Hypothalamic thyroid hormone deficiency underlies reversible anorexia in a mammalian hibernator

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Abstract

Mammalian hibernators survive prolonged periods of cold and resource scarcity by temporarily modulating normal physiological functions, but the mechanisms underlying these adaptations are poorly understood. The hibernation cycle of thirteen-lined ground squirrels lasts for 5–7 months and comprises weeks of hypometabolic, hypothermic torpor interspersed with 24–48-hour periods of an active-like interbout arousal (IBA) state. We show that ground squirrels, who endure the entire hibernation season without food, have negligible hunger drive during IBAs. These squirrels exhibit reversible inhibition of the hypothalamic feeding center, such that hypothalamic arcuate nucleus neurons exhibit reduced sensitivity to the orexigenic and anorexigenic effects of ghrelin and leptin, respectively. However, hypothalamic infusion of thyroid hormone during an IBA is sufficient to rescue hibernation anorexia. Our results reveal that thyroid hormone deficiency underlies hibernation anorexia and demonstrate the functional flexibility of the hypothalamic feeding center.

Hibernation was first documented in 350 BCE by Aristotle, who noted that some creatures cease eating and conceal themselves in a sleep-like state for many months to pass the winter[1]. Hibernation invokes a series of flexible adaptations that allow animals to thrive in inhospitable environments, where they experience thermal challenges and food scarcity[2]. Coordination of hunger and satiety is essential for hibernators to survive, as premature emergence from underground burrows to seek food may dysregulate dependent processes and increase the risk of predation.

In thirteen-lined ground squirrels *(Ictidomys tridecemlineatus)*, hibernation consists of repeated cycles of hypothermic torpor interspersed with brief periods of euthermic IBA (Fig. 1a, b). During torpor, animals enter a state of suspended animation by profoundly reducing their metabolic, heart and respiration rates, and lowering their body temperature to 2–4 °C. Every 2–3 weeks, squirrels arouse to spend ~24 hours in IBA, when their main bodily functions temporarily return to an active-like state (Fig. 1b)[3]. Ground squirrels do not depend on stored food for fuel during hibernation; instead, energy is supplied by body fat amassed during the summer. Thus, although hibernating squirrels resemble fasted animals metabolically, they demonstrate little interest in food despite enduring over seven months of starvation[4–7]. We sought to understand the mechanism underlying this remarkable example of reversible anorexia by comparing euthermic animals during the active season with euthermic animals during IBAs in the hibernation season. These experiments reveal that thyroid hormone deficiency in the hypothalamus underlies hibernation anorexia.

Results

Hibernating ground squirrels have negligible hunger drive

When squirrels were presented with food during the numerous IBAs throughout the hibernation season, they consumed approximately six times less food than during the active state (active: 14.0 ± 0.8 g/day; IBA: 2.4 ± 0.2 g/day; Fig. 1c). Their body weight continuously decreased throughout hibernation, reaching ~50% of their starting weight by the end of the season (Fig. 1c). During IBAs, we found reduced levels of serum glucose (active: 22.2 ± 2.9 mM; IBA: 12.8 ± 1.1 mM) and serum insulin (active: 44.4 ± 7.4 µU/mL; IBA: 8.4 ± 1.3 µU/mL), both of which decreased

throughout hibernation (Fig. 1d, e). Consistent with metabolism of fat being the primary energy source during hibernation, squirrels exhibited increased levels of serum β-hydroxybutyrate during IBAs compared to active squirrels (active: 0.23 ± 0.04 mM; IBA: 2.07 ± 0.25 mM; Fig. 1f). Thus, squirrels appear to lack hunger drive during hibernation, even during IBAs.

Hibernating squirrels demonstrate reversible resistance to ghrelin

During fasting, the stomach releases the orexigenic hormone ghrelin, which activates Agouti-Related Peptide/Neuropeptide Y (AgRP/NPY) neurons in the arcuate nucleus of the hypothalamus (ARC)[8–12]. To investigate whether low levels of ghrelin underlie lack of hunger in hibernating squirrels, we measured total and active ghrelin in blood plasma. There was no significant difference between IBA and active squirrels, and active ghrelin showed an increasing trend throughout hibernation, albeit insignificant (Fig. 2a-c). Because such high ghrelin levels are sufficient to induce feeding in active animals, we hypothesized that hibernating squirrels develop seasonal ghrelin resistance[4,13]. We tested this by monitoring food consumption in active and IBA squirrels after peripheral injection of ghrelin. As expected, ghrelin potentiated feeding in active squirrels to levels observed in mice and rats[14–16], exceeding those of active squirrels after 48 hours of food deprivation (Fig. 2d, e). In stark contrast, ghrelin failed to potentiate food consumption when injected during IBAs (Fig. 2d).

Immunohistochemical analysis of *c-FOS* expression showed that ghrelin injections activated a subset of ARC neurons in active, but not IBA, animals (Fig. 2f, g), suggesting that AgRP/NPY neurons have reduced sensitivity to ghrelin during hibernation. In normal physiological conditions, ghrelin binding to growth-hormone secretagogue receptors (*Ghsrs*) on AgRP/NPY neurons triggers release of AgRP from nerve terminals[8,17–21]. In contrast, we observed that AgRP accumulates in neuronal somas during IBAs (Fig. 2h), implying diminished release. Deep sequencing of the ARC showed similar levels of *Ghsrs* in active and IBA animals (Fig. 2i). Moreover, *de novo* cloning of the ARC *Ghsr* revealed that > 95% of transcripts in IBA animals represented the full-length functional *Ghsr* isoform[20] (Fig. 2j). Thus, the absence of a neuronal and behavioral response to peripheral ghrelin injection in IBA squirrels cannot be attributed to a lack of functional ghrelin receptors.

Hibernating squirrels have reduced leptin signaling

To investigate whether additional mechanisms are involved in the lack of hunger drive in hibernating squirrels, we asked if ARC neurons are sensitive to the satiety hormone leptin during IBAs. Plasma levels of leptin were slightly elevated in IBAs compared to active animals (Fig. 3a). In addition, deep sequencing of the ARC as well as *de novo* cloning revealed active-like levels of expression of both full-length and truncated transcripts of the long isoform leptin receptor (*Lepr*)[22] in IBA animals (Fig. 3b-c). However, despite the presence of leptin and its receptor during hibernation, we observed significantly lower expression of the phosphorylated form of the transcription factor signal transducer and activator of transcription 3 (pSTAT3) – a marker for leptin signaling in neurons[23] – in the ARC of IBA compared to active animals (Fig. 3d-e). These data imply that leptin signaling in ARC neurons is reduced in hibernating squirrels.

Hibernating animals demonstrate central hypothyroidism

Our findings that ARC neurons resist the orexigenic effects of ghrelin and exhibit reduced leptin signaling during IBAs suggested that the hypothalamic feeding center temporarily shuts down during hibernation. However, the mechanism responsible for this effect remained unclear. The action of the thyroid hormone triiodothyronine (T3) in the central nervous system (CNS) has been implicated in the control of feeding[24–28]. It has further been shown that the excitability of ARC AgRP/NPY neurons during fasting or ghrelin administration is increased via uncoupling protein 2 (UCP2)-dependent mitochondrial proliferation[29,30]. We therefore hypothesized that anorexia during IBA may be due to central T3 deficiency. In support of this, we found more than two-fold lower levels of hypothalamic T3 during IBA compared to active animals (active: 0.77 ± 0.1 pg/mg tissue; IBA: 0.32 ± 0.05 pg/mg tissue). Interestingly, we also detected a steady decline in hypothalamic T3 during the active season, reaching levels comparable to those observed during IBA by the end of the season (Fig. 4a). However, plasma levels of T3 were equivalent in both groups of animals and remained constant (Fig. 4b), indicating that thyroid hormone deficiency during hibernation was restricted to the CNS. We also found significantly higher blood serum levels of thyroxine (T4) – the precursor to T3 – during IBA (Fig. 4c), further supporting the

idea that hibernating squirrels exhibit central, but not peripheral, hypothyroidism. Thyroid hormones canonically exert their action by binding to nuclear thyroid hormone receptors alpha (*THRA*) and beta (*THRB*)[31]. Single-cell sequencing of ARC neurons revealed that THRA and THRB are expressed in AgRP- and pro-opiomelanocortin (POMC)-containing neurons (Fig. 4d), and that their expression levels are similar in both physiological states (Fig. 4e), suggesting that hypothyroidism in IBA animals is not caused by receptor downregulation. Together, these data indicate that central T3 deficiency may underlie the temporary anorexia associated with hibernation.

Central T3 infusion rescues hibernation anorexia

Given the difference between peripheral and central levels of T3, we wondered whether blood brain barrier (BBB) function might be impaired during hibernation. However, 3 kDa dextran and biocytin injections confirmed that ARC BBB function is equivalent in active and IBA animals (Fig. 5a, b and Extended Figure 1). Differential transcriptomics in the ARC subsequently revealed a significant downregulation of monocarboxylate transporter 8 (MCT8) – the thyroid hormone transporter – in IBA animals (Fig. 5c). Because MCT8 participates in the translocation of both T4 and T3 from the circulation to brain tissue, and then into glial cells and neurons[32–34], we hypothesized that hypothalamic T3 deficiency may be due to diminished transport of T3 and T4 into the ARC. To test this theory, we bypassed this transport step by infusing T3 directly into the mediobasal hypothalamus during IBA and measured its effect on feeding (Fig. 5a). Remarkably, even though T3 injection failed to induce feeding up to two hours post-injection (Fig. 5e), it resulted in robust potentiation of feeding over a 24-hour period (Fig. 5f), consistent with its role as a transcriptional regulator. Thus, the specific deficiency of T3 in the CNS appears to be the cause of hibernation anorexia.

Discussion

Anorexia is a severe disorder that impacts quality of human life and can cause death if symptoms are severe. In humans, it can be triggered by lack of hunger, for example during cancer cachexia, or in the psychiatric condition anorexia nervosa, in which subjects self-limit eating

despite of hunger. However, anorexia is also part of a normal physiological cycle for ground squirrels, who do not rely on stored food during hibernation. Because premature emergence from the safety of the underground burrow to search for food would pose a risk of predation, anorexia constitutes an important safety mechanism that increases survival. In this study, we found that squirrels exhibit T3 deficiency in the hypothalamus during IBA, and that restoration of T3 reverses anorexia, demonstrating that long-term suppression of hunger during hibernation is, at least partially, due to central hypothyroidism. Thyroid hormone deficiency is specific to the CNS and does not affect peripheral levels of T3 and T4, obviating the adverse effects of hypothyroidism on internal organs[35,36] and preserving the involvement of thyroid hormones in increasing thermogenic capacity prior to squirrels entering hibernation.

Hibernating squirrels present a naturalistic model in which central thyroid hormone function is depressed while essential peripheral thyroid function is preserved. Central thyroid hormone has been implicated in the seasonal shifts in reproduction and food intake in non-hibernating animals, including Siberian hamsters, sheep, photoperiodic F344 rats[37–40] and hibernating arctic ground squirrels[41]. Although the precise mechanism of central hypothyroidism remains to be determined in our model, the data presented here suggest that reduced levels of MCT8 may underlie hypothalamic thyroid hormone deficiency during hibernation. Future studies will provide clues to understand the contribution of neurophysiological pathways, including hormone transport, to human anorexia.

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Author contributions. Conceptualization: SMM, EOG, SNB. Data collection: SMM, RDP, MPP, VF, MS, LV, AK, HC, TLH. DKM supplied squirrels and provided advice on animal husbandry. Funding acquisition, project administration and supervision: EOG and SNB. Writing: SMM, RDP, EOG, SNB, TLH with contribution from DKM, MPP, HC.

Competing Interests. Authors declare that they have no competing interests.

Data and materials availability. All data are available in the main text or the supplementary materials. RNA sequencing data are deposited to the Gene Expression Omnibus, accession number XXXX. Single cell RNA sequencing data are deposited to the to the Gene Expression Omnibus, accession number XXXX.

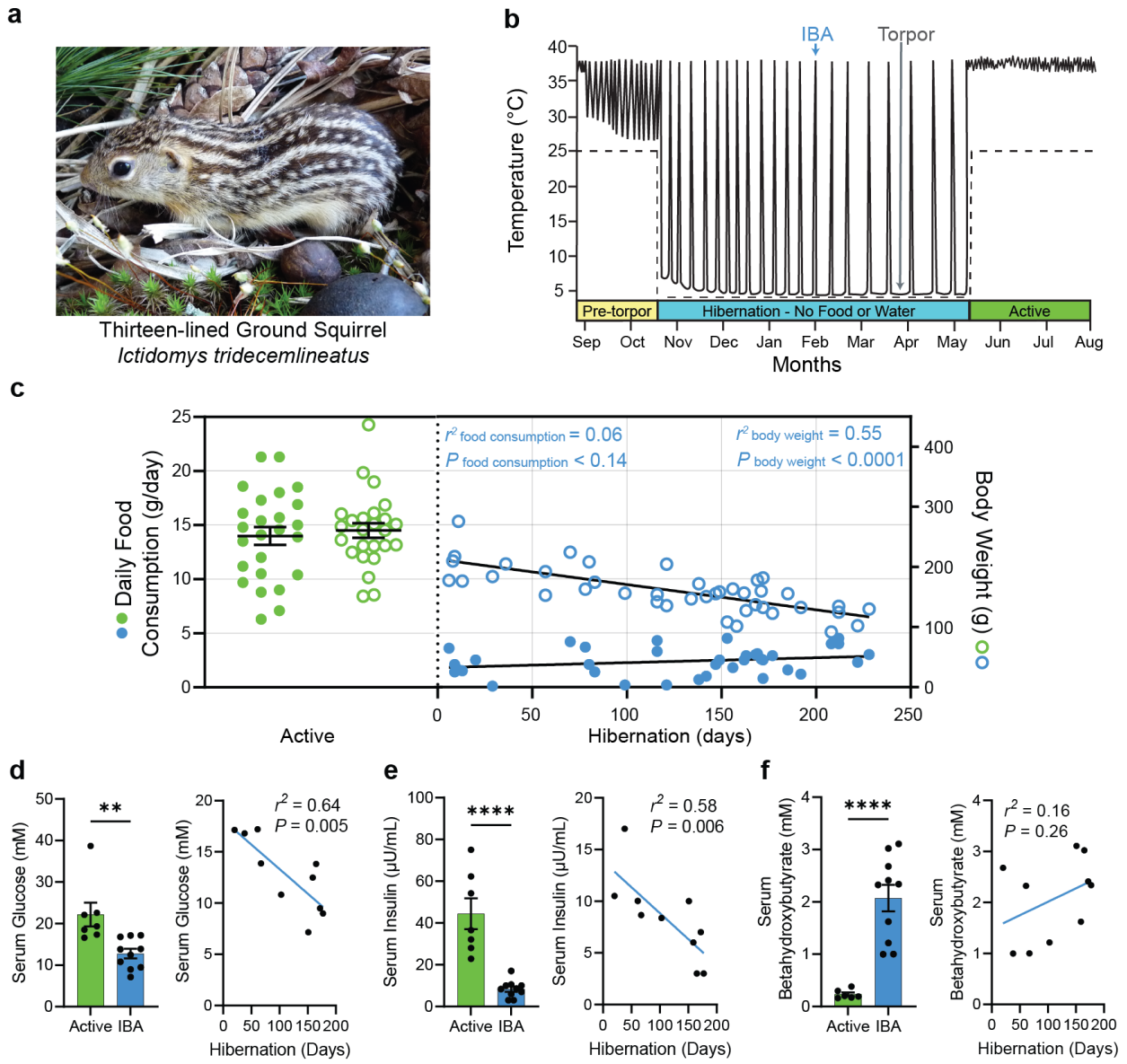


Figure 1: Hibernating ground squirrels have negligible hunger drive.

a, Image of a thirteen-lined ground squirrel, *Ictidomys tridecemlineatus.*

b, Schematic of ground squirrel core body temperature before, during and after hibernation. Dotted line, ambient temperature. Every temperature peak during hibernation represents an IBA.

c, Daily food consumption (left axis, solid points) and body weight (right axis, open circles) during the active (summer) season (green) and hibernation (winter) season (blue). Active feeding data is peak summer food consumption. N = 25 animals. Hibernation feeding and body weight is plotted by days in hibernation. $N_{IBA(Food)}$ = 36, $N_{IBA(Weight)}$ = 42 animals.

d *to* **f**, Blood metabolic indicators across active and hibernation states.

d, Serum glucose, N_{Active} = 7, N_{IBA} = 10 animals. **e**, Serum insulin, N_{Active} = 7, N_{IBA} = 10 animals. **f**, Serum beta-hydroxybutyrate, N_{Active} = 6, N_{IBA} = 10 animals.

Data represent mean ± SEM in histograms. Each point represents one animal. Student's t-test. Scatter plots are the same data plotted against days in hibernation and fitted with simple linear regressions. **P < 0.01, ****P < 0.0001.

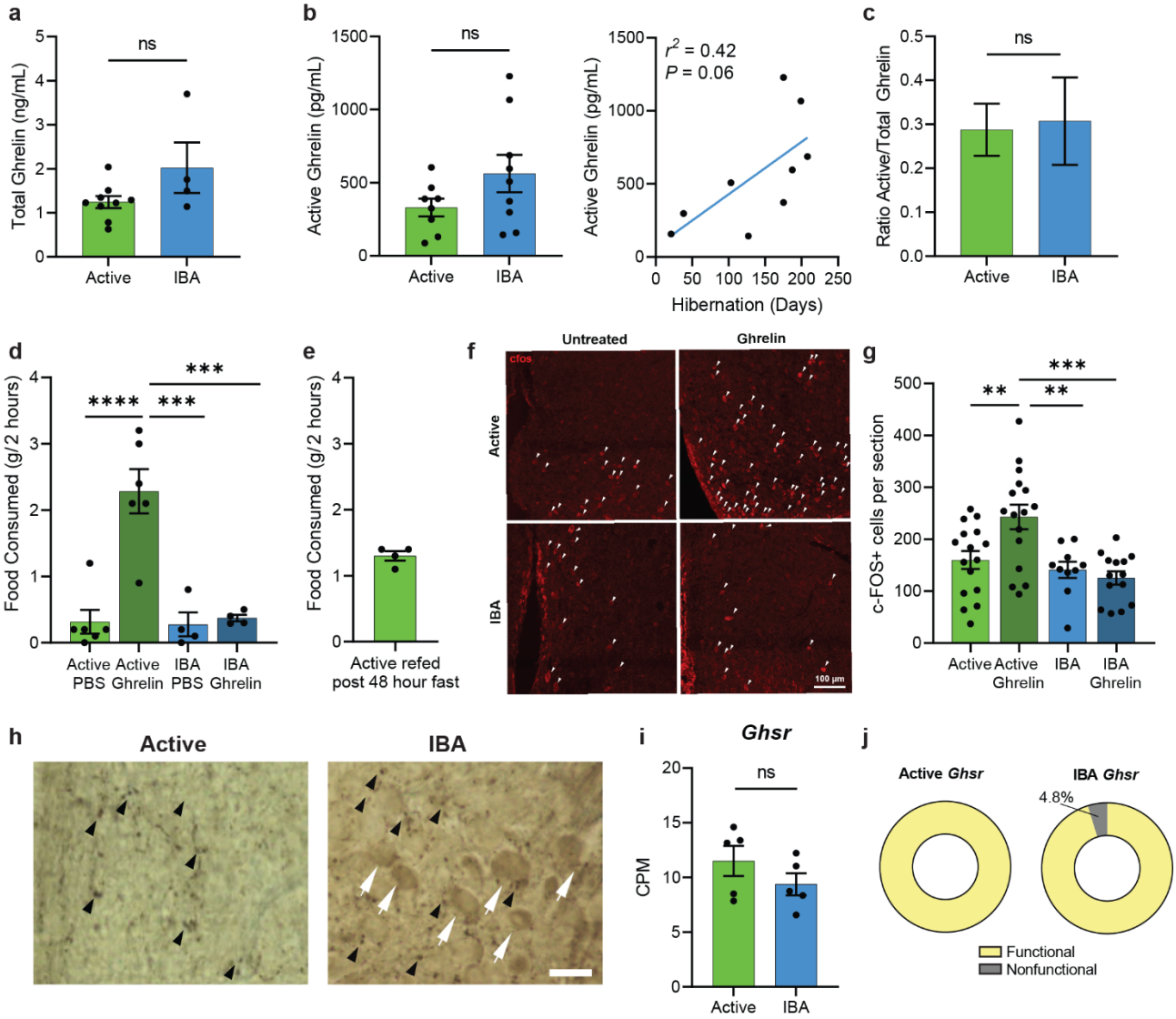


Figure 2: Hibernating squirrels demonstrate reversible resistance to ghrelin.

a, Plasma level of total and **b**, active (acylated) ghrelin across states *(left)* and throughout hibernation *(right)*. Total Ghrelin: $N \geq 4$ animals. Active Ghrelin: $N \geq 8$ animals. **c**, Ratio of active:total ghrelin. **d**, Two-hour food consumption after ghrelin or PBS injection across states. $N \geq 4$ animals.

e, Two-hour food consumption in active animals following 48-hour fast. $N = 4$ animals.

f, Representative images of ARC c-FOS staining of active and IBA squirrels injected with ghrelin or control. Arrowheads, c-FOS+ cells. Scale bar = 100 µm. **g**, Quantification of c-FOS+ cells per section across ARC volume. $N \geq 10$ sections, ≥ 2 squirrels.

h, Representative immuno-EM images of ARC from active and IBA animals. White arrows, neuronal soma; black arrowheads, fibers stained for AgRP. Scale bar = 20 µm.

i, Expression of *Ghsr* in the ARC. $N = 5$ animals.

j, Quantification of *Ghsr* isoforms across states. $N \geq 38$ clones, 2 squirrels.

(**a** – **i**) Data represented as mean ± SEM (**a** – **c**) Student's t-test. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{***}P < 0.0001$. (**d**, **g**) Two-Way ANOVA followed by Tukey's multiple comparisons. $^{**}P < 0.01$, $^{***}P < 0.001$. (**a** – **e**, **i**) Each point represents one animal. (**g**) Each point represents one section. (**i**) $FDR > 0.05$. n.s. = not significant.

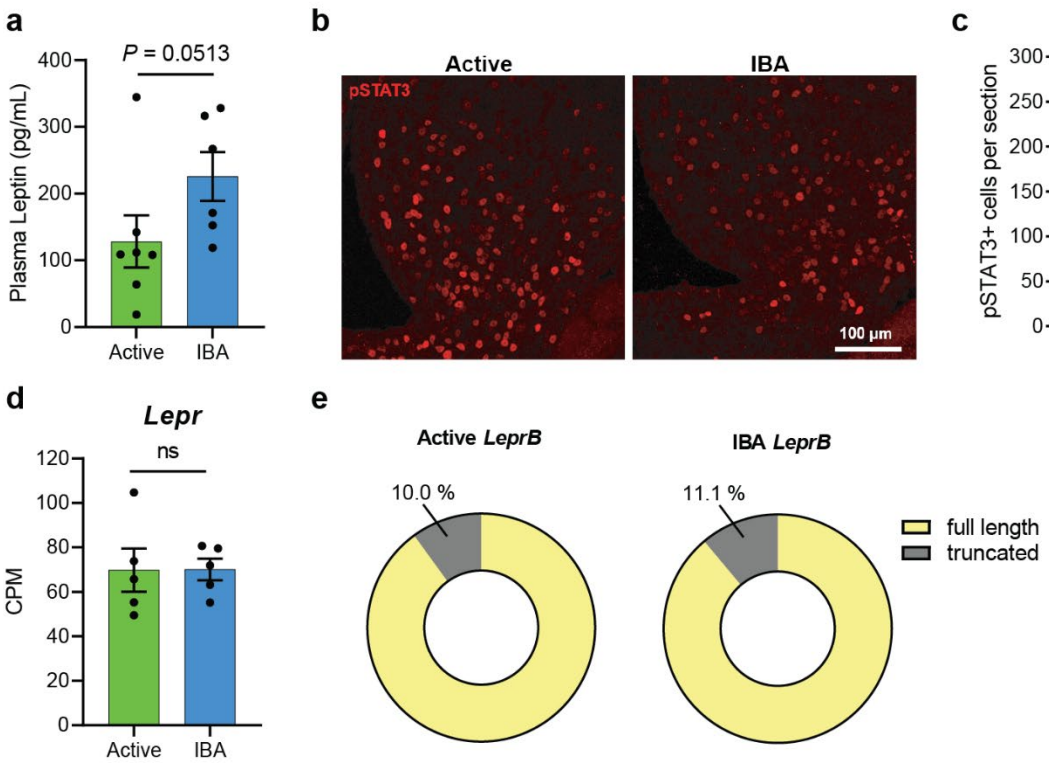


Figure 3: Hibernating squirrels have reduced leptin signaling.

a, Plasma leptin levels across states. Data are mean ± SEM. Mann-Whitney test, N_{Active} = 7 squirrels, N_{IBA} = 6 squirrels.

b, Expression level of *Lepr* in the ARC. Each point represents one squirrel. Data are mean ± SEM, *FDR* > 0.05, ns = not significant. N_{Active} = 5, N_{IBA} = 5.

c, Quantification of truncated versus full length long-form leptin receptor from full-length de novo cloning in active and IBA squirrels. N_{Active} = 10 clones from 2 squirrels, N_{IBA} = 9 clones from 2 squirrels.

d, Representative immunohistochemistry images of ARC in active and IBA squirrels using anti-pSTAT3 antibody. Scale bar, 100 µm.

e, Quantification of pSTAT3+ cells per section by state across ARC volume. Each point represents one section. Data are mean ± SEM. Student's t-test, $P < 0.01$, N_{Active} = 27 sections from 3 squirrels; N_{IBA} = 27 sections from 3 squirrels.

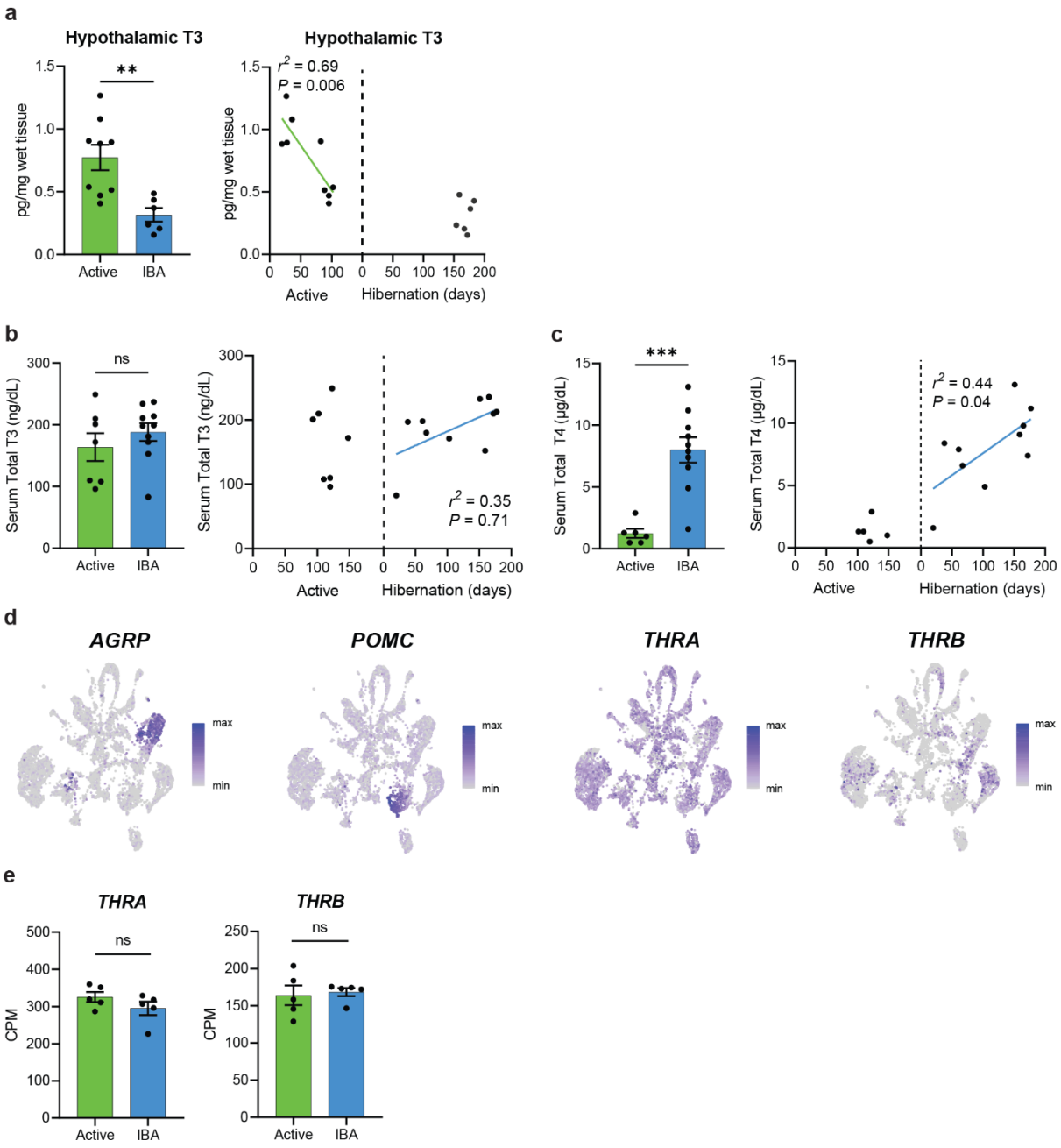


Figure 4: Hibernating animals demonstrate central hypothyroidism.

a, T3 content measured from homogenized hypothalamus from active and IBA squirrels. N_{Active} = 9, N_{IBA} = 6 animals.

b *to* **c**, Serum total thyroid hormone concentration across active and IBA seasons. **b**, Total T3. N_{Active} = 7, N_{IBA} = 10 animals. **C** Total T4 . N_{Active} = 6, N_{IBA} = 10 animals.

d, Single cell sequencing shows clusters expressing *Agrp, Pomc, Thra, Thrb. N* = 3 animals .

e, Transcript expression of *Thra* and *Thrb* assessed by RNA sequencing of ARC. N_{Active} = 5, N_{IBA} = 5 animals. (**a** – **c**) Data represent mean ± SEM in histograms. Each point represents one animal. Student's t-test. ***P* < 0.01, ****P* < 0.001, n.s. = not significant. The same data are plotted against days active and days in hibernation and fitted with simple linear regression. (**e**) *FDR* > 0.05, n.s. = not significant. Data represent mean ± SEM. Each point represents one animal.

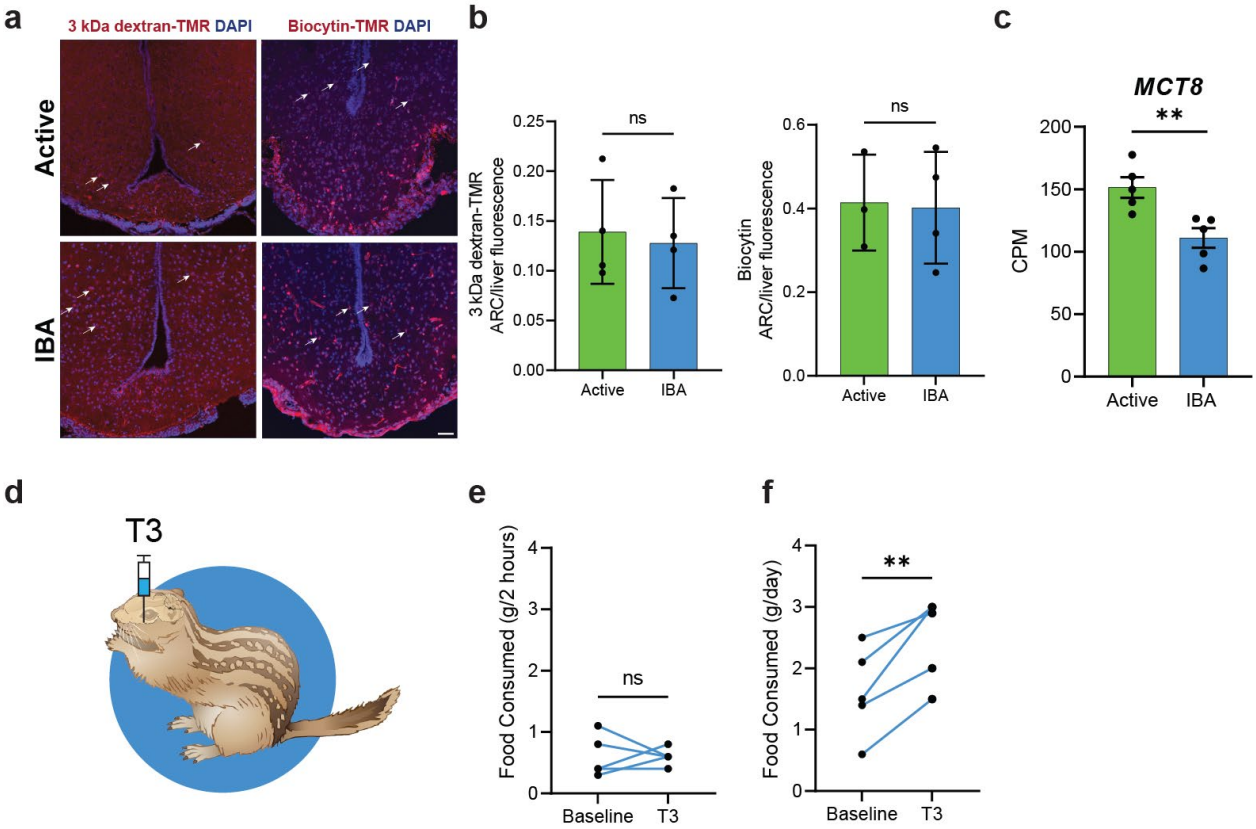
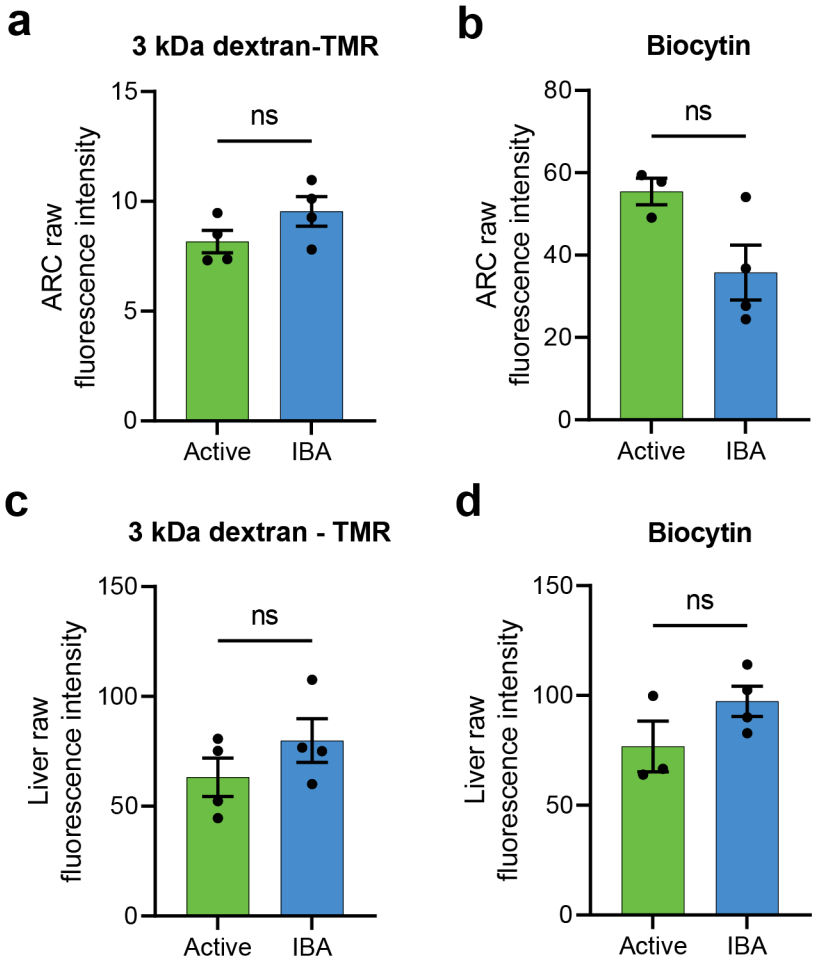


Figure 5: Central T3 infusion rescues hibernation anorexia.

a *to* **b**, Blood brain permeability assays by tail artery injection in active and IBA squirrels of 3kDa dextran-tetramethylrhodamine (TMR) and 860 Da biocytin-TMR results in **a**, deposition of dye (white arrows) within the median eminence and ARC. Scale bar = 50 µm. **b**, Quantification of normalized fluorescence of 3kDa TMR (left) and 860 Da biocytin-TMR (right). Dextran: N_{Active} = 4, N_{IBA} = 4 animals. Biocytin: N_{Active} = 3, N_{IBA} = 4 animals. **c**, Level of expression of *MCT8* in the ARC. N_{Active} = 5, N_{IBA} = 5 animals. **d** *to* **f**, Hypothalamic infusion of T3 during IBA and resulting food consumption. **d**, Schematic of hypothalamic T3 infusion during IBA after arousal from torpor. **e** *to* **f**, Paired food consumption at baseline and after T3 infusion at **e**, two-hours and **F** 24-hours. *N* = 5 animals.

(**b** – **c**) Data represent mean ± SEM. Each point represents one animal. (**b**) Student's t-test. n.s. = not significant. (**c**) ***FDR* < 0.01. (**e** – **f**) Data are paired with each point representing one animal. Student's Paired t-test. ***P* < 0.01; n.s. = not significant. *P* > 0.05.



Extended Figure 1: Raw fluorescence intensity of dyes.
Quantification of raw fluorescence intensity for 3kDa dextran-TMR and biocytin-TMR in **a** *to* **b**, ARC and **c** *to* **d,** liver. Data represent mean ± SEM with each point representing one animal. $N \geq 3$ per group. Student's t-test. $P > 0.05$. n.s. = not significant.

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Methods:

Animals

All experimental procedures were performed in compliance with the Institutional Animal Care and Use Committee of Yale University (protocol 2021-11497). Thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*; age 0.5 – 3 years) of both sexes were single housed in temperature- and humidity-controlled facilities at Yale University. All squirrels were implanted with an interscapular temperature transponder (IPTT-300, BMDS).

During the active season (May – August), squirrels were kept at 20 °C under a 12 hour:12 hour light:dark cycle at 40 – 60% humidity and maintained on a diet of dog food (IAMS) supplemented with sunflower seeds, superworms, and fresh vegetables (celery and carrots) with *ad libitum* access to water.

During hibernation (September – April), hypothermic squirrels (body temperature ~20 °C) were moved to 4 °C at 40 – 60% humidity under constant darkness, without access to food or water. In this study, "active" squirrels were those with a constant core body temperature (CBT) of 37 °C during the active season. "IBA" squirrels were those who had undergone at least one bout of hypothermic torpor during the hibernation season, but had achieved a CBT of > 32 °C for ≥ 60 minutes, or ≥ 20 minutes for the central thyroid hormone experiments.

Food Consumption and Body Mass Measurement

Food consumption and body weight of active adult animals (n = 25) were measured every 2 weeks during the active period (May-August). Active animals were moved to the behavioral room kept at 20 °C under a 12 hour :12 hour light:dark cycle and acclimated overnight. In the morning (9 – 11 AM), each was weighed, transferred to a clean cage, and allowed to habituate for 30 minutes. Food consumption measurements were performed with dog food only. Pre-weighed food was added to each cage, and animals were allowed to feed undisturbed. Food remaining 24 hours later was weighed and used to calculate daily food consumption. A separate bowl of dog food was kept in the behavior room to control for weight changes due to ambient humidity, but no difference was found so no correction was needed. The maximum food consumption per animal for the active season was reported.

Hibernating animals entered IBA spontaneously, so their food consumption and body weight measurements occurred between 11 AM – 8 PM. IBA animals were weighed, transferred to a clean cage in the hibernaculum kept at 4 °C under constant darkness and allowed to habituate for 30 minutes. Food consumption measurements were performed with dog food only. Pre-weighed food was added to each cage, and animals were allowed to feed undisturbed. Food remaining 24 hours later was weighed and used to calculate daily food consumption. IBA measurements occurred just once during the hibernation season, to ensure that animals remained naïve to food availability during the winter. A separate bowl of dog food was kept in the behavior room, kept at 4 °C in constant darkness, to control for weight changes due to ambient humidity, but no difference was found so no correction was needed.

Blood collection

Animals were euthanized by isoflurane overdose. The chest cavity was opened, the right atrium of the heart pierced, and trunk blood was collected with a 18G needle and syringe.

Serum hormone and metabolite measurements

Whole blood was allowed to coagulate at room temperature for 30 minutes, then centrifuged at 4 °C at 2000 rcf for 15 minutes. Serum was aliquoted and stored at -80 °C for later use. Serum glucose (Active: n = 7; IBA: n = 10), insulin (Active: n = 7; IBA: n = 10), beta-hydroxybutyrate (Active: n = 6; IBA: n = 10), total T3 (Active: n = 7; IBA: n = 10), and total T4 measurement (Active: n = 6; IBA: n = 10) were performed by Antech Diagnostics (Fountain Valley, CA).

Plasma ghrelin measurements

Whole blood was collected into chilled, pre-coated K_3EDTA tubes (MiniCollect, Grenier Bio-One) and immediately treated with Pefabloc (Sigma) to a final concentration of 1 mM. Blood was centrifuged at 1600 rcf for 15 minutes. Plasma was aliquoted and stored at -80 °C for later use. Plasma active (acylated) ghrelin (Active: n = 8; IBA: n = 9) was measured by mouse/rat ELISA (EZRGRA-90K, Millipore). Plasma total ghrelin (Active: n = 9; IBA: n = 4) was measured by mouse/rat ELISA (EZRGRT-91K, Millipore). All samples were run in duplicate. The ratio of active (acylated)/ total ghrelin was calculated by dividing the mean of the active (acylated) ghrelin concentration by the mean of the total ghrelin concentration per state. The SEM of the ratio was calculated by simple error propagation given by the formula:

$$\sigma_{ratio} = sqrt\ ((\sigma A/A)2\ +\ (\sigma B/B)2\ ))\ *\ A/B$$

where A and B are mean values of active (acylated) and total ghrelin, respectively.

Plasma leptin measurements

Whole blood was collected into chilled, pre-coated K_2EDTA tubes (BD Vacutainer, Lavender/H) and immediately treated with aprotinin (Millipore Sigma, 9087-70-1) to a final concentration of 0.02 mM. Blood was centrifuged at 1600 rcf for 15 minutes. Plasma was aliquoted and stored at -80 °C for later use. Plasma leptin (Active: n = 7, IBA: n = 6) was measured by mouse/rat ELISA (R&D Systems, MOB00). All samples were run in duplicate. A ROUT outlier test (Q = 1 %) was run to identify one outlier in the Active state and two outliers in the IBA state.

Intraperitoneal Ghrelin injections

Ground squirrels were acclimated in the behavioral room overnight. Animals were weighed, transferred to clean cages, and allowed to habituate for 30 minutes. Squirrels were immobilized with decapicones, injected with 2 mg/kg active (acylated) rat ghrelin (1465, Tocris) solubilized in PBS using an injection volume of 2 mL/kg body weight, and returned to their cages. Control injections were PBS injected at a volume of 2 mL/kg body weight. Pre-weighed food was added to the cage and animals allowed to feed for two hours. The food remaining after the feeding period was weighed and used to calculate food consumption.

Immunohistochemistry

Ground squirrels were deeply anesthetized by isoflurane inhalation and then subjected to intracardiac perfusion with PBS followed by fixative [4 % paraformaldehyde in PBS]. Brains were post-fixed overnight, and transferred to serial 10 %, 20 %, 30 % sucrose solutions after sinking. Brains were embedded in OCT, frozen on dry ice, and stored at -80 °C until use. Coronal brain sections of the arcuate nucleus were cut at a thickness of 40 µm on a cryostat (Leica, CM3050S). Sections were mounted onto SuperFrost Plus slides and stored at -80 °C with desiccant until the day of the immunohistochemistry procedure. Sections were dried in an incubator at 37 °C for 30 minutes. Slides were washed three times

with PBS for 10 minutes, and then washed with 1 % H2O2 and 1 % NaOH in PBS for 10 minutes. Slides were moved to 0.3 % glycine in PBS 1x for 10 minutes and washed with 0.03 % SDS in PBS. Sections were blocked for two hours at room temperature with 5 % normal goat serum in 0.5 % PBST.

For c-FOS immunohistochemistry after ghrelin injection (Active Untreated: n = 2 animals; Active ghrelin: n = 2 animals, 16 sections; IBA Untreated: n = 3 animals, 10 sections; IBA Ghrelin: n = 2 animals, 14 sections), sections were incubated with primary antibody (1:500, mouse monoclonal c-FOS C-10, Santa Cruz, sc-271243) at 4 °C for 48 hours. After incubation with the primary antibody, sections were washed four times with 0.1 % PBST for 15 minutes. Sections were incubated with secondary antibody (1:500, Alexa Fluor 647 goat anti-mouse, preadsorbed, Abcam, ab150119) for two hours at room temperature. Sections were washed four times with 0.1 % PBST for 15 minutes, followed by a wash with PBS.

For pSTAT3 immunohistochemistry (Active: n = 3, 27 sections; IBA: n = 3, 27 sections), sections were incubated with primary antibody (1:200, rabbit polyclonal Phospho-Stat3 (Tyr705), Cell Signaling Technology, 9131) at 4 °C for 24 hours. After incubation with the primary antibody, sections were washed five times with 0.1 % PBST for 10 minutes. Sections were incubated with secondary antibody (1:1000, Alexa Fluor 555 goat anti-rabbit, Abcam, ab150086) for 2 hours at room temperature. Sections were washed five times with 0.1 % PBST for 15 minutes, followed by a wash with PBS.

Slides were mounted using Vectashield with DAPI. Sections were imaged on a Leica SP8 Confocal Microscope at 20X using the LAS X software. Negative controls (primary antibody omitted) were performed for c-FOS and pSTAT3 immunohistochemistry and showed no non-specific fluorescent binding.

Immuno-electron microscopy

Ground squirrels were deeply anesthetized by isoflurane inhalation and were subjected to intracardiac perfusion. Free-floating sections (50 µm thick) were incubated with rabbit anti-AgRP antibody (Phoenix Pharmaceuticals) diluted 1:2000 in 0.1 M PB after 1 hour blocking in 0.1 M PB with 5% normal goat serum. After several washes with PB, sections were incubated in the secondary antibody (biotinylated goat anti-rabbit IgG; 1:250 in PB; Vector Laboratories Inc.) for 2 hours at room temperature, then rinsed several times in PB followed by incubation for 2 hours at room temperature with avidin–biotin–peroxidase (ABC; 1:250 in PB; VECTASTAIN Elite ABC kit PK6100, Vector Laboratories). The immunoreaction was visualized with 3,3-diaminobenzidine (DAB). Sections were then osmicated (1% osmium tetroxide) for 30 minutes, dehydrated through increasing ethanol concentrations (using 1% uranyl acetate in 70% ethanol for 30 min), and flat-embedded in Durcupan between liquid release-coated slides (product no. 70880, Electron Microscopy Sciences). After embedding in Durcupan (14040, Electron Microscopy Sciences), ultrathin sections were cut on a Leica Ultra-Microtome, collected on Formvar-coated single-slot grids, and analyzed with a Tecnai 12 Biotwin electron microscope (FEI) with an AMT XR-16 camera.

Bulk RNA Isolation and Sequencing

Total RNA was isolated from the arcuate nuclei of active (n = 5) and IBA (n = 5) animals that had been deeply anesthetized by isoflurane inhalation and subjected to intracardiac perfusion with ice cold PBS. The brain was rapidly dissected and a vibratome (Leica VT1200) was used to cut 300-600 µm coronal slices posterior to the separation of the optic chiasm. The area surrounding the third ventricle, including the arcuate nucleus and median eminence, were manually dissected out from the slices using 27G needles

and placed immediately into RNA lysis buffer from the Quick-RNA Microprep Kit (Zymo, R1050). Total RNA was isolated from tissue using the Quick-RNA Microprep Kit (Zymo, R1050). RNA concentration and integrity number (RIN) were assessed by an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). RNA concentrations were in the range of ~20 – 400 ng/µL and RIN values were in the range of 7.4 – 9.5.

Library preparation and sequencing were carried out at the Yale Center for Genome Analysis. Sequencing libraries were prepared using the Kapa mRNA Hyper Prep Kit (Cat# KR1352, Roche) including rRNA depletion. Libraries were sequenced on the Illumina NovaSeq6000 in the 100 bp paired-end mode according to manufacturer's protocols. A total of ~ 22 – 34 million sequencing read pairs per sample were obtained.

The sequencing data were processed on the Yale Center for Research Computing cluster. Raw sequencing reads were filtered and trimmed to retain high-quality reads using Trimmomatic v0.39 (*1*) with default parameters. Filtered high-quality reads from all samples were aligned to the *Ictidomys tridecemlineatus* reference genome using the STAR aligner v2.7.1a with default parameters (*2*). The SpeTri2.0 [GCA_000236235.1] reference genome and the gene annotation were obtained from the Broad Institute.

The gene annotation was filtered to include only protein-coding genes. Aligned reads were counted by the featureCounts programs from the Subread package v2.2.0 with default parameters (*3*). Read counting was performed at the gene level, i.e. the final read count for each gene included all reads mapped to all exons of this gene. Differential expression of genes was determined by EdgeR v 3.32.1 (*4*). Normalized read counts were obtained by normalizing raw read counts to effective library sizes of each sample and expressed as reads/million of total reads in a library (CPM). Statistical analysis was performed using the GLM approach and quasi-likelihood F-test, using a significance value of $FDR < 0.05$.

Primary cell dissociation and single-cell RNA Sequencing.

Primary neurons were isolated from the arcuate nucleus of hypothalamus and median eminence following a published protocol (*5*) with modification (n = 3). Animals were euthanized by isoflurane inhalation overdose followed by cardiac perfusion with brain perfusion solution (containing in mM: 196 sucrose, 2.5 KCl, 28 NaHCO3, 1.25 NaH2PO4, 7 Glucose, 1 Sodium Ascorbate, 0.5 CaCl2, 7 MgCl2, 3 Sodium Pyruvate, oxygenated with 95% O2/5% CO2, osmolarity adjusted to 300 mOsm with sucrose, pH adjusted to 7.4). The brain was dissected and slices were cut on a vibratome (Leica, VT1200). A brain slice containing the ARC and ME were identified by the presence of the third ventricle and separation of the optic chiasm. Three successive 600-µm slices containing the ARC were collected. The area around the third ventricle was microdissected from the brain slices using a micro-scalpel (Fine Science Tools, 10055-12).

Tissue was digested in Hibernate A medium (custom formulation with 2.5 mM glucose and osmolarity adjusted to 280 mOsm, BrainBits) supplemented with 1 mM lactic acid (Sigma, L1750), 0.5 mM GlutaMAX (ThermoFisher, 35050061) and 2% B27 minus insulin (ThermoFisher, A1895601) containing 20 U/ml papain (Worthington Biochemical Corporation, LS003124) in a shaking water bath at 34 °C for 30 min and dissociated by mechanical trituration through the tips of glass Pasteur pipettes with decreasing diameter (0.9 mm, 0.7 mm, 0.5 mm, 0.3 mm). Cell suspension was centrifuged over 8% bovine serum albumin (Sigma, A9418-5G) layer. Supernatant was removed leaving ~50 µl of suspension. Cell suspension was resuspended in 950 µl of Hibernate A medium (same formulation as above) and centrifuged at 300 rcf for 5 min. Supernatant was removed, leaving ~50 µl of cell suspension, which was gently mixed with a

glass pipette and stored on ice. A 10 µl aliquot of cell suspension was mixed with 10 µl of Trypan Blue stain, loaded into a hemocytometer, and used to assess cell concentration and viability.

Cell suspension was processed according to the 10X Genomics library preparation protocol at the Center for Genome Analysis/Keck Biotechnology Resource Laboratory at Yale University. Single cell suspension in RT Master Mix was loaded on the Single Cell G Chip and partitioned with a pool of about 750,000 barcoded gel beads to form nanoliter-scale Gel Beads-In-Emulsions (GEMs). The volume of cell suspension for loading was calculated based on cell concentration to capture 10,000 cells. Upon dissolution of the Gel Beads in a GEM, the primers were released and mixed with cell lysate and Master Mix. Incubation of the GEMs produced barcoded, full-length cDNA from poly-adenylated mRNA. Silane magnetic beads were used to remove leftover biochemical reagents and primers from the post GEM reaction mixture. Full-length, barcoded cDNA was amplified by PCR to generate sufficient mass for library construction. Enzymatic Fragmentation and Size Selection were used to optimize the cDNA amplicon size prior to library construction. R1 (read 1 primer sequence) was added to the molecules during GEM incubation. P5, P7, a sample index, and R2 (read 2 primer sequence) were added during library construction via End Repair, A-tailing, Adaptor Ligation, and PCR. The final libraries contained the P5 and P7 primers used in Illumina bridge amplification. Sequencing libraries were sequenced on an Illumina NovaSeq instrument with 150 bp reads according to the manufacturer's instructions at the depth of ~1.1-1.4 billion reads/sample.

Raw sequencing reads were processed using 10X CellRanger v.6.1.2 (10X Genomics, Pleasanton, CA). Custom genome reference for thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*) was built based on the reference genome sequence and annotation obtained from the Ensembl project (www.ensembl.org (*6*) Release 101; all files accessed on 11/20/2020):

Genome:
ftp://ftp.ensembl.org/pub/release-101/fasta/ictidomys_tridecemlineatus/dna/Ictidomys_tridecemlineatus.SpeTri2.0.dna.toplevel.fa.gz
Annotation:
ftp://ftp.ensembl.org/pub/release-101/gtf/ictidomys_tridecemlineatus/Ictidomys_tridecemlineatus.SpeTri2.0.101.gtf.gz

The gene annotation was filtered to include only protein-coding genes using cellranger mkgtf. 10X CellRanger was used to obtain transcript read counts for each cell barcode, filtered for cell barcodes called as cells based on the default parameters. Read count matrix was further processed using R 4.2.1, RStudio 2022.02.3, and Seurat 4.1.1 (*7*). Non-descriptive ground squirrel gene symbols (i.e. those starting with "ENSSTOG…") were replaced with gene symbols of mouse homolog genes, using the homolog conversion table from Ensembl. Initial set of cells/barcodes was further filtered to include only those with >= 500 features/cells, >= UMIs/cells, and <= 10% of mitochondrial genes (defined as those with gene symbol starting with "MT-"). This resulted in ~11,000-20,000 cells/sample included in the dataset for further analysis, with the sequencing depth of ~70-100k reads/cell. Read counts were processed according to the standard Seurat analysis workflow, including normalization, identification of variable features, scaling, PCA, clustering and visualization using tSNE plots. Graphs report normalized gene expression values.

***De novo* Receptor Cloning**

Total RNA from the arcuate nucleus of animals was collected as described above. The remaining RNA after sequencing was used for *de novo* cloning of *Ghsr* (Active: n = 3; IBA: n = 3) and long-form *Lepr* (Active: n = 2, 1 female; IBA: n = 2). cDNA was prepared (Invitrogen SuperScript III First-Strand Synthesus for RT-PCR, 18080-051) and the gene of interest amplified (Phusion High-Fidelity PCR Kit, E0553S) using the following primers for *Ghsr:* forward 5'- CCAACTTGATCCAGGCTCC -3', reverse 5'- CAAGTTCCGCTGTGCGATGG -3'; and *Lepr:* forward 5'- CAGGTACATGTCTCTGAAGTAAG -3', reverse 5'- GCCACGTGATCCACTATAATAC -3'. Gel electrophoresis was used to isolate the band of interest and DNA extracted using the Qiagen Gel Extraction Kit (28704). ORFs were then ligated to topo vector (StrataClone Blunt PCR Cloning Kit, 240207). cDNA was sent for Sanger Sequencing (Genewiz), and reference sequences compared the NCBI database.

Hypothalamic Infusions

Animals were implanted with abdominal transponders that measured core body temperature, calibrated between 4 – 40 °C (EMKA Technologies, M1-TA) during the active season, while animals were euthermic. Animals were allowed to recover for at least 2 weeks before implanted with hypothalamic infusion cannulas as described below.

In a subsequent surgery, infusion cannulas (10 mm 26 G guide, 11 mm 33 G internal, PlasticsOne) were implanted into the mediobasal hypothalamus during the active season while animals were euthermic. Briefly, animals were induced into, and maintained at, a stable anesthesia plane using isoflurane. Animals were administered 0.03 mg/kg preoperative buprenorphine subcutaneously. The scalp was shaved and the animal transferred to a stereotax, where the skin was sterilized by repeated applications of betadine and 70% ethanol. Sterile technique was used to expose the skull and drill a hole to allow for cannula implantation. The following stereotaxic coordinates were utilized for cannula implantation: 0.5 mm posterior bregma, 0.8 mm lateral midline, 8 mm ventral (guide cannula)/9 mm ventral (infusion cannula). Two bone screws (2mm long, 1.2 x 0.25 mm thread, McMaster-Carr) and dental cement (RelyX Unicem Resin, 3M, 56830) were used to anchor the guide cannula to the skull. A dummy cannula was placed in the guide cannula until experiments were performed. Animals received a dose of 0.4mg/kg meloxicam in 1.5 mL saline subcutaneously immediately after surgery. Animals received post-operative buprenorphine 0.01 mg/kg and meloxicam 0.2 mg/kg intraperitoneally every 12 hours for 48 hours. Animals were allowed to recover for at least 2 weeks before they were hibernated.

Two-hour and daily food consumption were measured in paired IBA animals in two separate experiments. In the first experiment, feeding was assessed after baseline (no injection) feeding (n = 5) and triiodothyronine (T3; Sigma, T2877) in the hibernaculum at 4 °C under constant darkness. Control experiments were performed once IBA patterns appeared stabilized and regular (corresponding to IBA 3 – 4). Animals were allowed to return to torpor after control experiments. Hypothalamic T3 infusion was performed in the same animals after at least 1 subsequent IBAs had elapsed (corresponding to IBA 5 – 7).

In the second experiment, performed in the subsequent year with a new cohort of animals, hypothalamic infusion of DMSO vehicle (control) (n = 6) (Sigma, D2650) and triiodothyronine (T3; Sigma, T2877) in the hibernaculum at 4 °C under constant darkness. Control experiments were performed once IBA patterns appeared stabilized and regular (corresponding to IBA 4 – 12). Animals were allowed to return to torpor after control experiments. Hypothalamic T3 infusion was performed in the same animals after at least 2 subsequent IBAs had elapsed (corresponding to IBA 9 – 15).

For both experiments, to reduce stress to the animals, infusions were performed while animals were in the process of arousing from torpor. Animals were identified as IBA candidates when abdominal temperature exceeded 8 °C. Squirrels were weighed and transferred to a clean cage in the hibernaculum, kept at 4 °C in constant darkness. When abdominal temperature exceeded 16 °C and interscapular temperature exceeded 26 °C, a connector assembly consisting of PE50 tubing attached to an infusion cannula was loaded with control DMSO vehicle or 15.3 mM T3 solubilized in DMSO. At this point, animals were responsive to touch, but remained curled in the stereotypical torpor position and were unable to move. An intrahypothalamic dose of either control DMSO or 15.3 nmol T3 was infused in a volume of 1µL over a time span of three minutes. The infusion cannula was allowed to remain in the guide cannula for two minutes to allow for the complete diffusion of the infusion solution The infusion cannula was removed and replaced with a dummy cannula.

During this time, animals continued to warm up. Once the abdominal temperature surpassed 28 °C and the interscapular temperature surpassed 35 °C, animals became mobile and explored their cages. Animals were allowed to habituate for 20 minutes. The timing of the rewarming process was variable and took on average around 45 minutes. Dog food was exclusively used for feeding consumption measurements. After habituation was complete, a pre-weighed amount of food was placed in the cage. Animals were allowed to feed for 2 hours, at which point the remaining food was removed, weighed, and returned to the cage. Retrieving and weighing the food took less than 10 minutes per animal. The remaining food was returned to the cage and the animal allowed to feed for a further 22 hours, to achieve a 24-hour food consumption measurement.

Hypothalamic Tissue Collection

Naïve animals (Active: n = 9; IBA: n = 6) that had not undergone any experiment were euthanized by isoflurane overdose and perfused with ice-cold PBS. The brain was removed from the skull and a ~6 mm thick section collected from the optic chiasm to the mamillary bodies using a rat coronal brain matrix (Electron Microscopy Sciences, 69083-C). The hypothalamus was isolated by removing brain matter above the top of the third ventricle and lateral to the optic tract. Tissue was flash-frozen in liquid nitrogen and stored at -80 °C until processing.

Measurement of Hypothalamic T3

Total triiodothyronine (T3) was extracted from frozen hypothalamus tissue (Active: n = 9; IBA: n = 6) and purified as reported previously (*8*). Briefly, hypothalamic tissue was homogenized in 100% methanol containing 1 mM 6-propyl-2-thiouracil (PTU) (Sigma, H34203) in a glass-glass tissue grind pestle (60mm, Kontes, KT885300-0002). Homogenized tissue was centrifuged at 3000 rpm and supernatant removed. The pellet was resuspended and washed twice more in 100% methanol containing 1 mM PTU. T3 was extracted from supernatants and purified through solid-phase chromatography using 200 – 400 anion exchange chloride resin (Bio-Rad, 140-1251) in Poly-Prep chromatography columns (Bio-Rad, 731-1550). Columns were developed with 70% acetic acid (Spectrum, AC110) and washed twice with water. Supernatants were passed through the column without vacuum. T3 bound to columns was purified through a series of washes with acetate buffer pH 7.0 and 100% ethanol. T3 was eluted with 2.5 mL 70% acetic acid. Extracts were evaporated to dryness under nitrogen. T3 concentration was measured by ELISA (Leinco Technologies, T181). Dried product was resolubilized in the zero-standard and the kit run according to the manufacturer's instructions.

Blood Brain Barrier Tracer Injections and Analysis

Naïve animals (n = 15) that had not undergone any experiment were anesthetized with isoflurane (4%) in medical air and injected in the tail artery with either biocytin-TMR (ThermoFisher, T12921) or 3kDa dextran-TMR (ThermoFisher, D3307) at 10 mg/kg. Animals were allowed to recover in their home cage for 30 minutes until perfusion fixation with 4% paraformaldehyde as described for Immunohistochemistry.

Brains were sectioned on a Leica cryostat at 40 μm and every tenth section was imaged for blood brain barrier permeability analysis. Sections were rinsed with PBS and coverslipped with Vectashield containing DAPI (Vector Labs, H-1200). Z-stack images of liver and arcuate nucleus were acquired on a confocal microscope (Zeiss, LSM-780) using ZEN Software. Maximum intensity projection images were used for quantification in FIJI.

Statistics, analysis, and data collection

Statistical analyses were performed in GraphPad Prism v9.0 or higher (GraphPad Software, San Diego, CA). Final figures were assembled in Adobe Illustrator. Data were tested for normality using the Shapiro-Wilk normality test. When normality was assumed, the Student's t-test was used to compare two groups and Two-Way ANOVA was used to compare multiple groups. Paired data were analyzed with a paired Student's t-test. Tukey's multiple comparisons test was used to find *post hoc* differences among groups. When data were not normal, the Mann-Whitney test was used. Sample sizes and statistical data are reported in the text and figure legends. In the text, values are provided as mean ± SEM, and $P < 0.05$ was considered statistically significant. No blinding was used for data collection. Individuals in experimental groups were chosen to best match body weight and to represent both sexes across groups.

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