Supporting Online Material for

Transgenic Mice with a Reduced Core Body Temperature Have an Increased Life Span

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Published 3 November 2006, *Science* **314**, 825 (2006)
DOI: 10.1126/science.1132191

This PDF file includes:

Supporting Online Material

Materials and Methods

Animals

Hcrt-UCP2 mice were generated on a C57BL/6 background using a linearized construct comprising the 7 kb region upstream of mouse hypocretin gene start site fused to the murine UCP2 cDNA and to the bovine growth hormone polyadenylation signal. A colony was established from one founder backcrossed for 7 generations on the C57BL/6 background. Hcrt-UCP2 was inherited by simple Mendelian segregation. Negative littermates were used as controls in all experiments (wt). Heterozygous Hcrt-UCP2 (tg) mice and wild-type littermates (wt) used as controls in all experiments were obtained by crossing heterozygous Hcrt-UCP2 males with wt females. Reproductive statistics were similar in Hcrt-UCP2 and C57/BL6 mice for average litter size (6.6 vs 6.5), percentage survival at weaning (90% vs 81%) and F:M sex ratio (1:1.01 and 1:1.04). The genome of Hcrt-UCP2 mice contained 7 copies of the Hcrt-UCP2 construct. Mice were fed *ad libitum* on sterilized breeder chow containing 17% crude protein, 11% of crude fat and 3.5% crude fiber.

In situ hybridization and immunohistochemistry

Animals were deeply anesthetized with sodium pentobarbital (120 mg/kg) and perfused transcardially with saline followed by 4% formaldehyde generated from paraformaldehyde in 0.1M phosphate buffer (PB; pH 7.2). The brains were post-fixed in the same solution for 1 hour and infiltrated with 30% sucrose overnight. Free-floating

sections (35 μm) were obtained on a cryostat. *In situ* hybridization was performed as previously described using ^{35}S labeled sense and antisense UCP2 riboprobes. For immunohistochemistry, sections were washed for 30 min in 0.1M PBS and permeabilized with 0.2% Triton X-100 in 0.1M PBS containing 1% bovine serum albumin (BSA). Sections were washed in PBS containing 0.5% BSA for 30 minutes and incubated overnight at 4 ºC with antibody against hypocretin (1: 1000; Santa Cruz Biotechnology, Santa Cruz, CA). Sections were washed in PBS-BSA and incubated for 1 hour with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) at 1:200. Immunopositive cells were visualized by DAB staining (ABC Vector).

For double-labeling immunohistochemistry, sections were co-incubated overnight in blocking solution containing UCP2 and hypocretin antibody (Santa Cruz, CA, 1:500). Sections were then processed to develop UCP2 immunoreactivity by sequential incubations with a biotinylated donkey anti-goat IgG (Jackson Immunoresearch, Inc., West Grove, PA) and avidin-biotinylated horseradish peroxidase complex (ABC; Vector Laboratories, Burlingame, CA). They were subsequently incubated in 1:100 fluorescein tyramide solution (TSA-direct) (NEN Life Science Products, Perkin Elmer Life Sciences, Inc, Boston, MA). For hypocretin immunoreactivity, sections were further incubated for 45 min in Alexa Fluor 594-conjugated goat anti-rabbit IgG (Molecular Probes, Inc., Eugene, OR). Sections were mounted and analyzed by confocal microscopy.

Radioimmunoassay.

Radioimmunoassay for the determination of hypocretin 1 and hypocretin 2 levels was carried out on protein extract obtained from hypothalamic tissue using a commercially

available RIA kit (Phoenix Pharmaceuticals, Belmont, CA) following the manufacturer's instructions.

Real Time PCR

Quantitative PCR was performed using Roche LightCycler equipment and LightCycler-Fast Start DNA Master SYBR Green I mix. Reactions were carried out in a 10 µL volume using 0.5 µM primers and 2 mM $MgCl_2$. The sequences of the primers are as follows: for β-actin, 5'- tac tcc tgc ttg ctg atg ca-3' and 5'- cat gta ccc agg cat tgc t -3'; for UCP2, 5'- gct ggt ggt ggt cgg aga ta -3' and 5'- aca gtt gac aat ggc att acg g -3'. PCR assays included an initial 10 min, 94 ºC step to activate Taq polymerase, followed by 35-45 cycles of denaturation at 94 ºC, 10 s, annealing 58 ºC, 10 s, and extension 72 ºC, 25 s. Standard curves were constructed using purified and sequenced UCP2 and β-actin PCR fragments. Before reverse transcription, RNA samples were subject to RNAse free-DNAse treatment. Results were analyzed by the second derivative methods and were expressed in arbitrary units normalized to the expression levels of the reference gene, β-actin, quantified simultaneously with the target gene.

Brain temperature

Brain temperature was measured on 3 month old mice previously implanted with radiotelemetry transmitters for core body temperature (CBT) monitoring. Thermocouple probes prepared from copper and constantin wires (W-TW-40P; diameter, ~125 µm,Physitemp Instruments,Clifton, NJ) were positioned by stereotactic surgical implantation. After mechanically removing 200-400 µm of insulation from the tip of each

wire, the tips were welded together and reinsulated with epoxy. The wires were connected to copper and constantin pins and fixed in a plastic connector with epoxy. Mice were anesthetized (induction 3-5%, maintenance 1-2% isoflorane) and placed into a stereotaxic apparatus. The probes were secured with dental cement to three stainless-steel screws threaded into the skull. Mice were allowed to recover from surgery for one week and to habituate to the experimental environment for at least 48 hours before recording. Probes were connected to the recording instrument (Thermes-16; Physitemp Instruments) via individual sockets, a common cord, and a custom-made electric swivel commutator (Model 50-6,Dragonfly R&D, Inc.). During the recording session, intrabrain temperatures were continuously recorded and data stored in a computer for off line analysis. The temperature in the room was maintained at ~25 ± 0.5 °C. Recording was carried out continuously for up to 24 hours. During this period digitized data was collected every 5 minutes. The significance of temperature differences was evaluated using ANOVA with repeated measures followed by Fisher LSD tests comparing 10 min intervals. The data were presented as changes in absolute temperature. After completion of the experiments, probe location was verified histologically. Only data from animals showing normal circadian temperature profile were used for analysis. Data from those animals where probe implantation in the POA resulted in disruption of temperature regulation as demonstrated by their CBT profile, were disregarded and did not differ in frequency between wt and tg.

Core body temperature and locomotor activity

Core body temperature (CBT) and locomotor activity were monitored through transmitter devices (TA10ETA-F20, Data Sciences, Inc.) surgically implanted into the peritoneal cavity under general anesthesia (induction 3-5%, maintenance 1-2% Isoflorane). After surgery each animal was singly housed in Plexiglas recording cages placed in environmentally controlled chambers (Tech/Serv model EPC-010). The ambient temperature was maintained at 25 ± 0.5 °C in a 12:12-h light-dark cycle controlled room (lights on 6:00 am). Food and water were available *ad libitum*. Data were recorded by placing a cage containing an animal implanted with a radiotransmitter on a receiver plate (RPC-1, Data Sciences, Inc). The DATAQUEST A.R.T. software (DataSciences, Inc.) was used for data collection and off-line analysis. Mice were allowed to recover from surgery for two weeks and to habituate to the experimental environment for at least 48h before recording.

Energy Expenditure and Food Intake

Mice were housed individually on white cellulose bedding (Harlan Teklad OMEGA-dri 6055) to facilitate spillage measurement. To determine whether Hcrt-UCP2 transgene expression grossly altered relative energy expenditure, we compared wild type (-/-) and transgenic *(+/-)* age- and weight-matched adult male (*n*=6 and 9, range=27-33 g) and female mice (*n*=9 and 9, range=23-36 g) for weight loss induced by acute food deprivation-induced weight loss. Food was withdrawn for 27 hours beginning 3 hours prior to the onset of the light cycle, and weight loss (to the nearest 0.01 g) was normalized for metabolic demands of body mass per Kleiber's power function (i.e., g

weight loss / g baseline weight$^{0.568}$ (*1*)). To determine whether Hcrt-UCP2 transgene expression influenced daily energy or fluid intake, we measured chow and water intake in nondeprived mice. We used the following two schedules of measurement: 1) every 3 hours for 24 hours beginning 3 hours prior to the light cycle, and 2) biweekly for two weeks at 3 hours prior to the light cycle. Differences in chow and water weight between time points (to the nearest 0.01 g) were the measures of intake.

Supporting text

Sleep

Sleep parameters were measured to determine if the reduction of hypocretin neurons in Hcrt-UCP2 mice could influence sleep and CBT. The states of vigilance during 24 hours were monitored in mice implanted with EEG and EMG electrodes (*2*). Unlike hypocretin deficient mice (*3*), Hcrt-UCP2 mice did not show major differences in sleep latency or the number of sleep episodes in any vigilance state (n=7, 7; $p>0.05$, Student t tests). Hcrt-UCP2 mice showed increased wakefulness ($M\pm$SEM: 39.00 ± 1.75 min vs 33.04 ± 1.30 min) and reduced NREM sleep ($M\pm$SEM: 18.61 ± 1.66 min vs 24.95 ± 0.96 min) 6 hours after dark onset, and increased REM sleep ($M\pm$SEM: 7.52 ± 1.09 min vs 2.31 ± 0.30 min) at the end of the light phase ($p<0.05$, Student t test). These differences cannot account for the reduction of CBT observed in other portions of the dark phase.

Figure Legend

Fig S1. Generation of Hcrt-UCP2 transgenic mice. **(A)** The construct consisted of the 7 kb region upstream of the mouse hypocretin gene start site fused to mouse UCP2 cDNA and the bovine growth hormone polyadenylation signal. Specific expression of UCP2 mRNA was assessed in Hcrt-UCP2 mice (tg) and in negative littermates (wt) by *in situ* hybridization using ^{35}S-labeled antisense riboprobe to murine UCP2. Shown are a film autoradiograph of a coronal brain section from Hcrt-UCP2 mouse **(B)** and a bright field photoemulsion of the lateral hypothalamus from a wt and an Hcrt-UCP2 mouse **(C)**. Specific expression of UCP2 in hypocretin neurons was demonstrated by double immunohistochemistry with antibodies for hypocretin (Hcrt) and UCP2 as indicated **(D)**. Bar 2 mm in A and 200 μm in C and D.

Fig. S2. Histograms showing that Hcrt-UCP2 and wt littermates have similar levels of UCP2 mRNA in peripheral tissues (spleen, lung, white adipose tissue and the liver as indicated). mRNA levels were determined by real-time PCR and are expressed as the ratio between UCP2 and beta actin, the internal standard. The value of UCP2/beta actin of the wt for each tissue was fixed to 1 to facilitate comparison. Data represent the mean (± SEM) of samples from 3 animals per group.

Fig. S3. Radiotelemetric temperature profile over 24 hours following injection of 100 μg/kg lipopolysaccaride (LPS). Hcrt-UCP2 and wt littermates mice showed similar fever response to LPS, indicating that thermogenesis is not compromised in Hcrt-UCP2 mice. (n=6 animals per group, *p<0.05 ANOVA with repeated measures followed by Fisher's

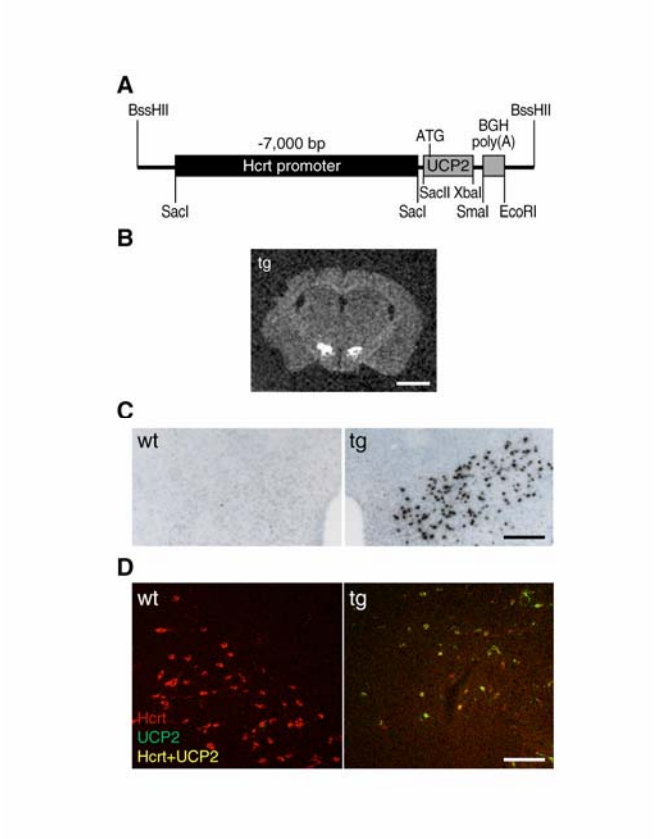
LSD tests comparing 10 min intervals). The temperature of the room was adjusted to 30 ± 0.5 °C, and mice were habituated for 72 hours before the fever experiment.

Fig. S4. Hcrt-UCP2 mice have a reduced number of Hcrt neurons. (**A**) Representative immunohistochemistry with hypocretin-specific antibody of brain coronal sections containing hypocretin neurons in wt and Hcrt-UCP2 (tg) mice; Bar is 1 mm for top and 200 μm for bottom panels. (**B**) Histogram showing the total cell number of hypocretin immunopositive neurons (± SEM). Cell number was determined after immunostaining by counting the neurons in contiguous 35 μm sections. Reduction was 22% in males and 30% in females.

Fig. S5. Selective loss of hypocretin neurons in orexin/ataxin 3 mice reduced locomotor activity but had no effects on CBT. Orexin/ataxin3 mice have hypocretin-specific expression of a truncated Machado-Joseph disease gene product (ataxin-3) with an expanded polyglutamine stretch generated on C57/BL6-DBA1 background that causes a progressive degeneration of hypocretin neurons. (**A**) Representative immunohistochemistry with hypocretin-specific antibody of brain coronal section containing hypocretin neurons in wt and orexin/ataxin 3 mice. Cell number was determined by counting hypocretin positive neurons in contiguous 20 μm sections after immunohistochemistry and was found to be reduced by 90% in the mice that were used for locomotor activity and temperature recordings; Bar is 1 mm for top and 200 μm for bottom panels. (**B**) Consistent with published data orexin/ataxin 3 mice showed reduction of locomotor activity. **(C)** CBT profile over 5 days of recording is similar in orexin/ataxin

3 mice and wt littermates. (**D**) Cumulative circadian CBT profile demonstrated that 90% loss of hypocretin neurons did not result in reduction of CBT (n=5 mice per group; *p< 0.05 ANOVA with repeated measures followed by Fisher's LSD tests comparing 10 min intervals).

Fig. S1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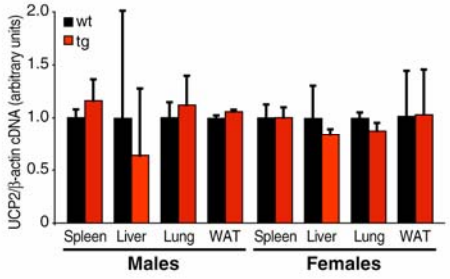
SmaI
EcoRI
B
tg
C
wt
tg
D
wt
tg
Hcrt
UCP2
Hcrt+UCP2

Fig. S2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1.5
1.0
0.5
0
wt
tg
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Liver
Lung
WAT
Spleen
Liver
Lung
WAT
Males
Females

Fig. S3

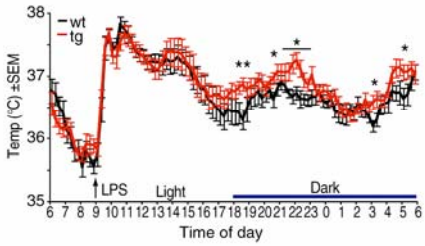
wt
tg
Temp (°C) ±SEM
38
37
36
35
LPS
Light
Dark
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 0 1 2 3 4 5 6
Time of day
**
*
*
*
*

Fig. S4

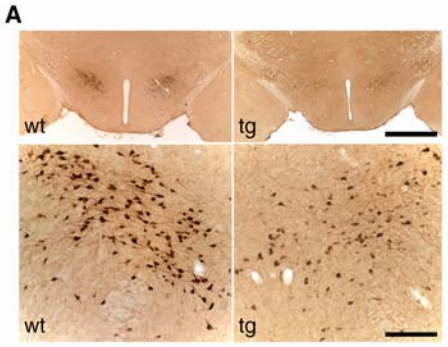
A
wt
tg
wt
tg
B
wt
tg
Number of hcrt-positive neurons
4000
3000
2000
1000
0
*
*
Males
Females

Fig. S5.

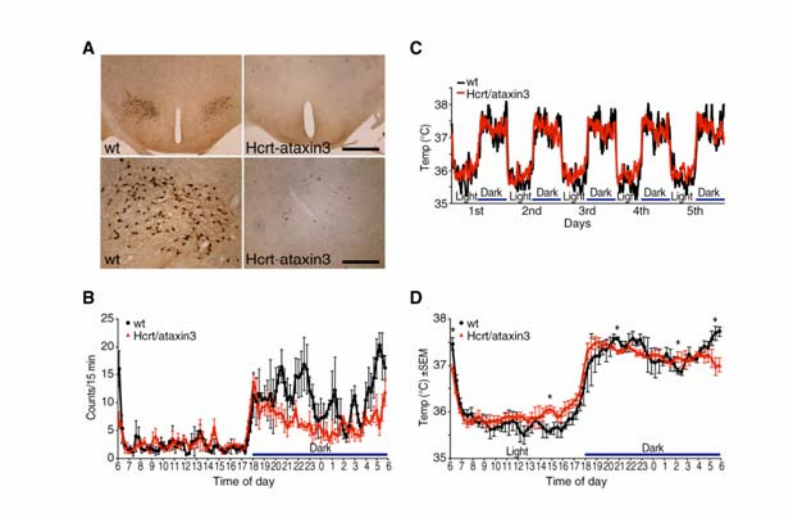
A
wt
Hcrt-ataxin3
wt
Hcrt-ataxin3
C
wt
Hcrt/ataxin3
Temp (°C)
38
37
36
35
Light
Dark
1st
2nd
3rd
4th
5th
Days
B
wt
Hcrt/ataxin3
Counts/15 min
25
20
15
10
5
0
Dark
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 0 1 2 3 4 5 6
Time of day
D
wt
Hcrt/ataxin3
Temp (°C) ±SEM
38
37
36
35
Light
Dark
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 0 1 2 3 4 5 6
Time of day

Table S1. Life table statistics

	Number animals		Survival Quartiles		Lifespan	
	Dead	Censored	Median, d	75%, d	Mean, d	Mean (s.e.)
Female						
Hcrt-UCP2	26	0	662	753	659.7	33.3
Wt	31	0	550	722	554.2	33.5
Male						
Hcrt-UCP2	53	6	805	920	786.9	24.6
Wt	36	0	716	806	695.2	34.7

d, days; s.e., standard error.

Table S2. Proportional Hazard Regression Statistics. Full model.

Effect Likelihood Ratio Tests

Source	Nparm	DF	L-R ChiSquare	Prob>ChiSq
genotype	1	1	7.884	0.0050
parent	14	14	45.07	0.0000
sex	1	1	17.62	0.0000
sex*genotype	1	1	0.0159	0.8997

Risk Ratios

Term	Risk Ratio	Lower CL	Upper CL
genotype[Hcrt-UCP2]	0.752402	0.616742	0.917286
sex[F]	1.69814	1.325591	2.181269
sex[F]*genotype[Hcrt-UCP2]	1.013092	0.826188	1.239182

Table S3. Statistics of time-dependent covariate analysis used to evaluate proportional hazards among genotypes through analysis of age as time-dependent covariate.

Males

Effect Likelihood Ratio Tests

Source	**Nparm**	**DF**	**L-R ChiSquare**	**Prob>ChiSq**
genotype	1	1	5.87254655	0.0154
age*genotype	1	1	2.67816581	**0.1017**

Risk Ratios

Term	**Risk Ratio**	**Lower CL**	**Upper CL**
genotype	0.482111	0.271421	0.867272
(age)*(genotype)	**1.002461**	0.999517	1.005468

Females

Effect Likelihood Ratio Tests

Source	**Nparm**	**DF**	**L-R ChiSquare**	**Prob>ChiSq**
genotype	1	1	3.64553351	0.0562
age*genotype	1	1	0.43429541	**0.5099**

Risk Ratios

Term	**Risk Ratio**	**Lower CL**	**Upper CL**
genotype	0.44881	0.186196	1.020948
(age)*(genotype)	**1.001445**	0.997194	1.00587

Supporting references

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2. S. Huitron-Resendiz, M. Sanchez-Alavez, D. N. Wills, B. F. Cravatt, S. J. Henriksen, *Sleep* **27**, 857 (Aug 1, 2004).
3. T. Mochizuki *et al.*, *J Neurosci* **24**, 6291 (Jul 14, 2004).