Neuronal UCP1 expression suggests a mechanism for local thermogenesis during hibernation

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Hibernating mammals possess a unique ability to reduce their body temperature to ambient levels, which can be as low as −2.9 °C by active down-regulation of metabolism. Despite such a depressed physiologic phenotype, hibernators still maintain activity in their nervous systems, as evidenced by their continued sensitivity to auditory, tactile, and thermal stimulation. The molecular mechanisms that underlie this adaptation remain unknown. We report, using differential transcriptomics alongside immunohistologic and biochemical analyses, that neurons from thirteen-lined ground squirrels (Ictidomys tridecemlineatus) express mitochondrial uncoupling protein 1 (UCP1). The expression changes seasonally, with higher expression during hibernation compared with the summer active state. Functional and pharmacologic analyses show that squirrel UCP1 acts as the typical thermogenic protein in vitro. Accordingly, we found that mitochondria isolated from torpid squirrel brain show a high level of palmitate-induced uncoupling. Furthermore, torpid squirrels during the hibernation season keep their brain temperature significantly elevated above ambient temperature and that of the rest of the body, including brown adipose tissue. Together, our findings suggest that UCP1 contributes to local thermogenesis in the squirrel brain, and thus supports nervous tissue function at low body temperature during hibernation.

Significance

Mammalian hibernators can reduce their metabolic rate by 95% and body temperature to 2 °C. However, their central and peripheral nervous systems retain activity even in cold, through unknown mechanisms. We report here that neurons from hibernating squirrels express uncoupling protein 1 (UCP1), a protein known as a heat generator in brown adipose tissue. We show that squirrel UCP1 acts as the typical thermogenic protein and is up-regulated during torpor, suggesting its thermogenic capability is important during hibernation. Accordingly, we found that the temperature of squirrel brain during the deep torpor associated with hibernation is warmer than the surrounding tissues. We hypothesize that neuronal UCP1 allows squirrels to withstand the long hibernation season and tolerate temperatures prohibitively low for survival and neuronal function in nonhibernating species.

Deeply hibernating animals can keep their brain temperatures elevated above ambient by several degrees (9). This may reflect a potential mechanism to support the functionality and integrity of the nervous system in the cold. In the search for a molecular basis for this process, we investigated the expression of uncoupling protein 1 (UCP1). UCP1 is known to be expressed in the inner mitochondrial membrane of brown adipose tissue (BAT) (10, 11), where it generates heat by dissipating the proton gradient set up by the electron-transport chain (12, 13). In hibernating mammals, as well as in human infants and small rodents, UCP1-mediated nonshivering thermogenesis plays a crucial role in the maintenance of core body temperature (14–16). Although UCP1 was originally thought to be restricted to the BAT of placental mammals, recent studies have challenged phylogenetic and tissue distributions, with it being found in marsupials, monotremes, and nonmammals (17–20). Intriguingly, UCP1 mRNA up-regulation was observed in the nervous tissue of cold-exposed common carp (Cyprinus carpio) (19). It was suggested that this temperature-induced UCP1 expression in carp may support local cranial endothermy necessary to survive winter dormancy. Trace amounts of UCP1 mRNA have been detected in mouse cortex (21, 22), but no expression was observed in peripheral nervous tissues (23).

Here, using differential transcriptomics alongside immunohistologic, biochemical, and functional analyses, we show that squirrel neurons from central and peripheral nervous system...
express a functional ortholog of UCP1. UCP1 protein is localized in neuronal mitochondria and is up-regulated during torpor compared with the summer active state. Functional analysis showed that squirrel UCP1 is capable of decoupling the electron transporting chain, suggesting a role in neuronal thermogenesis. Finally, we show that squirrel brain is warmer than the surrounding tissues, including BAT, in torpid hibernating animals. Our findings suggest a previously unexplored role for UCP1 in maintaining functionality of the nervous system in mammals during hibernation.

**Results**

Squirrel Nervous Tissue Expresses UCP1. In hibernating squirrels, we detected *UCP1* transcripts not only in BAT but also in central (cortex, cerebellum, hippocampus, spinal cord) and peripheral (trigeminal and dorsal root ganglia) nervous tissues of torpid animals (Fig. 1A). We confirmed *UCP1* identity by direct sequencing of PCR products from each sample. In contrast, we did not detect *UCP1* in kidney and liver, even though we amplified transcripts for the housekeeping gene hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) (24) from all tissues (Fig. 1A).

UCP1 Protein Is Present in Neuronal Mitochondria of Torpid Squirrels. Transcriptome analysis of trigeminal ganglia (TG) and dorsal root ganglia (DRG) from summer active and hibernating torpid squirrels revealed a dramatic up-regulation of *UCP1* during torpor (13- and 68-fold increase in TG and DRG, respectively) (Fig. 1B). The expression of the control *HPRT1* transcript remained stable in all conditions (1.1-fold increase in torpor vs. active state for TG and DRG). Notably, we did not detect molecular markers that are normally present in BAT and involved in the regulation of UCP1 expression and activation pathways (15, 25–27) (Table S1). Examination of cDNAs encoding UCP1 using both conventional cloning and de novo transcript assembly did not reveal the presence of isoforms in central and peripheral nervous tissues. To explore *UCP1* expression at the cellular level, we examined the distribution of *UCP1* transcripts by RNA in situ hybridization. *UCP1* was consistently detected in somatosensory neurons of torpid squirrels, but not in the surrounding tissue (Fig. 1C). Taken together, these observations demonstrate that *UCP1* transcripts are present in the neurons of hibernating squirrels.

Having established the presence of *UCP1* transcripts in torpid squirrel nervous tissues, we next performed immunohistochemical analyses using an antibody targeting a conserved region between mouse and squirrel UCP1. As expected, we observed *UCP1* labeling in both squirrel and mouse BAT. Strikingly, we also detected UCP1 protein in squirrel, but not mouse, cortex, hippocampus, TG, and DRG (Fig. 2A and S1). In squirrel cortex, UCP1 expression partially overlapped with the neuron-specific microtubule-associated protein 2 (MAP2) (28). We observed the same labeling pattern using a different antibody raised against a conserved UCP1 region (Fig. S2). Notably, we did not detect overlap between UCP1 and astrocyte-specific glial fibrillary acidic protein (Fig. 2B). Together, these data demonstrate the presence of UCP1 protein in squirrel neurons.

To achieve its thermogenic capabilities by uncoupling the electron transport chain, UCP1 must be present in mitochondria (29). Therefore, we conducted colocalization studies in dorsal root ganglia and BAT, using antibodies to both UCP1 and cytochrome *c* oxidase (COX IV), a major component of the inner mitochondrial membrane electron transport chain (30). We observed mitochondrial localization of UCP1 in both squirrel neurons and BAT (Fig. 2C).

We tested the presence of UCP1 protein in mitochondrial lysates from different tissues by immunoblot analysis, using an antibody raised against a conserved epitope between mouse and squirrel UCP1. We detected several bands migrating at positions close to the predicted molecular weights of both proteins at 33 kDa in mitochondrial lysates from mouse and torpid and active squirrel BAT (Fig. 3A). We also detected robust staining in mitochondria from squirrel, but not mouse, cortex. These results agree with our immunohistochemical analyses, using the same (Fig. 2 and S1) or an alternative (Fig. 2A) UCP1 antibody. We also detected robust staining in mitochondria from torpid squirrel whole-brain lysate, spinal cord, and pons (Fig. 3A). In contrast, we did not detect any signal in mouse whole brain or spinal cord or in squirrel kidney, liver, or lung (Fig. 3A and S3A). Together, these results are in agreement with the pattern of UCP1 mRNA expression (Fig. 1) and immunohistochemical analyses (Fig. 2 and Figs. S1 and S2) and strongly suggest the detected bands correspond to different UCP1 species, which are known to migrate on immunoblot at slightly different levels in different preparations and tissues, possibly as a result of post-translational modifications (31–35).

Notably, compared with tissues from summer active squirrels, we detected significant up-regulation of UCP1 protein in mitochondrial lysates from torpid brain, cortex, and spinal cord (*P* = 0.0194, *P* = 0.0158, and *P* = 0.016, respectively; unpaired t test; *n* ≥ 3 experiments) (Fig. 2B).

**Fig. 1.** *UCP1* is expressed in neurons of torpid squirrels. (A) PCR amplification of *UCP1* and housekeeping gene *HPRT1* transcripts from cDNA isolated from various tissues of torpid squirrels. Transcripts were amplified by 35-cycle PCR reactions. The RNA integrity number (RIN) was calculated to assess RNA quality in each sample. TG, trigeminal ganglia; SpC, spinal cord; Cer, cerebellum; Hip, hippocampus; BAT, brown adipose tissue; DRG, dorsal root ganglia; *HPRT1*, hypoxanthine-guanine phosphoribosyltransferase. (B) Differential transcriptome analysis of TG and DRG from torpid and active squirrels shows up-regulation of *UCP1* expression during torpor; the control *HPRT1* gene remained unchanged (*n* = 3 animals for torpor state; *n* = 2 animals for summer active state). (C) RNA in situ hybridization in TG and DRG from torpid squirrels, showing neuron-specific *UCP1* staining (*n* = 32 sections for TG; *n* = 40 sections for DRG; *n* ≥ 2 animals). (Scale bar, 100 μm.)
Functional Characterization of Squirrel UCP1 Protein. Squirrel UCP1 cloned from nervous tissue showed 84% and 80% identity to its mouse and human orthologs, respectively (Fig. S4). Because UCP1 function in squirrel neurons is unknown, we investigated its activity in vitro through studying uncoupling properties of UCP1 protein is expressed in the mitochondria of squirrel neurons. (Fig. 2A) Representative images of mouse and torpid ground squirrel BAT and cortex sections immunolabeled for UCP1 protein (ab10983, green) and for the neuronal marker MAP2 (red). (Fig. 2B) Representative images of torpid ground squirrel cortex sections immunolabeled for UCP1 (ab10983, green) and the astrocyte marker glial fibrillary acidic protein (red). (Fig. 2C) Representative images of torpid squirrel DRG and BAT sections immunolabeled for UCP1 (ab10983, green) and the mitochondrial marker COX IV (red). (Scale bars, 20 μm.) See also Figs. S1 and S2. n ≥ 14 sections for each condition.

We tested the subcellular localization of squirrel UCP1, using a mitochondria-enrichment assay. A comparison of the total and mitochondria-enriched preparations from squirrel spinal cord showed substantial increases in UCP1 protein, together with the mitochondria-specific ATP synthase (ATP5A). In contrast, we did not detect UCP1 in mitochondrial preparations from mouse spinal cord (Fig. S3B). Thus, immunohistologic and biochemical data demonstrate the expression of UCP1 protein in the mitochondria of squirrel neurons.

To test uncoupling properties, we isolated mitochondria from HEK293 cells expressing squirrel or mouse UCP1 and analyzed their function using a high-sensitivity polarographic respirometry assay. Mitochondria were incubated in an isolated chamber, and the rates of oxygen consumption in response to the sequential addition of various reaction components were measured (Fig. 4A). We found that UCP1-containing mitochondria exhibited significantly higher basal oxygen consumption rates compared with nontransfected (control) cells when incubated in nonphosphorylating (state 2) conditions in ADP-free media containing pyruvate/malate [normalized O2 flux (pmol), mean ± SEM (n ≥ 7); sqUCP1-HA, 1,088 ± 68; mUCP1-HA, 1,166 ± 157.3; control, 367.7 ± 24.2; sqUCP1 vs. control, P < 0.0001; mUCP1 vs. control, P < 0.0001; two-way ANOVA with Tukey’s post hoc test].

Mouse and human UCP1 are activated by free fatty acids and inhibited by purine nucleotides such as GDP (12, 29, 36–38). Along these lines, addition of GDP specifically reduced the oxygen flux of UCP1 containing mitochondria to such an extent that mUCP1-HA became indistinguishable from control, which remained unchanged (Fig. 4B; sqUCP1-HA, 740.8 ± 65.4; mUCP1-H, 705.4 ± 101.9; control, 381.9 ± 29.9). When ADP was added to induce phosphorylating (state 3) respiration, oxygen consumption rates for all groups increased to similar levels, suggesting the presence of active mitochondria in all three samples (sqUCP1-HA, 1,899 ± 92.9; mUCP1-HA, 1,943 ± 269.3; control, 1,654 ± 102.6). Subsequent titration of oligomycin was sufficient to poison ATP synthase and return the system to nonphosphorylating respiration levels (state 4o) (Fig. 4B; sqUCP1-HA, 625.8 ± 46.3; mUCP1-HA, 606 ± 66.8; control, 406.9 ± 25.3). To test whether squirrel UCP1 is activated by free fatty acids, we finally added 100 μM palmitate, which significantly
increased respiration rates of UCP1-containing mitochondria over that of the control (sqUCP1-HA, 1.362 ± 113.5; mUCP1-HA, 1.254 ± 177.5; control, 448 ± 32.3; sqUCP1 vs. control, P < 0.0001; mUCP1 vs. control, P < 0.0001).

When ATP synthase is not active, such as during state 2 or in the presence of oligomycin, respiration rates mainly reflect attempts by the electron transport chain to maintain the proton motive force in the face of proton leak back into the matrix (leak respiration). Thus, the significantly higher oxygen fluxes observed in UCP1-containing mitochondria under basal state 2 conditions and after palmitate addition likely result from increased proton leak through UCP1 and subsequent attempts by the electron transport chain to compensate for this leak. Together, these data show that squirrel UCP1 is indistinguishable from the mouse ortholog and functions as a mitochondrial uncoupling protein.

To analyze the uncoupling abilities of mitochondria isolated from mouse and torpid squirrel brains, we performed a respirometry assay to quantify palmitate-induced uncoupling. Although we did not detect the presence of UCP1 in the mouse brain (Figs. 2A and 3A and Figs. S1 and S2), we detected some level of palmitate-induced uncoupling in this tissue, which likely reflects the presence of other uncoupling proteins, such as UCP2 (39).

However, we found that mitochondria isolated from squirrels displayed significantly higher levels of uncoupling compared with BAT (Fig. 3B). Pair-wise comparisons within each animal showed that cortex was consistently warmer than BAT (P = 0.009, ANOVA with Holm-Sidak’s multiple comparisons test; n = 14) and WAT (P = 0.002; Fig. 5B), which agrees with the hypothesis that UCP1 expression in neurons contributes to heat generation during torpor.

**Discussion**

Here, we demonstrate the expression of a functional UCP1 protein in neuronal mitochondria of ground squirrels. We show that UCP1 expression is significantly up-regulated during hibernation. These findings provide a molecular explanation for the heat production in the nervous systems of hibernators during torpor. Until now, the prevailing view was that BAT was the sole significant source of heat. However, this notion is inconsistent with our observation that cortex temperature is significantly elevated compared with BAT and other tissues during torpor. Furthermore, the dramatic reductions in heart rate [down to three beats per minute (4)] and cerebral blood flow [~90% reduction, from 62 ± 16 mL/100 g per minute to 7 ± 4 mL/100 g...
per minute (40)], in torpid animals pose formidable obstacles to efficient heat exchange between BAT and nervous tissue. Although we do not exclude a significant contribution of BAT-mediated heating, our findings offer an additional nervous tissue-autonomous mechanism. We hypothesize that mammalian hibernators make use of neuronal UCP1 to supply heat, which aids in maintaining basal activity of both central and peripheral nervous systems during hibernation. Our study demonstrates seasonal changes in neuronal UCP1 expression. In the future, it would be interesting to investigate the dynamics of UCP1 levels in different intrahibernation cycles: interbout arousal and early and late torpor, as well as arousal phases.

It is tempting to speculate that other mammalian hibernators may use a similar mechanism. For example, extreme hibernators such as arctic ground squirrels experience torpor at very low ambient temperatures (≤ −10 °C). These unique animals experience zero to subzero temperatures in several internal organs and tissues (41), but brain temperature is maintained at a constantly elevated level of 4 °C (9). Similar to thirteen-lined ground squirrels (their close relative), arctic ground squirrels may potentially rely on UCP1 to support the integrity and function of the nervous system during deep torpor. In this case, however, UCP1 expression should be seasonal, as shown here for the thirteen-lined ground squirrels, because an earlier study did not detect UCP1 transcripts in the brain of active arctic ground squirrels (42). It will be interesting, therefore, to evaluate the expression of UCP1 transcripts and protein in torpid animals.

During arousal, hibernators significantly increase oxygen consumption rates and rapidly perfuse torpid tissues. In contrast, there may be danger of ischemia during entrance into and arousal from torpor (2). How mammalian hibernators are able to adapt to rapidly fluctuating oxygen levels without incurring oxidative damage, and how they avoid reperfusion injury in the brain and other sensitive tissues, is not well understood. Multiple studies have shown that hibernators display resistance to ischemia/reperfusion injury and, during torpor, increase expression of molecules that help regulate oxidative stress, such as superoxide dismutase 1 and 2, catalase, and glutathione peroxidase (2, 43–46). Interestingly, uncoupling proteins have been linked to reduction of reactive oxygen species formation (47). Although such a role for UCP1 is more controversial than for some other uncoupling proteins, there is still evidence to suggest that UCP1 may indeed function to regulate reactive oxygen species levels (48–50). Further studies are needed to explore the role of UCP1 in regulation of reactive oxygen species level during hibernation cycles.

Notably, we did not detect BAT-specific molecular markers other than UCP1 in the squirrel nervous tissue (Table S1) (15, 25–27). This suggests that ground squirrels evolved alternative molecular mechanisms for the regulation of UCP1 expression in their neurons that differ from those in BAT. However, it has been demonstrated that levels of nonesterified serum fatty acids are elevated during torpor in different mammalian hibernators, including ground squirrels (9, 51), hamsters (52), and black bears (53). This pool of free fatty acids in the blood may promote activation of UCP1 in the torpid brain. Recently, adipocyte-specific fatty acid binding protein 4 (FABP4) was identified in different regions of the thirteen-lined ground squirrel brain. Moreover, FABP4 was found to be up-regulated in nervous tissue during hibernation, offering a potential pathway for the uptake of fatty acids by torpid brain (54). This study, however, did not identify UCP1 mRNA in torpid brain, even though we detected significant amounts of UCP1 protein in this tissue.

Identification of UCP1 protein in the ground squirrel brain is interesting from an evolutionary point of view. To our knowledge, this report is the first demonstration of UCP1 protein in neuronal mitochondria of a mammal. Earlier reports did not identify UCP1 in mouse TG and DRG (23) and detected only trace amounts of UCP1 transcript in mouse cortex (~0.01% of UCP1 level in BAT) (21, 22), suggesting neuronal UCP1 expression could be a specific feature pertaining to animals that have to withstand prolonged periods of extreme hypothermia.

In support of this hypothesis, UCP1 was previously identified in the brain of common carp (C. carpio) (19). In carp, UCP1 transcripts are twofold up-regulated in different brain regions after cold acclimation. Similar to the conclusions drawn here, this molecular adaptation was suggested to support nervous tissue function and thermal adaptation of brain metabolism in cold water, and potentially to support winter dormancy.

Cranial endothermy was also discovered in lamnid sharks, billfishes, tunas, and opah and was proposed as a physiologic adaptation to deep dives in cold water (Fig. 6) (55–57). However, these divergent groups of fish evolved this capability through UCP1-independent mechanisms. Sharks use a vascular heat exchange system (56), whereas billfish developed heater organs derived from modified, noncontractile extraocular muscles that can increase local cranial temperature 13 °C above ambient water condition (58).

Recently, localized heat production was described in the Lesser hedgehog tenrec (Echinops telfairi), a protoendothermic mammal that regulates its core body temperature only during the reproductive season. UCP1-containing fat deposits were identified in the abdomen of these animals, just adjacent to the gonads. Therefore, tenrecs may support their reproductive function using the strategy of localized endothermy (18).

Our findings provide a molecular explanation for the increased heat production in the nervous system of thirteen-lined ground squirrels during torpor, supporting the notion that localized endothermy is an essential prerequisite for survival despite cold core body temperatures, a strategy that has evolved independently multiple times over the course of vertebrate evolution.

**Methods**

Animals were housed in a pathogen-free facility at Yale University. All animal procedures were performed in compliance with the Institutional Animal Care and Use Committee of Yale University. Animal handling, tissue collection, temperature measurements, deep sequencing, cloning, in situ hybridization, immunohistochemistry, generation of stable cell lines, mitochondrial isolation, western blotting, and respirometry were carried out following the detailed protocols described in SI Materials and Methods.
ACKNOWLEDGMENTS. We thank Eve Schneider for comments throughout the study. Michael DePonte for help with animal imaging, and Joseph DePonte for coordinating the construction of the hibernaculum. We are grateful to Oporobors Instruments for the loan of an Oxygraph-2k. This work was supported by fellowships from the Beckman Foundation and Alfred P. Sloan Foundation (to E.O.G.), National Institutes of Health Grants T32 HG-31918-10 (to W.J.L.) and R01 DK-40936 (to G.I.S.), and startup funds from Yale University (to S.N.B. and E.O.G.).

13. Riouvier D (2011) Uncoupling protein 1 of brown adipocytes, the only uncoupler: A historical perspective. Front Endocrinol (Lausanne) 2:85.
Animals. Animals were housed in a pathogen-free facility at Yale University. All animal procedures were performed in compliance with the Institutional Animal Care and Use Committee of Yale University. Thirteen-lined ground squirrels were maintained on a diet of dog food (Iams) supplemented with sunflower seeds, superworms, and fresh vegetables. Daily monitoring of core body temperatures was accomplished telemetrically, using transponders implanted subcutaneously in the back of the animals (IPTT-300; Bio Medic Data Systems). When body temperatures dropped to ambient levels in the late summer, animals were transferred to a dark, humidity- and temperature-controlled hibernation chamber (hibernaculum) set to 3.8 °C without access to food and water. Hibernating animals are housed in enclosed hibernation chambers to simulate underground burrows as described (1). Experiments on active squirrels were performed in May and June. Mice were housed on a 12-h light/dark cycle under standard laboratory conditions with ad libitum access to food and water. During the summer active period, squirrels were also housed on a 12-h light/dark cycle with ad libitum access to food and water.

Temperature Measurements. For direct temperature measurements of different tissues, animals (6–8 mo old) with core body temperatures at ≈3–4 °C were killed during deep torpor bouts (defined as >1 wk from the most recent interbout arousal). Tissues were rapidly excised by incision, and their respective temperatures were measured by the insertion of an electronic thermometer (Sper Scientific) in the following sequence: BAT, cortex, WAT. The whole procedure from decapitation to taking final measurements took less than a minute for each animal. Temperature collection occurred in a 3.8 °C environment, and laboratory personnel wore insulated gloves to prevent passive warming of tissues. Each animal was 2–3 mo in hibernation and at least 7 d in torpor without interbout arousals.

Tissue Collection and Processing. Active squirrels were killed by CO2 inhalation, and torpid squirrels were killed by decapitation. Tissues for RNA extraction were dissected into TRIzol Reagent (Invitrogen). RNA was extracted according to manufacturer's instructions, and RNA integrity numbers were determined using 2100 bioanalyzer (Agilent Technologies). For in situ hybridization and immunohistochemistry, tissues were dissected and fixed overnight in 4% (vol/vol) paraformaldehyde in PBS. For immunoblots, tissue was dissected, flash-frozen, and used immediately or stored at −80 °C.

Deep Sequencing and Analysis. Total RNA was isolated from three torpor and two active samples for each of the trigeminal and dorsal root ganglion. Sequencing libraries were prepared from poly(A)+ RNA, using the Illumina TruSeq Stranded total RNA Prep Kit (RS-122-2301) according to the manufacturer's instructions. Libraries were then sequenced on the Illumina HiSeq2000 by standard protocols. Between 8 million and 15 million read pairs per sample. Sequences were aligned to the annotated UCP1 transcript ENSSTOT00000003103 and the pool was analyzed by 100 raw reads on average across all 10 samples. Expression changes were analyzed using the DESeq package and a generalized linear modeling framework to test for ganglion- and torpor-dependent changes as well as significant interactions between ganglion and torpor status. The UCP1 transcript (Ensemble transcript ID ENSTOG00000003103) showed a false discovery rate-adjusted P value (q-value) <1e−99 for ganglion-specific expression differences and for ganglion-specific, torpor-induced expression changes. Automated and manual methods were used to search for alternative UCP1 transcripts. Transcriptome assemblies for individual samples were performed and merged together using Cufflinks v2.0.1, guided by spetri2 gene annotations. Additionally, all read pairs where at least one read aligned to the annotated UCP1 transcript ENSSTOT00000003103 were extracted to search for evidence of alternate mRNA isoforms.

Cloning. Mouse UCP1 cDNA was obtained as a glycercol stock from Thermo Scientific (clone 4191276). Functional cDNAs from squirrels were amplified from first-strand cDNA generated by reverse transcription, using the following primers: squirel UCP1 (forward: 5′-CCACCTGGAGGGTTGGACCC-3′; reverse: 5′-GGGTCAATCAAGCATTCC-3′) and squirrel HPR1 (forward: 5′-TGATAGATCCATCTCATTGAGTGA-3′; reverse: 5′-CAACATCAAGCATCTTCCA-3′).

In Situ Hybridization. Cryostat sections (12 μm) were processed and probed with a digoxigenin-labeled cRNA. Probes were generated by T7/T3 in vitro transcription reactions using full-length UCP1 cDNA. Signal was developed with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments according to the manufacturer's directions (Roche).

Immunohistochemistry. Cryostat sections (12 μm) were permeabilized with Triton X-100, blocked with 2% (wt/vol) BSA, and incubated overnight at 4 °C with primary antibody [rabbit anti-UCP1 (ab10983, 1:800; Abcam), rabbit anti-UCP1 (ab155117, 1:250; Abcam), chicken anti-glial fibrillary acidic protein (ab4674, 1:750; Abcam), mouse anti-MAP2 (ab11268, [AP-20], 1:800; Abcam), and mouse anti-COXIV (ab33985, 1:100; Abcam)]. Bound antibody was detected using Alexa Fluor-488 goat anti-rabbit, Alexa Fluor-568 goat anti-chicken, and Alexa Fluor-568 donkey anti-mouse (1:500; Life Technologies). Slides were imaged using an UltraView VoX (Perkin-Elmer) spinning disk confocal microscope equipped with Volocity image analysis software (Improvision) and an Axio Observer microscope equipped with AxioCam MRC camera (Zeiss).

Mitochondrial Isolation. Mitochondria were isolated according to the manufacturer's instructions, using the mitochondria isolation kits for tissue or cultured cells (AbCam). Mitochondrial isolation was verified by staining for COX IV or ATP5A.

Preparation of Protein Lysates. Protein lysates were prepared using a homogenizer to rupture cells, isolated mitochondria, or tissue in lysis buffer. Samples were incubated on ice for 30 min and centrifuged at 12,000 × g for 15 min at 4 °C. Supernatants were processed immediately or saved and stored at −80 °C.

Western Blot Analysis. Protein lysates (40 μg) were diluted with Laemmli sample buffer (Bio-Rad). Proteins were transferred to PVDF membranes, using a semidry transfer cell (Bio-Rad). Membranes were incubated in nonmammalian Odyssey blocker (Li-Cor) for 1 h at room temperature before incubation overnight at 4 °C with primary antibody diluted in TBST. The following
antibodies were used: rabbit anti-UCP1 (ab10983, 1:1,000; Abcam), mouse anti-ATP5a ([15H4C4], 1:1,000; Abcam), mouse anti-HA (HA7, 1:5,000; Sigma), mouse anti-COXIV (ab33985, 1:1,000; Abcam), Odyssey goat anti-rabbit IRDye 680 (1:10,000; Li-Cor), and goat anti-mouse IRDye 800 (1:10,000; Li-Cor). Membranes were imaged using an Odyssey Infrared Imaging System (Li-Cor), and Li-Cor Image Studio was used for analysis and quantification.

**Generation and Culturing of Inducible Stable Cell Lines.** The coding sequences for the mouse and squirrel UCP1 homologs were cloned into a pcDNA5/FRT/TO vector containing a single FRT site (Invitrogen). In addition, an HA-tag was introduced at the N terminus of each gene, using site-directed mutagenesis. The Flp-In T-REx 293 HEK host cell line was used to generate tetracycline-inducible stable cell lines according to manufacturer's instructions (Invitrogen). Protein expression was induced by incubating cells with media supplemented with 1 μg/mL tetracycline (Invitrogen) for 48 h. Induction was verified by immunoblot.

**Respirometry.** Mitochondria were isolated from nervous tissue or HEK293 cells, using a mitochondria isolation kit, according to the manufacturer's instructions (Abcam). Mitochondrial protein concentrations were determined by Bradford assay. Citrate synthase activity levels were calculated using the citrate synthase assay kit, according to manufacturer’s instructions (Sigma). Respiration was assessed using a high-resolution polarographic respirometer maintained at 37 °C (Oroboros Oxygraph). Isolated mitochondria (HEK293 cells, 0.08 mg/mL; nervous tissue, 0.04 mg/mL) were incubated in MiR01 media (100 mM sucrose, 50 mM KCl, 20 mM TES, 1 mM EDTA, 4 mM KH2PO4, MgCl2, 0.1% wt/vol fatty acid-free BSA at pH 7.2). Reaction components were added sequentially to the chamber with no washing in-between. Basal nonphosphorylating respiration (state 2) was initiated by the addition of 5 mM pyruvate and 2 mM malate. After reaching a steady level, 750 μM GDP was added to mitochondria preps from HEK293 cells to inhibit UCP1-mediated proton leak. In contrast, no GDP was used for experiments with mitochondria isolated from nervous tissue. Phosphorylating respiration (state 3) was induced by titration of 1 mM ADP. ATP synthesis was then halted by treatment with 2 μg/mL oligomycin to inhibit ATP-synthase (state 4). Addition of the free fatty acid palmitate (100 μM) stimulated UCP1-dependent proton leak. Oxygen flux data are presented after normalization to units of citrate synthase activity for the HEK293 experiments or as a percentage of oligomycin-induced state 4 respiration for nervous tissue mitochondria. Data were processed using DatLab software (Oroboros) and analyzed by two-way ANOVA with Tukey’s post-hoc test (HEK293 experiments) or unpaired t-test (nervous tissue mitochondria).

**Fig. S1.** Immunohistochemical analysis of UCP1 in squirrel and mouse tissues. (Left) DAPI (blue) and UCP1 (green) colabeling (ab10983) of mouse and torpid squirrel brown adipose tissue (BAT), dorsal root ganglia (DRG), trigeminal ganglia (TG), hippocampus, and cerebral cortex (Cortex). (Right) (Secondary AB only): Specificity of the secondary antibody was verified by labeling tissues not exposed to primary antibody. (Scale bar, 100 μm.)
Fig. S2. Immunohistochemical analysis of UCP1 in squirrel and mouse tissues using an alternative anti-UCP1 antibody. UCP1 (ab155117, green), and neuronal MAP2 marker (red) colabeling mouse and torpid squirrel BAT and cortex. (Scale bar, 20 μm.)

Fig. S3. Immunoblot analysis of mitochondrial fractions (Mito) from mouse and torpid squirrel tissues. (A) Mitochondrial lysates from torpid squirrel brown adipose tissue (BAT), lung, and liver. (B) Mitochondrial enrichment analysis of mouse (m) and torpid squirrel (sq) spinal cord (SpC). Mitochondrial enrichment in all fractions was verified using the ATP synthase loading control (ATP5A).
**Fig. S4.** Sequence comparison of squirrel, mouse and human UCP1. Amino acid sequence alignment between squirrel, mouse, and human UCP1 showing predicted transmembrane domains (blue bars), regulatory nucleotide binding region (NBD, red bar), and amino acid residues implicated in activation by fatty acids (red stars). Identical amino acids are highlighted in green.

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<th>Gene</th>
<th>Squirrel TG/DRG</th>
<th>Mouse TG/DRG</th>
<th>Mouse BAT</th>
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List of molecules previously implicated in the function of brown adipocytes and their expression in mouse and squirrel somatosensory system.