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Hypothalamic gene expression underlying pre-hibernation satiety

C. Schwartz[†], M. Hampton[‡] and M. T. Andrews[†,*]

[†]Department of Biology, and [‡]Department of Mathematics and Statistics, University of Minnesota Duluth, Duluth, MN, USA

**Corresponding author: M. T. Andrews, Department of Biology, University of Minnesota Duluth, 1035 Kirby Drive, Duluth, MN 55812-3004, USA. E-mail: mandrews@d.umn.edu*

Prior to hibernation, 13-lined ground squirrels (*Ictidomys tridecemlineatus*) enter a hypophagic period where food consumption drops by an average of 55% in 3 weeks. This occurs naturally, while the ground squirrels are in constant environmental conditions and have free access to food. Importantly, this transition occurs before exposure to hibernation conditions (5°C and constant darkness), so the ground squirrels are still maintaining a moderate level of activity. In this study, we used the Illumina HiSeq 2000 system to sequence the hypothalamic transcriptomes of ground squirrels before and after the autumn feeding transition to examine the genes underlying this extreme change in feeding behavior. The hypothalamus was chosen because it is known to play a role in the control and regulation of food intake and satiety. Overall, our analysis identified 143 genes that are significantly differentially expressed between the two groups. Specifically, we found five genes associated with feeding behavior and obesity (*VGF, TRH, LEPR, ADIPOR2, IRS2*) that are all upregulated during the hypophagic period, after the feeding transition has occurred. We also found that serum leptin significantly increases in the hypophagic group. Several of the genes associated with the natural autumnal feeding decline in 13-lined ground squirrels show parallels to signaling pathways known to be disrupted in human metabolic diseases, like obesity and diabetes. In addition, many other genes were identified that could be important for the control of food consumption in other animals, including humans.

Keywords: Adiponectin, food intake, hibernation, hypophagia, hypothalamus, Illumina HiSeq, leptin, obesity, satiety, seasonal behavior

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Obesity is a serious epidemic worldwide, with over 10% of adults characterized as obese (WHO 2014). Obesity stems from an imbalance between energy intake and expenditure, which is regulated by the hypothalamus. The hypothalamus receives information about the body's energy status from peripheral systems, including, most notably, leptin from white adipose tissue (WAT) and insulin from the pancreas. Neurons within the arcuate nucleus receive and integrate these signals to affect feeding behavior and body weight by producing either agouti-related protein (AGRP) or neuropeptide Y (NPY), which promote food intake, or cocaine- and amphetamine-regulated transcript (CART) and pro-opiomelanocortin (POMC), which decrease food intake (Bell *et al.* 2005). The arcuate nucleus also interacts with other hypothalamic areas, including orexin (HCRT)-producing neurons of the lateral hypothalamus, which promote food intake, and thyrotrophin-releasing hormone (TRH)-producing neurons of the paraventricular nucleus, which promote satiety. Single gene mutations in *POMC*, leptin and the leptin receptor (*LEPR*) have all been associated with human obesity (Choquet & Meyre 2011). In mice, mutations or transgene expression of leptin, *LEPR*, insulin receptor substrate 2 (*IRS2*), *AGRP, NPY, CART, POMC* and *HCRT*, among many others, are all associated with body weight phenotypes (Rankinen *et al.* 2006), indicating that the molecular basis of obesity is very complex.

The 13-lined ground squirrel (*Ictidomys tridecemlineatus*) is a valuable animal for examining signaling in the brain underlying obesity because of its natural and extreme changes in feeding behavior and body weight. Thirteen-lined ground squirrels are obligate hibernators, seasonally entering a period of depressed metabolism to survive periods of low food availability (Carey *et al.* 2003). Amazingly, ground squirrels eat little to no food during hibernation, despite periodic arousals with increased metabolism (Torke & Twente 1977). Instead of eating, they survive on their massive WAT stores, which are accumulated during the hyperphagic summer months (Carey *et al.* 2003). The weight gain before and weight loss after hibernation is almost exclusively due to changes in adiposity (Dark *et al.* 1989). Previous work investigating satiety-related gene expression during hibernation in the hypothalamus showed low expression of *AGRP*, *NPY* and *HCRT*, and high expression of CART prepropeptide (*CARTPT*) when compared with active, non-hibernating ground squirrels (Schwartz *et al.* 2013), indicating that satiety signals are in place to facilitate this lengthy fasting.

Even more remarkable than the extensive hibernation fast is that during early autumn, while animals are still active, food consumption shows a dramatic decrease, despite constant housing conditions and free access to food in the laboratory (Fig. 1a). This phenotype is particularly relevant to human obesity because it occurs in active, obese ground squirrels with unrestricted food. One issue in addressing obesity in human patients is controlling food intake (Blundell & Gillett 2001),

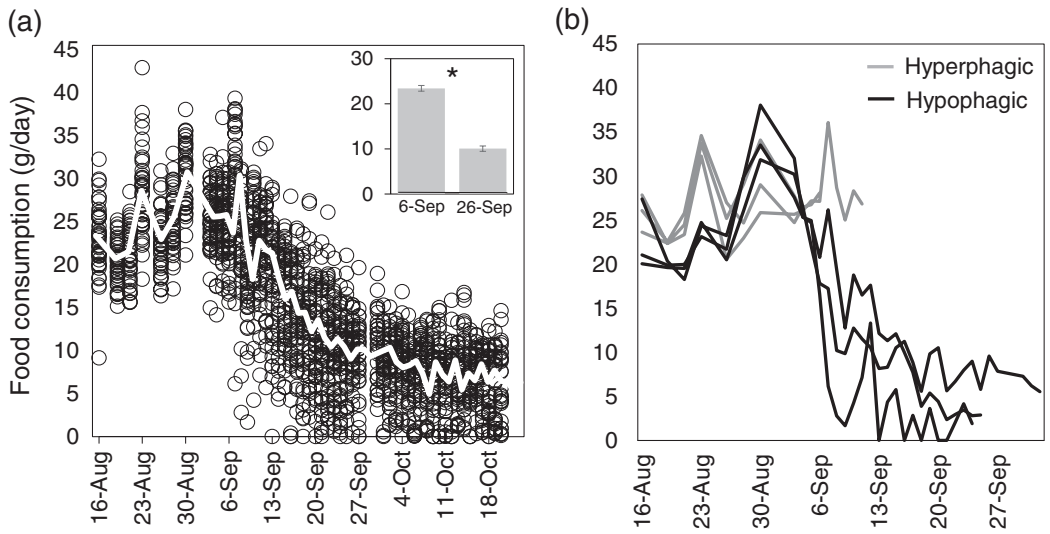

Figure 1: Autumnal feeding transition in seasonally hibernating 13-lined ground squirrels. (a) Ground squirrel food consumption (g/day) from their August arrival to the onset of hibernation in the laboratory ($n = 27–43$). Each open circle represents the daily consumption of one individual. The white line represents the colony average. The inset illustrates that the colony food consumption is statistically different between the hyperphagic and the hypophagic phases ($P < 0.0001$*). Error bars represent standard error of the mean. (b) Food consumption of the six animals used for transcriptome analysis. The hyperphagic animals are represented by gray and the hypophagic animals are represented by black.

Table 1: Ground squirrel food consumption and body weights of animals used for Illumina sequencing

	Arrival body weight (g)	Final body weight (g)	Body temperature (°C)	Final day food consumption (g/day)	Final week food consumption (g/day)	Maximum consumption (g/day)
Hyperphagic ($n = 3$)	123.13 ± 9.62	241.45 ± 14.60	36.8 ± 0.15	26.92 ± 0.74	28.24 ± 0.65	34.93 ± 0.59
Hypophagic ($n = 3$)	121.27 ± 3.34	188.15 ± 13.67	36.4 ± 0.33	3.41 ± 1.06	4.15 ± 1.66	34.49 ± 1.85
Statistical analysis	$t(4) = 0.18$, $P = 0.86$	$t(4) = 2.66$, $P = 0.06$	$t(4) = 1.18$, $P = 0.30$	$t(4) = 18.19$, $P = 5.37E{-}05$*	$t(4) = 13.47$, $P = 0.0002$*	$t(4) = 0.23$, $P = 0.83$

Data are represented as means ± standard error. For the statistical analysis, *P*-values were obtained with a Student's *t*-test between the two groups. The statistical results are presented as 'statistical test (df) = *t*-statistic, *P*-value'. *$P < 0.05$ was considered significant.

particularly in environments with abundant food (Swinburn *et al.* 2011). Here, we examine hypothalamic gene expression in naturally hyperphagic and hypophagic ground squirrels using Illumina HiSeq 2000 sequencing. The genes underlying this feeding transition in ground squirrels support previous findings related to feeding and body weight in humans and other mammals, and also contribute new ideas.

Materials and methods

Animals

Wild-caught 13-lined ground squirrels (*I. tridecemlineatus*) were used in these experiments. Because they are wild-caught, their ages are not known. All animals were captured in early August in central Minnesota. All ground squirrels were housed upon arrival in the Animal Care Facility located in the University of Minnesota Duluth Medical School. Animals were housed individually in standard plastic rat cages (7.5 × 16 × 8 in.) with wire tops containing aspen bedding. The ground squirrels were kept at room temperature (set to 21°C) with a 12:12 light/dark cycle and provided Purina Laboratory Rodent Diet 5001 (pellet form) and water *ad libitum* from their arrival in August through October. Experimental animals were weighed upon arrival, at 2 weeks post-arrival, and at tissue collection (Fig. S1, Table 1). The remaining colony animals were weighed in mid-October, prior to hibernation in an environmental chamber. All experimental procedures reported here were approved by the University of Minnesota Institutional Animal Care and Use Committee (protocol #1103A97712).

Experimental collection points

Early work with ground squirrels showed seasonal changes in food consumption prior to hibernation (Pengelley & Fisher 1963). Similarly, we previously showed a rapid decrease in ground squirrel food consumption over the course of approximately 1 month in early autumn, preceding the onset of the hibernation season (Schwartz & Andrews 2013). This small pilot study used animals that had been captive for over a year, indicating that this feeding behavior transition would still occur reliably in the lab without external environmental

Table 2: Differentially expressed genes associated with feeding and obesity

Gene	Expression profile	Specific references	Associated gene ontology (GO) terms
VGF	↑ Hypophagic	Hahm *et al.* (2002), Watson *et al.* (2009) and Jethwa *et al.* (2007)	GO:0002021~response to dietary excess
TRH	↑ Hypophagic	Suzuki *et al.* (1982) and Steward *et al.* (2003)	GO:0007631~feeding behavior
LEPR	↑ Hypophagic	Furusawa *et al.* (2010) and Lautier *et al.* (2003)	GO:0007631~feeding behavior; GO:0031667~response to nutrient levels
IRS2	↑ Hypophagic	Lin *et al.* (2004) and Lautier *et al.* (2003)	GO:0043467~regulation of generation of precursor metabolites and energy
ADIPOR2	↑ Hypophagic	Bjursell *et al.* (2007) and Diez and Iglesias (2003)	GO:0031667~response to nutrient levels

cues. However, wild-caught animals were chosen for this current experiment in an attempt to minimize any influence of long-term captivity on hypothalamic gene expression.

Ground squirrel food consumption for the entire captive colony ($n = 43$) was monitored by weighing each individual animal's food at least three times weekly starting at their arrival in the lab (Fig. 1a). Weighing food to determine consumption is used commonly in other rodents, including rats (Hsieh *et al.* 2014) and mice (Parks *et al.* 2013). The food weight in grams was divided by the number of days since the last weighing to calculate grams per day. Food was weighed every day during the autumn transition period for maximal data coverage. This food weighing method does introduce some variability, with a small amount of food likely lost during eating or weighing, but weighing was performed this way every time to minimize and control the issue. Any animal that pulled pellets down into the cage or ground food into powder without eating it was not included. Any measureable food spillage was collected and factored into the consumption.

Two time points were used for this experiment: hyperphagic and hypophagic. These time points were based on food consumption. Hyperphagic animals, defined as ground squirrels eating at least 20 g of food per day, were euthanized in the beginning of September. All animals were monitored closely during this time, and the ground squirrels ultimately chosen for this group were specifically selected because they were very close to the beginning of the feeding transition. Importantly, if these animals had not been euthanized, they would have transitioned into hypophagic animals within a few weeks like the rest of the colony (Fig. 1a). Hypophagic animals, defined as former hyperphagic animals that were currently eating less than 10 g of food per day, were euthanized at the end of September. These animals were also closely monitored to ensure that their food consumption had leveled off for at least 1 week. Final food consumption (day) was the amount of food consumed on the day before tissue collection. Final food consumption (week) was the average food consumption over the final week before tissue collection. Max consumption was the highest recorded food consumption. Statistical differences in food consumption and body weight were determined using a Student's *t*-test. *P*-values less than 0.05 were considered statistically significant.

Tissue preparation and Illumina HiSeq procedure

All animals were fully anesthetized with Isoflurane and then euthanized by decapitation 1–3 h after the 12 h light period began. The hypothalami were removed and RNA was prepared as described previously (Schwartz *et al.* 2013). RNA from three individuals (two females, one male) was prepared for each of the two experimental groups for Illumina sequencing, making a total of six individual samples. Individual samples were sent to the University of Minnesota Genomics Center (St. Paul, MN, USA) for sequencing on the Illumina HiSeq 2000 (Illumina, Inc., San Diego, CA, USA). Transcriptome sequencing was performed as described previously (Schwartz *et al.* 2013) on each of the six individual samples. Retroperitoneal WAT pads were also extracted and immediately frozen in liquid nitrogen. Blood was also collected at tissue collection via heart puncture. Samples clotted at room temperature (21°C) for 20 min and were centrifuged to collect serum. Serum and WAT were stored at −80°C until use.

Bioinformatics and data analysis

Raw reads generated by Illumina sequencing were identified using a set of *I. tridecemlineatus* contigs that were assembled in Trinity (Grabherr *et al.* 2011; Haas *et al.* 2013) from previous hypothalamic transcriptome data (Schwartz *et al.* 2013). Briefly, Trinity predicted coding domains within the contigs to specifically select for protein-coding transcripts. These contigs were trimmed to include the coding domain plus up to 100 bases on both ends. Due to differences in transcription and polyadenylation in the mitochondrial vs. nuclear genome, the 13 protein-coding mitochondrially encoded genes were screened out. The contigs were identified by comparison to the human RefSeq nucleotide database (National Center for Biotechnology Information, Bethesda, MD, USA) using Blastn (*E*-value = 0.00001). The *I. tridecemlineatus* genome was also used to supplement this identification for any contig that needed species-specific sequence verification. Raw reads from each experimental sample were identified using these custom contigs, and then quantified into counts for each gene. Resulting counts were upper quartile normalized and then fitted to a negative binomial distribution using DESeq v1.6.1 (Anders & Huber 2010).

The animals in the hypophagic group showed behavioral evidence of shallow torpor in addition to reduced food consumption, resulting in a more complicated phenotype transition than a simple and discrete change in feeding behavior. Behavioral evidence of torpor included exhibiting the curled torpor body posture and building a deep, dome-like nest (Merriman *et al.* 2012). Short, shallow torpor bouts prior to cold exposure have been described in 13-lined ground squirrels previously (Russell *et al.* 2010). During the autumn transition, the ground squirrels in this study were kept at room temperature (21°C), and because body temperature does fall below the ambient temperature, they were only capable of shallow torpor during this time. In order to address this complication and focus directly on gene expression likely associated with the change in feeding behavior, the resulting 143 differentially expressed genes were entered into the Database for Annotation, Visualization and Integrated Discovery (Huang *et al.* 2008), the Online Mendelian Inheritance in Man database (Hamosh *et al.* 2005), and literature searches to specifically highlight the genes associated with feeding behavior and obesity (Table 2).

Western blot analysis

For western blot analysis, we only used animals that had both a serum sample and a WAT sample to assay. White adipose tissue (WAT) and serum from four animals were run for each experimental group, for a total of eight animals. This included five of the animals used for Illumina sequencing and three additional animals with similar feeding phenotypes. One hypophagic animal used for Illumina sequencing did not have a remaining WAT sample, so this animal was not used in this analysis. The average final day food consumption for the animals used in the western blot analysis was 27.85 ± 1.59 g/day for the hyperphagic animals and 2.1 ± 0.73 g/day for the hypophagic animals. In addition,

for comparison in the adiponectin western blots, we included four post-hibernation April animals with an average food consumption of 22.16 ± 1.21 g/day.

WAT samples were homogenized in radioimmunoprecipitation assay (RIPA) buffer with protease inhibitors (1:1000, P8340; Sigma-Aldrich Corp., St. Louis, MO, USA) and phosphatase inhibitors (1:1000, P5726, P0044; Sigma-Aldrich Corp., St. Louis, MO, USA). The homogenized samples were kept for 30 min on ice with periodic vortexing. They were centrifuged at 10 000 × g for 20 min at 4°C. The liquid protein layer was then extracted, avoiding the upper fat cake and the lower pellet. Serum was diluted 1:1 in RIPA buffer with inhibitors. Serum and WAT protein concentrations were determined using a bicinchoninic acid assay (Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer's instructions. 50 μg of serum protein and 20 μg of WAT protein were loaded onto 15% gels for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Electrophoresis was run at 180 V until the tracking dye front ran off the gel. Proteins were then transferred onto a nitrocellulose membrane at 75 V for 3 h. The membrane was blocked in 3% bovine serum albumin for 1 h and then incubated with primary antibody (anti-leptin: 1:4000, ab16227; Abcam, Cambridge, MA, USA; anti-adiponectin: 1:2000, ab59687; Abcam) overnight. The membrane was incubated in secondary antibody (Leptin: anti-rabbit, 1:20 000, Immunopure; Thermo Fisher Scientific, Rockford, IL, USA; Adiponectin: anti-chicken, 1:5000; Life Technologies, Grand Island, NY, USA) for 1 h. White adipose tissue (WAT) was also used as the positive control that verified the specific band at ~16 kDa (leptin) and ~26 kDa (adiponectin). The resulting western blots were quantified using the 'Analyze gels' function in ImageJ to obtain relative densities (Rasband 1997). Total protein stain with amido black was used as a loading control (Aldridge *et al.* 2008).

Statistical analysis

For Illumina transcriptome sequencing, differential expression between experimental groups was determined using DESeq (command: nbinomGLMTest), which generated a test statistic (*P*-value). Importantly, DESeq was designed to accommodate datasets with small numbers of biological replicates and provide good statistical power (Anders & Huber 2010). The computed *P*-values were independently filtered (Bourgon *et al.* 2010), by restricting to those with at least a 25% change and at least 100 or more average counts. The Benjamini–Hochberg method was then used to correct for multiple comparisons, which provided false discovery rate (FDR) control. The FDR sets a *P*-value cutoff for significance. Differentially expressed genes had to have an FDR of <0.05, which was a *P*-value <0.0135 in this experiment. For statistical differences in body weight, food consumption, body temperature and relative density in leptin western blots, Student's *t*-test was used. $P < 0.05$ was considered significant. For statistical differences in the adiponectin western blots, an analysis of variance (ANOVA) was used with a *post hoc* Tukey's test.

Results

Food consumption phenotype

This experiment examined hypothalamic gene expression underlying a natural, yet drastic and relatively rapid change in food consumption. Figure 1a illustrates the decline in food consumption in the entire captive ground squirrel colony ($n = 27–43$). The average food consumption of the colony on 6 September was 23.43 ± 0.63 g/day, dropping to 10.22 ± 0.07 g/day on 26 September (Fig. 1, inset). In the span of just 20 days, food consumption significantly dropped by over 55% [$t(71) = 15.21$, $P < 0.0001$]. This decline in food consumption occurred in all animals, with some individual variability in both the timing to hypophagia and the total decline in consumption. Males and females showed similar transition profiles in both timing and overall consumption (Fig. S2). Behavioral observations during this time, specifically noting animal posture and activity level, indicated that the ground squirrels were beginning short, shallow torpor bouts during this transitional period and during hypophagic status. No observations of torpor-like behavior were seen in hyperphagic animals.

Figure 1b displays the food consumption of the six individual ground squirrels used for Illumina sequencing. The hyperphagic ground squirrels had an average final consumption of 26.92 ± 0.74 g/day, while the hypophagic ground squirrels were eating 3.41 ± 1.06 g/day (Table 1). The difference in consumption was statistically significant [$t(4) = 18.19$, $P < 0.0001$]. However, importantly, all animals in the hypophagic group were formerly hyperphagic and exhibited similar peak consumption values [Table 1; $t(4) = 0.23$, $P = 0.83$]. The hyperphagic group weighed more on average at the time of tissue collection compared with the hypophagic group, which was euthanized approximately 3 weeks later, but this difference was not statistically significant (Table 1). Both groups had similar body weights upon arrival and gained weight during the hyperphagic phase, similar to the entire captive colony (Table 1, Fig. S1).

Gene expression overview

Overall, 15 223 unique protein-coding transcripts were identified in the ground squirrel hypothalamus, which is similar to our previous work (Schwartz *et al.* 2013). Of these, 143 were differentially expressed according to the criteria outlined in the Methods and Procedures (Table S1). In the hyperphagic group, 85 genes showed significantly higher expression, while 58 genes had significantly higher expression in the hypophagic group.

Functional analysis

Five of the differentially expressed genes identified in this analysis are specifically associated with feeding behavior or obesity, supporting the underlying phenotypic change in food consumption (Table 2). *VGF* (non-acronymic), *TRH*, *LEPR*, *IRS2* and adiponectin receptor 2 (*ADIPOR2*) were all significantly higher in the hypophagic group (Fig. 2). Interestingly, there is no significant change between groups in anorexigenic *CARTPT* and *POMC*, or orexigenic *AGRP*, *NPY* and *HCRT* (Fig. S3).

One of the differentially expressed genes, *LEPR*, encodes for the leptin receptor, which receives leptin from WAT. Serum analysis of circulating leptin showed that leptin protein levels are significantly higher in the serum of hypophagic animals [Fig. 3a, $t(6) = 2.45$, $P < 0.01$], but there is some individual variability in the hyperphagic group (Fig. 3c). There was not a significant difference in WAT leptin between groups [Fig. 3b,d; $t(6) = 2.45$, $P = 0.22$, ns]. However, WAT leptin is low in the hypophagic group. This suggests that the leptin produced in WAT in the hypophagic group is likely ending up in the serum, where it can serve as a signal to the hypothalamus. These data suggest that leptin signaling is very important during the autumnal transition period and could therefore be a key satiety signal needed for the transition to hypophagia. The variability in serum leptin levels in the hyperphagic

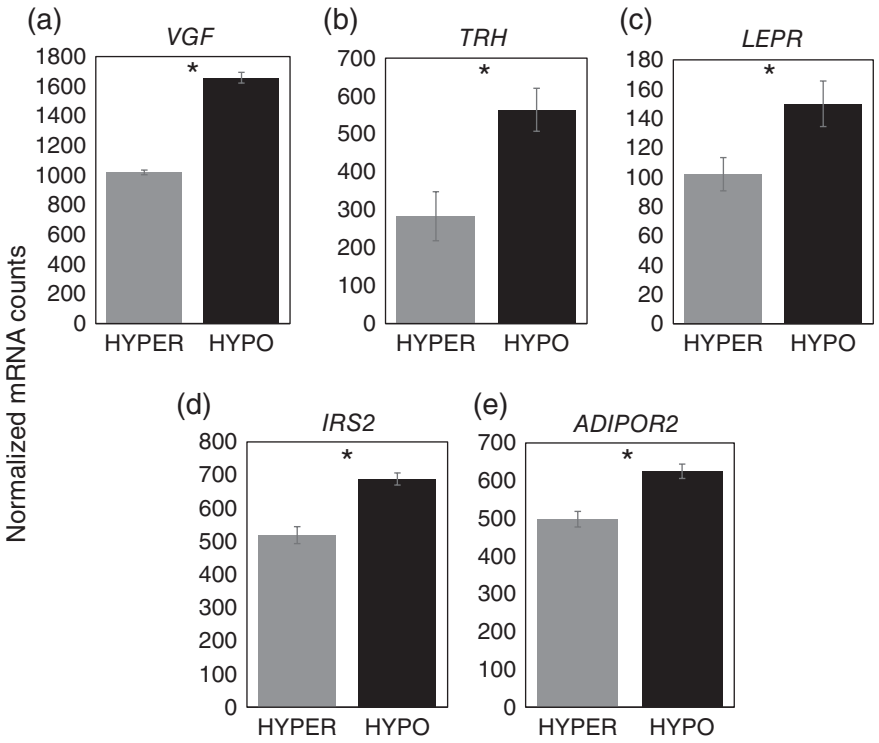

Figure 2: Feeding and obesity-related genes show higher expression in hypophagic group. Hypophagic-associated changes in *VGF* (a), *TRH* (b), *LEPR* (c), *IRS2* (d) and *ADIPOR2* (e) in the hypothalamus of 13-lined ground squirrels as determined by Illumina HiSeq transcriptome sequencing. Error bars represent standard error of the mean. *FDR < 0.05.

group also suggests that upregulation of the LEPR in the hypothalamus could be integral for this satiety to occur.

In addition to leptin, another adipokine receptor gene (*ADIPOR2*) had higher expression in the hypophagic group (Fig. 2e). Interestingly, adiponectin protein expression in WAT is significantly increased in April compared with the hypophagic group [Fig. 4; $F_{2,9} = 4.26$, $P < 0.01$; Tukey's HSD April:Hypophagic, $P < 0.01$; April:Hyperphagic, ns; Hyperphagic:Hypophagic, ns]. However, there was no difference between the hyperphagic and hypophagic animals. We were unable to quantify adiponectin protein in the serum in any of the three points assayed because the levels were too low.

Discussion

Overview

We sequenced the hypothalamic transcriptomes of ground squirrels at the beginning and end of an extreme and rapid feeding behavior transition (Fig. 1) to uncover gene expression potentially underlying this change.

Central integration of peripheral signaling

Leptin, an adipokine produced by WAT in relation to the amount of fat stored in the body, is thought to act as a satiety signal, circulating through the blood to receptors in the hypothalamus to regulate food consumption (Klok *et al.* 2007). We show here that *LEPR* expression in the hypothalamus increases in the hypophagic group (Fig. 2c), and that serum leptin is also elevated during this time (Fig. 3a). There was some variability in serum leptin in the hyperphagic group (Fig. 3c), suggesting that upregulation of the *LEPR* is important and vital for autumn satiety to occur. Previous work showed that leptin administration decreased food intake and body weight in naturally hyperphagic Arctic ground squirrels (Ormseth *et al.* 1996). In Siberian hamsters, serum leptin was low in torpid animals and leptin administration prevented torpor (Freeman *et al.* 2004). This conflicts with our findings because the hypophagic animals in our study showed behavioral signs of using shallow torpor, but exhibited high serum levels of leptin. However, Siberian hamsters only spontaneously enter daily torpor when WAT stores are low and thus leptin could be regulated differently.

In addition to leptin signaling, insulin and adiponectin receptor signaling are likely important for the autumn feeding transition. Insulin is produced by beta cells in the pancreas and travels through the bloodstream to receptors throughout the body, including the hypothalamus. *IRS2* encodes an insulin receptor which is significantly upregulated in the hypophagic group (Fig. 2d). *IRS2* knockout in the hypothalamus results in increased body weight and food intake in mice (Lin *et al.* 2004). Single nucleotide polymorphisms in *IRS2* are associated with severe obesity in humans (Lautier *et al.* 2003), which suggests a role in food intake and body weight regulation. Adiponectin, like leptin, is released into the serum by WAT, and while the role of adiponectin is not well understood, it has been associated with obesity, type 2 diabetes and inflammation (Diez & Iglesias 2003). The gene for an adiponectin receptor, *ADIPOR2*, is upregulated in the hypothalamus in the hypophagic group (Fig. 2e). Adiponectin in WAT was not different between the hyperphagic and

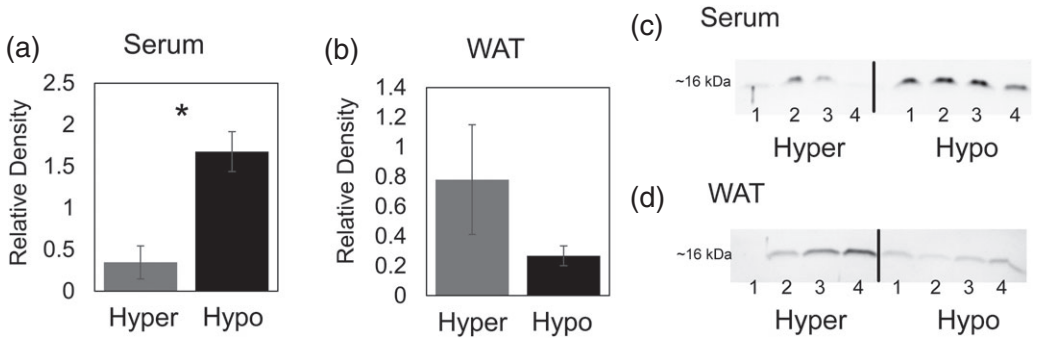

Figure 3: Leptin signaling in seasonally hibernating ground squirrels. (a) Relative leptin protein expression in the serum of hyperphagic and hypophagic ground squirrels ($P < 0.01$*) as quantified from the western blot shown in (c). (b) Relative leptin protein expression in the WAT of hyperphagic and hypophagic ground squirrels as quantified from the western blot in (d). Error bars represent standard error of the mean. (c) Western blot showing leptin expression in serum of hyperphagic (1–4) and hypophagic (1–4) animals. This blot is quantified in (a). (d) Western blot showing leptin expression in WAT of hyperphagic and hypophagic animals. This blot is quantified in (b).

hypophagic groups, but was significantly increased in April (Fig. 4a). It is unclear why this receptor would be important for pre-hibernation satiety, but *ADIPOR2* knockout mice are resistant to high fat diet-induced obesity, lean, have a higher energy expenditure and activity level (Bjursell *et al.* 2007), so it is possible that this receptor plays a role, even without detectable adiponectin levels.

Overall, the hypophagic group exhibited increased hypothalamic expression of three receptors involved in metabolic regulation, specifically regulation through integration of peripheral signals, indicating the importance of these signaling pathways in the control of food intake and satiety during this natural transition to hypophagia. All of these receptors are found in humans and disruption of these signaling pathways results in a variety of metabolic disorders, including obesity (Furusawa *et al.* 2010; Lautier *et al.* 2003) and diabetes (Blüher *et al.* 2006; Civitarese *et al.* 2004). These data support the idea that the pre-hibernation ground squirrel is a useful and valuable experimental animal for investigating natural regulation of these signaling pathways.

Hypothalamic feeding and satiety signals during the autumn transition

In addition to receptors for peripheral signals, there are two additional differentially expressed genes also associated with food intake and satiety, *VGF* and *TRH*. *VGF* expression increases significantly in the hypophagic group (Fig. 2a). *VGF* knockout mice are hypermetabolic and had decreased fat storage, but do not have altered food consumption (Hahm *et al.* 2002; Watson *et al.* 2009). Intracerebroventricular administration of a VGF-derived peptide decreased food intake in Siberian hamsters (Jethwa *et al.* 2007). In addition, *VGF* is associated with seasonality in hamsters (Barrett *et al.* 2005). Our previous work showed that this gene was highly

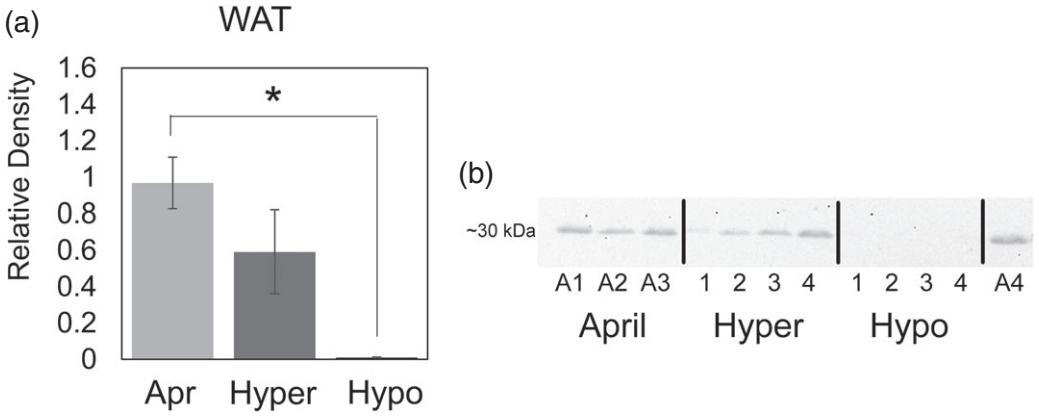

Figure 4: Adiponectin signaling in seasonally hibernating ground squirrels. (a) Relative adiponectin protein expression in the serum of April, hyperphagic, and hypophagic ground squirrels ($P < 0.01$*) as quantified from the western blot shown in (b). (b) Western blot showing adiponectin expression in April (A1–A4), hyperphagic (1–4), and hypophagic (1–4) ground squirrels. This blot is quantified in (a).

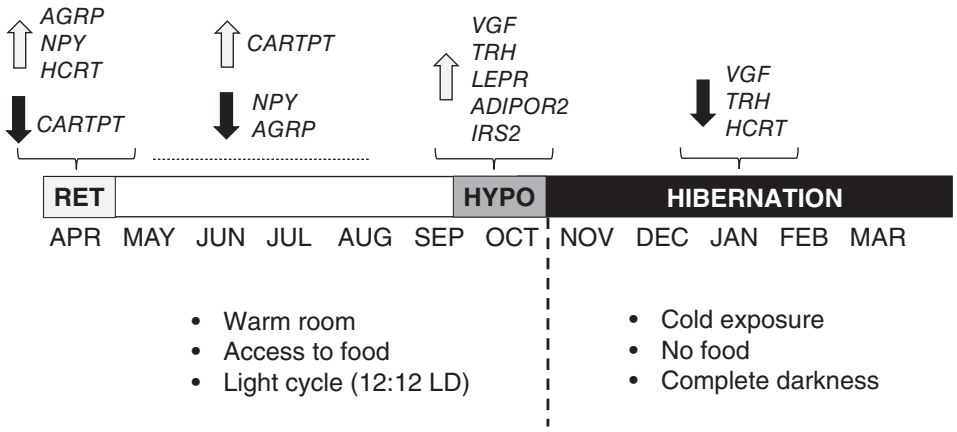

Figure 5: Working model of seasonal changes in hypothalamic feeding-related gene expression. This figure presents a model of the seasonal control of changes in satiety and food-intake-related gene expression in the hypothalamus of 13-lined ground squirrels. This model is based on transcriptomic data from the study presented here and a previous study (Schwartz *et al.* 2013). This model represents only the transcriptional level that was investigated, not the protein level. RET refers to the spring period following the post-hibernation return to a normal lab environment. HYPO represents the hypophagic, pre-hibernation phase of the year when food consumption drops prior to housing in hibernation conditions.

expressed in autumn, but not during hibernation (Schwartz *et al.* 2013), suggesting a seasonal timing and hibernation initiation role.

TRH was the only member of the well-known feeding regulation signals that was differentially expressed between groups. *TRH* expression was increased in the hypophagic group (Fig. 2b), which fits previous findings that TRH is anorexigenic. Microinjection of TRH into the hypothalamus decreased starvation-induced food intake in rats (Suzuki *et al.* 1982). Similarly, intracerebroventricular administration of TRH also decreased food intake in hamsters (Steward *et al.* 2003).

Interestingly, expression of *HCRT*, *CARTPT*, *NPY* and *AGRP* did not change between groups (Fig. S3), despite an extreme change in feeding phenotype and differential expression of these same genes during hibernation (Schwartz *et al.* 2013). However, the expression levels seen here suggest that hibernation-related changes in *AGRP*, *NPY* and *CARTPT* may have occurred prior to the collection of the hyperphagic group. The expression level of anorexigenic *CARTPT* seen here is similar to what was found in hibernation (Fig. S3e). This suggests that *CARTPT* is not playing a role in the autumn transition to hypophagia and that the ground squirrels may actually be resistant to this anorexigenic signaling in autumn, at least initially. Similarly, hypothalamic *NPY* and *AGRP* expression during the autumn transition mirrors the low levels previously seen in hibernation (Fig. S3b,d), even though the hyperphagic animals exhibit high food consumption behavior. This suggests that *NPY* and *AGRP* are not playing a role in hyperphagia during autumn but are important for food intake in the spring. *HCRT* expression does not change in the autumn transition either, and appears to decline during hibernation (Fig. S3a).

To help summarize our findings, we have combined the results of this study and our previous hypothalamic transcriptome study into a working model of the seasonal changes in hypothalamic signaling in the hibernator (Fig. 5). This model suggests that the classic hypothalamic signals (*AGRP*, *CARTPT*, *NPY*, *HCRT*) are important for regulating re-feeding after hibernation, but not for the autumn transition to hypophagia. Our model suggests that *VGF* and *TRH* are very important for initiating the autumnal transition, and that leptin, adiponectin and insulin receptor signaling are critical during this transition period. Importantly, this model is based only on transcriptional data, not protein data. There have been many examples of changes in mRNA that correspond to changes in proteins in hibernating mammals (Buck *et al.* 2002; Epperson *et al.* 2010; Squire *et al.* 2003). However, it can be difficult to investigate proteins in a non-model species because of suitable antibody availability (Epperson & Martin 2002). Therefore, this model should be treated as a preliminary hypothesis.

Importance for hibernation research

It is important to note that behavioral observations indicated that the hypophagic group was periodically entering shallow torpor. Physiological parameters were not measured in this study, but it is well known that heart rate, metabolic rate and body temperature are reduced during torpor, which could contribute to the reduced food consumption. In addition, examination of body temperature and food consumption in Arctic ground squirrels showed that, in some cases, a decline in body temperature preceded a food consumption decline (Olson *et al.* 2013). Similarly, a small decline in body temperature was seen prior to torpor onset in hamsters (Arai *et al.* 2005). We found no significant difference in body temperature at tissue collection between groups (Table 1), and no behavioral evidence of torpor was seen in the hyperphagic group. However, the sample size used here is small and is unlikely to detect a difference if one existed.

However, it is clear that this autumn feeding transition is very important for hibernation preparation. Several transcripts

upregulated in the hypophagic group, including RNA binding motif protein 3 (*RBM3*) and aggrecan (*ACAN*) (Table S1), were previously shown to be highly expressed during hibernation in the hypothalamus (Schwartz *et al*. 2013). A gene potentially involved in seasonal timing is D site of albumin promoter binding protein (*DBP*), which is downregulated in the hypophagic group (Table S1), showing a similar expression pattern in the seasonal hypothalamic transcriptome (Schwartz *et al*. 2013). *DBP* expression follows a robust circadian rhythm, and mice homozygous for a DBP-null allele exhibit less motor activity and have a shorter free-running period than wild-type mice (Lopez-Molina *et al.* 1997). The hypophagic animals are less active and undergoing a seasonal transition generally associated with decreasing day length, even though they are kept in constant conditions, suggesting a seasonal timing role for *DBP*. These findings illustrate the importance of this feeding transition as an indicator of hibernation readiness.

Therefore, in addition to the important gene expression data generated by this study, we have also demonstrated a simple and inexpensive method to track the autumn torpor status of hibernating species in the lab without relying on costly telemetry. Weighing food is an easy and reliable way to estimate when individual animals start transitioning into a torpor-ready state.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1: Colony and individual body weight changes from arrival to tissue collection. (a) Body weight changes of the entire captive ground squirrel colony ($n = 43$). Animals were weighed three times: upon arrival, after 2 weeks and before lab hibernation onset. (b) Body weights changes of hyperphagic (dark gray) and hypophagic (black) animals. Each individual symbol type represents one animal. Animals were weighed three times: upon arrival, after 2 weeks and at sacrifice.

Figure S2: Food consumption in males and females. (a) Food consumption changes of the entire captive ground squirrel colony ($n = 43$), with males represented in gray ($n = 14$) and females represented in black ($n = 29$). Each open circle represents the daily consumption of one individual. (b) Average food consumption for males (gray) and females (black). Error bars represent standard error of the mean.

Figure S3: Hypothalamic expression of well-known feeding and satiety-related genes. Expression of *HCRT*(a), *AGRP*(b), *POMC*(c), *NPY*(d) and *CARTPT*(e) from our previous seasonal hypothalamic Illumina transcriptome data (Schwartz *et al.* 2013) (light gray bars) and current hyperphagic (dark gray bars) and hypophagic (black bars). Error bars represent standard error of the mean. The letters represent *post hoc* pair-wise comparison to determine significance between seasonal collection points. Any collection point not connected by the same letter is significantly different (FDR <0.05).Sets of bars without letters represent genes that were not differentially expressed. Data from the two transcriptomes were normalized to each other using upper quartile normalization.

Table S1: All differentially expressed genes. Table provides the mean ($n = 3$) and standard error for all the differentially expressed genes in the hypothalamus of hyperphagic and hypophagic 13-lined ground squirrels. Genes were considered differentially expressed if they had at least 100 average counts in at least one group, they had at least a 25% change between groups, and they had an FDR <0.05. Gene names listed are the official HGNC designations. FDR, false discovery rate; HYPER, hyperphagic group; HYPO, hypophagic group; SE, standard error.