀ versus EH♂ support the prior GO terms, with an enrichment of the “complement cascade” pathway. In EH♂ versus EH♀, enriched pathways were related to “chromatin organization” and “chromatin modification.” No ReactomePA pathways were significant for genes upregulated in LH♀ versus LH♂ or LH♂ versus LH♀. All pathway enrichment results are provided in Supplemental File S3.

Functional enrichment of our differentially expressed genes in females revealed terms related to the transport of solutes, anions, and nucleosides. Seasonal retrograde signaling relies on the transport of factors from the pars tuberalis through tanycyte processes to the ARC (23), and tanycytes have also been shown to traffic anorectic peripheral signals to the CSF and ARC to maintain energy balance (45). We therefore examined whether any of the genes intersecting with these functional enrichment terms are thought to aid in these processes. One intersecting gene, sortilin-related receptor-A 1 (*Sorl1*), which was expressed at higher levels in LH♀ versus EH♀ (Fig. 4A), is involved in endosomal transport within cells (46) and exhibits strong mRNA expression along the base of the 3V in the hypothalamus of mice based on the Allen Brain Atlas (Fig. 4B) (47). Our immunohistochemical analysis in AGS confirmed SORL1 was expressed in ependymoglial cells along the ventral floor of the 3V in close proximity to vimentin-positive (VIM; a protein marker for tanycytes) cell bodies (Fig. 4C); SORL1 also shows low levels of expression in the surrounding hypothalamus (Fig. 4C). This labeling pattern of SORL1 and VIM also occurred in LH♂ and EH♂ (Supplemental Fig. S3).

Finally, we measured circulating levels of steroids at late hibernation and early hibernation in both sexes. In females, a trend for a modest increase in estradiol was observed at late hibernation compared with early hibernation ($F_{1,5} = 4.886$, $P = 0.078$) (Supplemental Fig. S4). Although testosterone

activation is hypothesized to drive the earlier vernal termination of hibernation in males (48), at the time point tested we found no evidence for an increase in testosterone levels in LH♂ versus EH♂; if anything, testosterone levels showed a trend toward a slight decrease at late versus early hibernation ($F_{1,5} = 4.047$, $P = 0.100$) (Supplemental Fig. S5).

Upstream Regulators Involved in ARC Signaling across Hibernation

We performed transcription factor binding site enrichment analyses using CiiiDER to reveal broader regulatory processes occurring across hibernation within and between the sexes. We first examined whether any binding sites were enriched across hibernation. Within the set of genes that were significantly upregulated in both sexes at late hibernation versus early hibernation, we found only two significantly enriched transcription factor binding sites: diencephalon/mesencephalon homeobox 1 (*Dmbx1*) and Glial cells missing transcription factor 2 (*Gcm2*) (Supplemental Table S1). Seven transcription factor binding sites were enriched in the set of genes that were downregulated in both LH♂ versus EH♂ and LH♀ versus EH♀; most of these transcription factors belong to the HOX-related factor family (e.g., *Hoxc11*, *Hoxd12*, *Hoxd11*, *Hoxc12*, *Hoxa11*, and *Hoxd10*) (Supplemental Table S1). Gene targets for these enriched transcription factor binding sites are shown in Supplemental Table S2.

We subsequently examined the enriched transcription factor binding sites in our set of genes that were differentially expressed within the sexes. Considering both GO and ReactomePA pathway analyses in females revealed terms related to thyroid signaling and neurogenesis, we predicted that 1) enriched transcription factor binding sites in females would reveal upstream regulators with identities and functions related to these terms and 2) genes intersecting with these GO and ReactomePA terms would contain binding sites for the enriched transcription factors.

Of the transcription factor binding sites significantly enriched in the set of upregulated genes in LH♂ (relative to EH♂), we found only one, sterol regulatory element binding

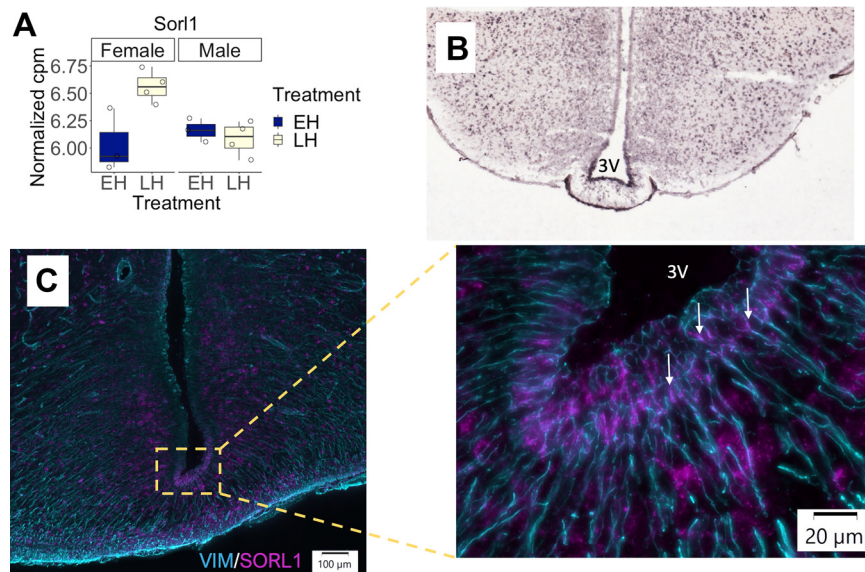


Figure 4. A: normalized cpm of sortilin-related receptor-A 1 (*Sorl1*) at early and late hibernation, faceted by sex. Boxplots of normalized cpm for each gene contain a center line representing the median, boundaries representing the first and third quartile, and whiskers representing minimum and maximum values. B: in situ hybridization coronal image of *Sorl1* in a C57BL/6J male mouse at 56 days old, acquired online from the Allen Brain Atlas (<https://mouse.brain-map.org/experiment/show/74511924>). C: representative immunohistochemical image of the ARC from an EH female AGS using anti-VIM and an anti-SORL1 antibody ($\times 10$ objective). Yellow dashed box highlights SORL1 and VIM staining at the base of the third ventricle (3V). White arrows point to SORL1 within VIM-positive tanycyte processes ($\times 40$ objective). AGS, Arctic ground squirrel; ARC, arcuate nucleus; cpm, counts per million; EH, early hibernation; VIM, vimentin.

transcription factor 1 (*Srebf1*), that was also enriched in upregulated genes at LH♂ relative to LH♀ (Supplemental Table S3). Predicted binding sites for *Srebf1* occurred in the regulatory regions of 30% (6/20) of upregulated genes in LH♂ versus EH♂ but in only 12% [292/2347] of genes in the background set and 12% (5/39) of the upregulated genes in LH♂ versus LH♀ (vs. 4.3% [143/3291] in the background set) (Supplemental Table S4). In females, we found three significantly enriched transcription factor binding sites in LH♀ versus EH♀ that were also enriched in LH♀ relative to LH♂: Glial cells missing transcription factor 1 (*Gcm1*), V-Maf avian musculoaponeurotic fibrosarcoma oncogene (*Maf*), and Myocyte enhancer binding factor 2 b (*Mef2b*) (Supplemental Table S3). Binding sites for *Gcm1* occurred in the regulatory regions of 23% (3/13) of the upregulated genes in LH♀ versus EH♀ compared with only 3% (91/3123) of genes in the background and 24% (6/25) in upregulated genes in LH♀ versus LH♂ [vs. 8.5% (279/3292) in the background set]. Binding sites for *Maf* occurred in regulatory regions of 15% (2/13) of the upregulated genes in LH♀ versus EH♀ [vs. 1.5% (47/3,123) in the background set] and 20% (5/25) in upregulated genes in LH♀ versus LH♂ [vs. 6.7% (223/3,292) in the background set]. Binding sites for *Mef2b* occurred in regulatory regions of 7% (1/13) of upregulated genes in LH♀ versus EH♀ [vs. 0.2% (5/2,123) in the background set] and 8% (2/25) in upregulated genes in LH♀ versus LH♂ [vs. 1.2% (42/3,292) in the background set] (Supplemental Table S4).

Enriched transcription factor binding sites in LH♀ versus EH♀ tended to be associated with differentially expressed genes involved in hormone/solute transport (e.g., *Slc16a2* and *Slc24a4*), and these genes also intersected with our GO and ReactomePA results. In addition, enriched transcription factor binding sites in LH♀ versus EH♀ were associated with differentially expressed genes known to be involved in neurogenesis (e.g., *Rax*, *Neurog2*, *Nrep*, and *Rorb*) (Supplemental Table S3). Cross-referencing these genes with the Allen Brain in situ hybridization (ISH) Reference Atlas (47) revealed localized mRNA expression to known regions along the 3V of the hypothalamus and ARC of the hypothalamus (Fig. 3, E–G).

DISCUSSION

Our analyses of RNA-sequencing data from hypothalamic micropunches identified substantial sex differences in processes and regulators modulated across hibernation in Arctic ground squirrels (AGS). Specifically, we found differences in how hypothalamic gene expression profiles changed in males versus females across hibernation, with greater sex differences in expression profiles occurring at late hibernation. Based upon prior work (11, 14, 49), we hypothesized that processes related to thyroid hormone signaling and neurogenesis would be important in regulating the seasonal rheostat of AGS. In females, although we did not observe a significant change in the expression of key genes in the thyroid hormone signaling pathway, functional enrichment analyses of genes differentially expressed across hibernation provided evidence that processes and pathways related to the transport/signaling of hormones, as well as neurogenesis, were activated. Many of the differentially expressed genes across hibernation in females exhibited localized

expression in the walls and floors of the 3V, supporting the purported role of tanycyte cells in orchestrating seasonal changes within the hypothalamus (17).

Although males end hibernation earlier than females, and we observed a similar number of differentially expressed genes across hibernation in males relative to females, we did not identify processes or pathways that are important for hypothalamic remodeling in males. Rather, we observed a number of predicted X-linked genes (*Usp9x*, *Zfx*, *Kdm6a*, *Kdm5c*, *Zrsr2*, and *Eif1a*), that were consistently overexpressed in males. These genes have been implicated in hypothalamic development and function and are linked to playing a role in energy balance (50–53). The results we present support a need for more concerted efforts to understand sex differences in the control mechanisms that underlie seasonal life-history transitions.

Cellular Hormone Transport Processes Are Upregulated across Hibernation in Female AGS

We provide evidence that female AGS show concerted changes in gene expression during hibernation, indicating activation of molecular pathways that lead to hypothalamic remodeling. In seasonal vertebrates, increases in thyroid hormones modulate the functioning of the reproductive axis, which has previously been shown via the implantation of T3 into the hypothalamus (54). We anticipated that processes and pathways related to T3 signaling would be revealed in our gene expression data set. Although we observed changes in thyroid hormone transporter expression, we did not see changes in the expression of key genes in the retrograde thyroid hormone signaling pathway (e.g., *Nes*, *Kiss*, *Dio2*, *Dio3*, *Eya3*, and *Tshb*) that met our significance threshold—these genes had previously been identified as differentially expressed across hibernation based on in situ hybridization data in an earlier AGS study (14). The differences between studies may relate to differences in the time at which animals were sampled across hibernation. For instance, sampling of individuals at late hibernation in the earlier AGS study only occurred after an individual's torpor bouts had begun shortening (14), whereas, in the present study, all animals were sampled based on calendar date. Alternatively, the differences may simply be related to differences in the approaches used (i.e., in situ hybridization vs. RNA-sequencing).

Regardless of our inability to detect changes in the expression of key genes associated with hypothalamic T3 signaling, both GO and ReactomePA analyses of our global gene expression set provide support for our prediction that annual changes in gene expression are mirrored by the upregulation of processes related to hormone transport. Many of the genes intersecting with GO and ReactomePA terms related to hormone transport (e.g., “SLC-mediated transport,” “export from cell,” “calcium:sodium antiporter activity,” “transport of organic anions”) were localized to tanycytes. This supports both our hypotheses and aligns with earlier evidence (17, 55, 56) that annual changes in metabolism are dictated by tanycytic regulation of seasonal transport of signals into the hypothalamus.

The transmembrane transport and trafficking of signals is likely an important component of seasonal metabolic reprogramming. Ground squirrels are completely aphagic

across hibernation and do not consume food or water during interbout arousal periods, even if available (57). Seasonal alterations in signal transport across cellular membranes may determine seasonal differences in sensitivity to peripheral signals. For instance, it has been suggested that T3 signaling is responsible for rescuing the food suppression ground squirrels exhibit during hibernation; hypothalamic infusions of T3 during periodic arousals from torpor cause minor increases in food intake (57). Indeed, we found that female AGS upregulated the expression of solute carrier family 16 member 2 (*Slc16a2*) across hibernation. *Slc16a2* encodes the monocarboxylate transporter protein 8 (MCT8), a bidirectional transporter of thyroid hormones in cells (58). Although previous research in AGS did not reveal any substantial changes in the expression of MCT8 (14), work in Siberian hamsters (*Phodopus sungorus*) has shown seasonal changes in its expression corresponding to seasonal fluctuations in body weight (59). We also show an upregulation of *solute carrier family 24 member 4* (*Slc24a4*), which encodes the sodium/potassium/calcium exchanger 4 (NCKX4), which is involved in neuronal development and excitability. NCKX4 is required for melanocortin-mediated satiety—NCKX4 knockout mice become hypophagic and lose weight (60). *Slc24a4* is expressed in the ARC and other hypothalamic nuclei (60); the upregulation of *Slc24a4* at late hibernation in female AGS may occur in anticipation of the resumption of feeding in the springtime.

Tanycytes are the proposed shuttle of TSH β between the pars tuberalis and hypothalamus, where signals are retrogradely transported to the cerebrospinal fluid of the 3V (61); tanycytes also transport peripheral anorectic signals (e.g., leptin) to the cerebrospinal fluid and ARC (62). We found sorbitin-related receptor-A 1 (*Sorl1*)—a gene that sorts endosomal cargo to either the cell surface via retrograde transport to the trans-Golgi network or for degradation through the endo-lysosomal network (63)—is upregulated at late hibernation in females. *SORL1* expression occurred along the base of the 3V; this subpopulation of tanycytes is known to function in the retrograde transport of signals from the median eminence to the ARC (64), suggesting a potential role of *SORL1* in modulating seasonal signaling.

Overall, our results suggest that changes in hypothalamic cellular transport processes across the hibernation season may be important in progressively regaining sensitivity to peripheral signals in anticipation of the resumption of springtime physiological processes, like feeding and reproduction.

Functional Enrichment and Upstream Regulators Are Linked to Neurogenesis in Female AGS

Previous research in AGS highlighted that, as part of the modulation of the reproductive axis, markers indicative of structural remodeling and neurogenesis/gliogenesis (e.g., nestin and vimentin) were altered across hibernation in tanycytes (14). This structural plasticity may seasonally alter the ability to transduce signals to the ARC, as access of signals from the periphery to the ARC is dependent on structural changes in tanycytes (65). Furthermore, changes in markers of structural remodeling may also be important in

the seasonal regulation of neurogenesis and/or gliogenesis (66). In our gene expression data set, GO enrichment analysis in females revealed upregulated terms related to neurogenesis and cell proliferation (e.g., “neural precursor cell proliferation” and “negative regulation of neurogenesis”), suggesting that the regulation of neurogenic processes is occurring across hibernation.

Although hypothalamic remodeling of neuroendocrine pathways has been previously described in AGS (14) and other rodents across the year (67), the regulators responsible for these changes have not been identified. CiiiDER enrichment analysis revealed transcription factors with enriched binding sites that are known to play a role in cell differentiation, neuronal development, survival, and plasticity: glial cells missing transcription factor 1 (*Gcm1*), V-maf avian musculoaponeurotic fibrosarcoma oncogene (*Maf*), and myocyte enhancer binding factor 2 b (*Mef2b*) (68–70). Furthermore, these enriched transcription factors contain binding sites in genes that intersect with GO terms related to neurogenesis (*Vax1* and *Slc16a2*) as well as in other genes implicated in neurogenesis that are localized to tanycytes (*Rax*, *Neurog2*, *Nrep*, and *Rorb*) (71–74). For instance, *Rax* is required for tanycyte differentiation (75), and *Neurog2* is a proneural factor that promotes neuronal differentiation in progenitor cells (76).

In rodents that are not sensitive to changes in day length, levels of neurogenesis in the adult hypothalamus appear to be low (77). In contrast, recent work in seasonal mammals suggests that neurogenesis in the adult hypothalamus is seasonally regulated. This appears physiologically important for animals that undergo seasonal periods of sexual activity and quiescence, but may also be an important component for rheostatic shifts in energy balance (22, 23). Most of the seasonal neurogenic changes in the hypothalamus are localized to tanycytes (78–80), which have been shown to proliferate into neuronal or glial cells both seasonally and in response to changes in nutritional state (16, 81). In seasonal mammals, it has been theorized that seasonal fluctuations in T3 induce the formation or degradation of neurons in the ARC; declines in T3 in the fall (before torpor entrance) lead to neuronal cell death, whereas increased T3 during the transition from winter to summer results in the proliferation of tanycytes that replace ARC neurons (23). We saw increased expression of the thyroid hormone transporter, *Slc16a2*, across hibernation, suggesting that increased seasonal thyroid hormone transport may result in an alteration of neurogenic processes and regulators across hibernation in female AGS, though functional studies are needed to evaluate this hypothesis. Although our bulk sequencing approach cannot identify the specific neuronal populations changing in the hypothalamus during the hibernation season, cell-type specific methods (e.g., single-cell/nuclei sequencing) would be useful in delineating the underlying cellular phenotypes responsible for these metabolic and neurogenic shifts.

Male AGS Exhibits Reduced Functional Gene Expression Coordination across Hibernation

Gene expression data in females supported the enrichment of processes related to hormone transport and neurogenesis, with many of these genes being localized to

tanocytes. In contrast, evidence of hypothalamic remodeling across hibernation was less supported in males, despite a similar number of differentially expressed genes at late hibernation versus early hibernation. We suggest that the differences in results between males and females may have occurred due to phenological differences in hibernation timing. Nevertheless, we suggest there is a need for hibernation studies to focus more on sex differences in seasonal hypothalamic remodeling, and more frequent sampling across the hibernation season may be beneficial to capture potential subtle changes in gene expression.

Sex differences in spring phenology are common across vertebrates (82). In AGS, reproductive males enter hibernation ~1–2 wk after females and terminate hibernation ~3–4 wk before females emerge (27). Sex differences in spring phenology occur because males need to spend several weeks sequestered in their hibernacula undergoing testicular maturation so they can mate with females that can become pregnant within 48 h of emergence (83). Given the earlier termination of hibernation by males, we anticipated that they might show greater activation of signaling processes related to increases in hypothalamic thyroid hormone availability in late hibernation. Indeed, activation of the reproductive axis, triggered by increased T3 availability, is hypothesized to drive an increase in testosterone, which governs the earlier termination of hibernation by males (84, 85). We observed little change in the expression of components of the hypothalamic thyroid hormone signaling pathway in either sex across hibernation, contrary to our prediction. However, we found evidence that genes expressed in late hibernation females are enriched for solute transport processes to a greater degree, relative to the early hibernation sampling timepoint. The increased expression of thyroid hormone transporters and the enrichment of biologically relevant processes in late hibernation females may relate to their need to become reproductively competent immediately after terminating hibernation in the spring. Why we see a muted response at late hibernation in males is unclear.

We initially anticipated that the major drivers of gene expression at late hibernation would be driven by sex hormones in male AGS. However, there was no significant change in testosterone levels nor thyroid hormone signaling components. Instead, we saw that the genes consistently different between the sexes at both early and late hibernation time points were predicted to be linked to the X chromosome. Predicted X-linked genes that were upregulated in males are involved in RNA splicing (*Zrsr2*), translation (*Eif1a*), neuronal development (e.g., *Usp9x*, *Zfx*, *Kdm6a*, *Kdm5c*) (51–53, 86, 87). Of note, lysine demethylase 6A (*Kdm6a*) has been shown to control the differentiation of hypothalamic neurons involved in energy balance in a sex-specific manner; knockdown of *Kdm6a* in mice affects the expression of appetite regulatory neuropeptides (e.g., *Pomc* and *Npy*) in females but not in males (50). Although we cannot tease apart a seasonal effect of these predicted X-linked genes, our findings provide insight into systemic sex differences in hypothalamic gene expression.

Conclusions

Our study highlights substantial sex differences in the modulation of regulatory processes controlling energy

balance, appetite, and neurogenesis across hibernation in AGS. Changes in gene expression in females across hibernation were associated with neurogenic and cellular transport processes. We hypothesize that these changes may be required during hibernation to prime neuronal circuitry before springtime emergence and are important to facilitate the resumption of appetite and reproductive preparedness once hibernation ends. In males, by contrast, GO and pathway analyses identified equivocal functional responses within the hypothalamus, despite similar changes in the number of differentially expressed genes across hibernation. This was surprising given males end hibernation more than 3 wk before females in this species. Overall, we suggest that disregarding sex as a factor in gene expression studies of hibernators is problematic, particularly given known sex differences in the timing of life history transitions. In the future, transcriptomic studies are needed that use larger sample sizes in both sexes and take a single-cell or single-nuclei approach. This would help to delineate the role of different hypothalamic cell types in the seasonal plasticity and function of the hypothalamus.

DATA AVAILABILITY

All data and code included in the analysis are publicly available on GitHub (https://github.com/ckdeal/AGS_transcriptomics).

SUPPLEMENTAL MATERIAL

Supplemental Figs. S1–S4: <https://doi.org/10.6084/m9.figshare.28789901>.

Supplemental Tables S1–S4: <https://doi.org/10.6084/m9.figshare.28789868>.

Supplemental Files S1–S3: <https://doi.org/10.6084/m9.figshare.28789931>.

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DISCLAIMERS

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

DISCLOSURES

K.L.D. has a financial interest in Be Cool Pharmaceuticals LLC. None of the other authors have any conflicts of interest, financial or otherwise, to disclose.

AUTHOR CONTRIBUTIONS

C.K.D. and C.T.W. conceived and designed research; C.K.D., M.H.S., K.L.D., and C.T.W. performed experiments; C.K.D. analyzed data; C.K.D. and C.T.W. interpreted results of experiments; C.K.D. prepared figures; C.K.D. drafted manuscript; C.K.D., M.H.S., K.L.D., and C.T.W. edited and revised manuscript; C.K.D., M.H.S., K.L.D., and C.T.W. approved final version of manuscript.

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