and frozen in isopentane as described[10]. Sections (10–30 μm) were prepared using a cryostat and stained with haematoxylin and eosin. Muscle fibre numbers were determined from sections taken from the widest part of the tibialis cranialis muscle. Muscle fibre sizes were measured from photographs of sections of tibialis cranialis and gastrocnemius muscles.

Carcasses were prepared from shaved 4-month-old mice by removing all of the internal organs and associated fat and connective tissue. Fat content of carcasses was determined as described[11]. For protein and DNA analysis, muscles from 11–14-week-old males were dissected free of attachments, weighed and homogenized in 150 mM NaCl, 100 mM EDTA. Protein concentrations were determined using the Biorad protein assay. DNA was isolated by adding SDS to 1%, treating with 1 mg ml^{-1} proteinase K overnight at 55 °C, extracting 3 times with phenol and twice with chloroform, and precipitating with ammonium acetate and EtOH. The samples were then digested with 40 μg ml^{-1} RNase for 1 h at 37 °C, and following proteinase K digestion and phenol and chloroform extractions, the DNA was precipitated twice with ammonium acetate and EtOH.

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Mice lacking mitochondrial uncoupling protein are cold-sensitive but not obese

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The mitochondrial uncoupling protein (UCP) in the mitochondrial inner membrane of mammalian brown adipose tissue generates heat by uncoupling oxidative phosphorylation[1]. This process protects against cold[2] and regulates energy balance[3]. Manipulation of thermogenesis could be an effective strategy against obesity[4–9]. Here we determine the role of UCP in the regulation of body mass by targeted inactivation of the gene encoding it. We find that UCP-deficient mice consume less oxygen after treatment with a β3-adrenergic-receptor agonist and that they are sensitive to cold, indicating that their thermoregulation is defective. However, this deficiency caused neither hyperphagia nor obesity in mice fed on either a standard or a high-fat diet. We propose that the loss of UCP may be compensated by UCP2, a newly discovered homologue of UCP; this gene is ubiquitously expressed and is induced in the brown fat of UCP-deficient mice.

The *Ucp* gene was inactivated by homologous recombination in embryonic stem cells (ES) with a targeting vector that replaced a *BamHI/BglII* fragment carrying exon 2 and part of exon 3 with the *neo*^{r} gene, thereby deleting an essential membrane-spanning domain[10–12] (Fig. 1). One correctly targeted D3 ES cell clone produced a chimaera that transmitted the targeted allele (*Ucp*^{tm1}) to progeny. Homozygous and heterozygous mice were recovered in the proportions expected for a single gene mutation. Northern blot analysis demonstrated that −/− mice lack *Ucp* messenger RNA of the correct size (Fig. 2a). A faint signal from RNA products, both larger and smaller, can be detected on overexposed films; however, the absence of any UCP by immunoblot analysis indicates that this RNA does not contribute to any detectable immunoreactive protein (Fig. 2b). Mice with only one intact copy (+/−) of the gene produce *Ucp* mRNA at levels comparable to wild type (+/+) mice, as expected for an inducible gene required for thermoregulation. No differences were seen in the levels of transcripts for mitochondrial-localized cytochrome oxidase, the adipocyte-specific aP2 protein (Fig. 2a), cytoplasmic or mitochondrial glycerol-3-phosphate dehydrogenases, the β3 adrenergic receptor or leptin (data not shown).

The brown adipose tissue of UCP-deficient mice has enlarged lipid vacuoles (Fig. 3), but in all other respects it appears normal, including in the shape of its mitochondria (data not shown). The increased lipid deposition is to be expected in the absence of an uncoupling mechanism to dissipate the protonmotive force across the mitochondrial membrane[13].

Given the importance of UCP in the regulation of energy expenditure in rodents and the ability of β3-adrenergic agonists to stimulate this energy expenditure through their exclusive effects on brown and white adipose tissue[14], we studied oxygen consumption in resting UCP-deficient mice before and after injection of the β3-adrenergic agonist CL 316,243. We found no significant differences in resting oxygen consumption (that is, preinjection values) between wild-type (+/+), heterozygous (+/−) and UCP-deficient (−/−) mice at 28 °C (Fig. 4a). The oxygen consumption after treatment with CL 316,243 was significantly blunted in UCP-deficient mice, however, compared to wild-type and heterozygous controls. Whereas there was an approximate doubling of resting oxygen consumption in the latter two groups, which is normal for rodents[15], in UCP-deficient mice stimulation was only 34%. These results indicate an abnormal response to β3-adrenergic stimulation, like that found for *Ucp*-DTA mice (*Ucp*-diphtheria toxin A chain transgenic mice), when the same protocol and equipment were used[7].

The effects of UCP deficiency on thermoregulation were assessed by exposing 41 (−/−) mice (23 males and 18 females, ranging in age from 27 to 85 days) to cold (5 °C). The time required to lose 10 °C of body heat was used as a measure of sensitivity to cold. Thirty-five (−/−) mice (85%) were cold-sensitive and six (−/−) mice (15%) were resistant to cold, that is, they maintained body temperature after 24 h in the cold (Fig. 4b). Within the cold-sensitive group, the onset of temperature loss varied from 1.5 to 9.5 hours and was independent of sex or age. The ten heterozygous mice tested were all resistant to cold. These results show that brown-fat thermogenesis is important for protecting both young and

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mature mice against cold. Although the role of non-shivering thermogenesis in protecting against cold was expected, the sensitivity of the majority of mature UCP-deficient mice is surprising, contrasting with the resistance to cold of *Ucp*-DTA transgenic mice in which 60–70% of brown adipose tissue had been inactivated by diphtheria toxin[7,16].

It is assumed that a role for brown fat in energy balance is determined by UCP-derived thermogenesis. Therefore, as UCP-deficient mice are more sensitive to cold than *Ucp*-DTA mice and consume less oxygen after stimulation with β3-adrenergic agonists, the UCP-deficient mice should be as, or more obese than, *Ucp*-DTA mice. We evaluated susceptibility to obesity by feeding mice with either a low-fat laboratory chow or a high-fat diet. UCP-deficient mice showed no significant increase in the development of obesity on the basis of measurements in their body mass, selected fat-pad mass or total body triglyceride content (Table 1). The mass of brown fat was significantly greater in UCP-deficient mice, presumably because of the increased deposition of triglyceride. Food intake was not significantly different in 10 determinations each on groups of 10 male +/+, +/− and −/− mice fed a laboratory chow containing 6% fat.

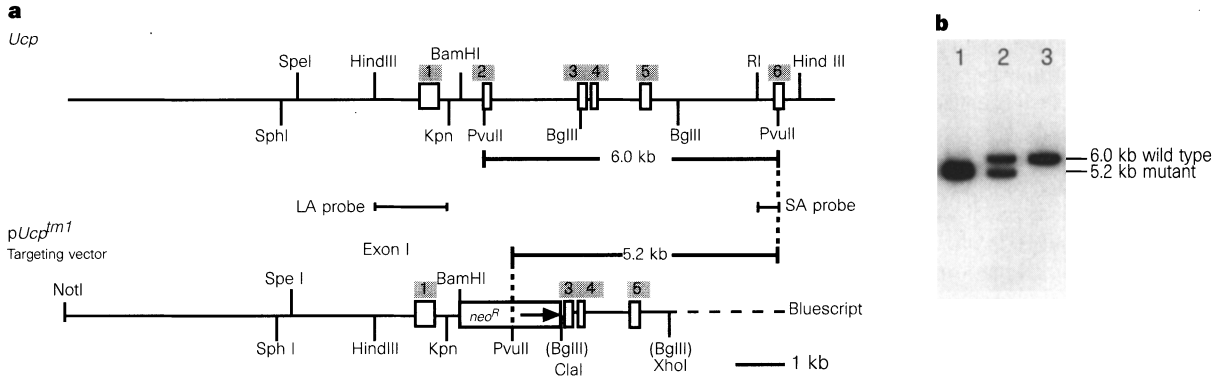

Figure 1 Targeted disruption of the *Ucp* gene. **a**, Restriction enzyme map of the *Ucp* gene showing the relevant sites and the locations of the six exons. **b**, Southern blot analysis of DNA isolated from lane 1, a mouse homozygous for the targeted allele; lane 2, a heterozygous mouse; and lane 3, a mouse homozygous for the wild-type allele. The wild-type and mutated genes gave 6.0- and 5.2-kb fragments, respectively, after *Pvu*II digestion and hybridization with the short-arm (SA) probe. *Bam*HI-cut DNA of the wild-type and targeted genes gave the same predicted-size fragment of 20 kb when hybridized with the long-arm (LA) probe and a probe for the *neo*^{r} gene hybridized only to the 5.2-kb *Pvu*II fragment (data not shown).

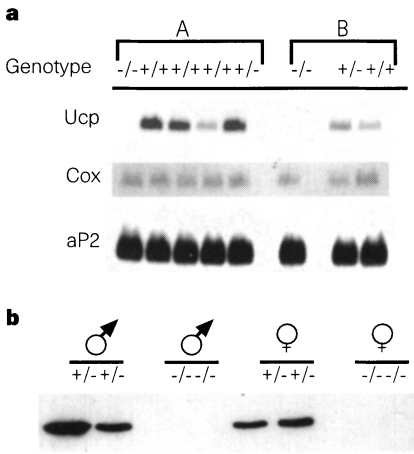

Figure 2 Targeted mice are deficient in *Ucp* mRNA and protein. **a**, Northern blot analysis of brown fat RNA from mice homozygous (−/−) and heterozygous (+/−) for the targeted allele and wild-type mice (+/+). Filters were hybridized sequentially with probes for Ucp, cytochrome oxidase (COX) and aP2 mRNA. Animals in A and B were 18 d and 35 d old, respectively. **b**, Immunoblot of UCP, showing the absence of immunoreactive UCP in −/− mice.

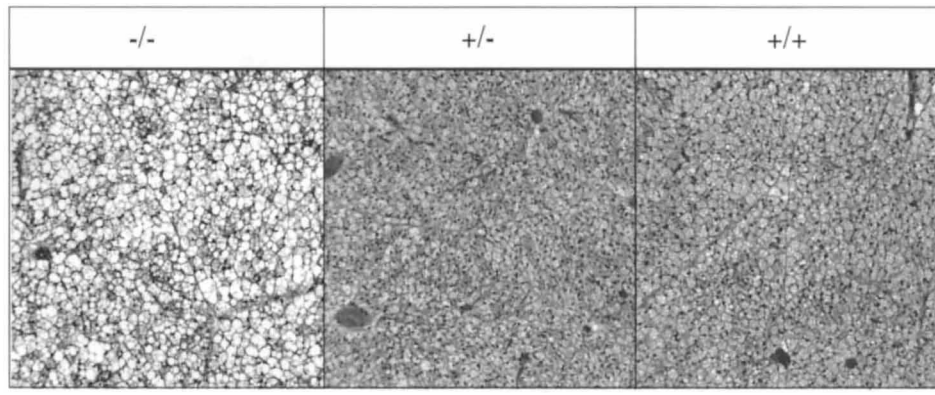

Figure 3 Histology of brown fat shows lipid accumulation in adipocytes. Interscapular brown fat was removed from 3 month-old-male mice of −/−, +/− and +/+ genotype at *Ucp* and fixed in Bouin's solution. Paraffin-embedded sections were stained with haematoxylin and eosin.

The absence of obesity in UCP-deficient mice is unexpected. UCP in mammals exists specifically to produce heat: this function provides a mechanism for controlling energy expenditure under conditions of either reduced or enhanced caloric intake and is supported by complex regulation from the central nervous system through neurohormones that modulate feeding behaviour as well as brown-fat thermogenesis[17]. The elimination of this system in *Ucp*^{tm1} mice indicates that an alternative mechanism for maintaining body mass must exist that cannot protect against the cold: the thermogenic glycerol phosphate cycle, which is more prevalent in brown fat than in other tissues[18], or *Ucp2*, a recently described homologue of the brown-fat-specific *Ucp* gene, might be involved.

The UCP2 protein shares 58% overall sequence identity with UCP and their secondary structures, as predicted from hydrophobicity plots, are extraordinarily similar; both have the nucleotide-binding domain. If *Ucp2* can become part of a compensatory mechanism for regulating energy balance in the absence of *Ucp* expression, then its expression might be upregulated in *Ucp*^{tm1} mice. Using northern blot analysis of RNA, we compared the expression of *Ucp2* in UCP-deficient mice and in normal controls. Whereas *Ucp1* is expressed exclusively in brown fat, *Ucp2* is broadly expressed in different mouse tissues, suggesting that thermogenic mechanisms involving mitochondrial proton leaks may be occurring in many tissues (Fig. 5). *Ucp2* was only upregulated in brown fat (Fig. 5a); this fivefold increase in *Ucp2* mRNA expression in the brown fat of UCP-deficient mice makes it comparable to that in white fat. As UCP-deficient mice accumulate fat in their brown adipocytes, we investigated whether the induction of *Ucp2* mRNA might be related to this phenotype. We measured *Ucp2* mRNA in the brown adipocytes of transgenic mice overexpressing the gene encoding cytoplasmic glycerol-3-phosphate dehydrogenase, because these mice also accumulate fat in their brown adipocytes[6]. *Ucp2* mRNA was also increased in these mice (data not shown), suggesting that the accumulation of fat in the adipocyte increases *Ucp2* expression. *Ucp2* is the only gene in UCP-deficient mice to show significant alterations is expression; however, this induction may be secondary to the accumulation of lipid in the cells.

The resistance in *Ucp*^{tm1} mice to developing obesity is striking. This observation is underscored by other mutations affecting brown fat thermogenesis: like *Ucp*^{tm1} animals, *Ucp*-DTA transgenic mice and mice lacking the β3-adrenergic-receptor gene both have limited energy expenditure[7,14], although it is only the *Ucp*-DTA transgene that abolishes brown fat deposition and causes obesity. A recent study showing that hyperphagia and obesity are absent when Ucp-DTA mice are reared at thermoneutrality[19] indicates that even in this model there is no obesity without hyperphagia. Accordingly, the mechanisms that conserve a normal body mass are important in mice, as they are in humans[20], so the phenotypes of thermogenesis mutants reflect the efficient maintenance of a normal body mass. As adult humans have very low brown-fat thermogenesis[21], the UCP-deficient mouse is likely to be a useful model for investigating the relation between energy balance and obesity.

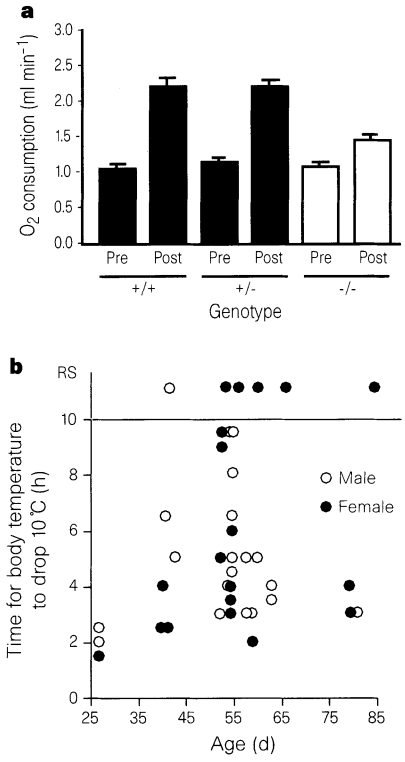

Figure 4 a, UCP-deficient mice have normal resting oxygen consumption, but show a blunted response to a β3-adrenergic agonist. Results are presented as the mean ± s.e. for the following numbers of male mice: 8 (+/+), 7 (+/−) and 7 (−/−). **b**, Thermoregulation of UCP-deficient mice is defective. Body temperature was measured every 30 min until it was reduced to between 26 to 28 °C. Males and females and both immature and mature mice were sensitive to the cold. RS, mice resistant to cold.

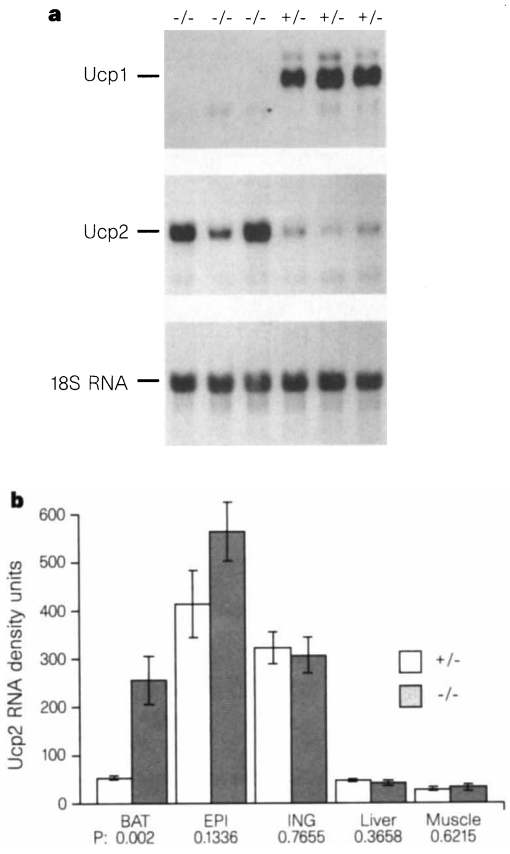

Figure 5 a, Northern blot showing that levels of *Ucp2* mRNA are increased in brown adipose tissue of mice homozygous for the allele *Ucp*^{tm1}. **b**, Bar graph showing that Ucp2 mRNA is raised only in brown adipose tissue. Data are presented as the mean ± s.e. of estimates for 6 male mice of 2–3 months old for brown adipose tissue (BAT), epididymal fat (EPI) and inguinal fat (ING), and for 3 mice for liver and muscle. *P* values were determine using the Students *t*-test with Statview 4.1.

Table 1 Body and fat-pad mass determination indicate that mice homozygous for *Ucp*^{tm1} are not obese

(**a**) Standard laboratory diet

Genotype	Sex		Body mass	BAT	RPF	GF	IF
+/−	M		29.12 ± 2.18	0.10 ± 0.01	0.14 ± 0.02	0.57 ± 0.10	0.37 ± 0.06
−/−	M		27.91 ± 1.47	0.14 ± 0.02	0.11 ± 0.03	0.40 ± 0.08	0.34 ± 0.06
		P:	0.6540	0.2035	0.4530	0.1966	0.7481
+/−	F		21.90 ± 0.86	0.07 ± 0.01	0.07 ± 0.01	0.37 ± 0.07	0.27 ± 0.04
−/−	F		21.63 ± 0.93	0.11 ± 0.01	0.08 ± 0.01	0.48 ± 0.05	0.28 ± 0.04
		P:	0.8374	0.0292	0.4342	0.2137	0.8943

(**b**) High-fat diet

Genotype	Sex		Body mass	TG (% body mass)	BAT	RPF	GF	IF	MF
+/−	M		45.77 ± 2.77	34.2 ± 1.7	0.25 ± 0.03	0.46 ± 0.04	2.51 ± 0.24	1.74 ± 0.24	0.98 ± 0.10
−/−	M		37.21 ± 2.99	29.9 ± 3.8	0.28 ± 0.06	0.32 ± 0.04	1.45 ± 0.32	1.09 ± 0.20	0.55 ± 0.10
		P:	0.0918	0.2275	0.5885	0.0649	0.0260	0.1265	0.0259
+/−	F		30.98 ± 1.93	28.4 ± 2.5	0.11 ± 0.01	0.25 ± 0.04	1.24 ± 0.22	0.90 ± 0.14	0.41 ± 0.07
−/−	F		28.92 ± 3.00	23.4 ± 4.6	0.21 ± 0.04	0.22 ± 0.07	1.20 ± 0.45	0.82 ± 0.22	0.38 ± 0.11
		P:	0.5532	0.3151	0.0279	0.7748	0.7207	0.7479	0.8184

Data are expressed as means (in g) ± s.e., except triglyceride (TG), which was assayed with a Sigma kit and is expressed as a percentage of total body mass. *P* values were calculated by using a *t*-test (Statview 4.1). Experiment A contained six mice in each group; Experiment B contained 12 (+/−) males, 5 (−/−) males, 10 (+/−) females and 7 (−/−) females. In **a**, mice were between 68 and 77 d old at the time of killing; in **b**, mice were fed standard laboratory chow until they were 2 months old and then fed on a high-fat diet (5 kcal % in fat; Research Diets, NJ) for three months until they were killed. BAT, brown fat, RPF, retroperitoneal fat; GF, gonadal fat; IF, inguinal fat; MF, mesenteric fat.

Although reduced thermogenesis does not cause obesity, more relevant to the obesity problem is that genetic alterations that enhance the levels of UCP[8,9] or futile cycling[6] prevent obesity when mice are either fed a high fat diet or carry an 'obesity' gene. Thus, strategies to reduce obesity should be aimed at enhancing thermogenesis to assist homeostasis during an obesity threat.

Note added in proof: Mice deficient in noradrenaline due to inactivation of dopamine β-hydroxylase fail to induce *Ucp* expression and are cold-sensitive but not obese[31]. □

Methods

Gene targeting. The targeting vector, p*Ucp*^{tm1}, a derivative of pPGKneobpA[22], contains an 8-kb fragment of 129/SvJ DNA from the *Not*I site in the multiple cloning site at 5′ end of the *Ucp* gene to the *Bam*HI site in intron 1, a *neo*^{r} gene under the control of the phospoglycerate kinase gene promoter, a bovine poly(A) signal and a 2-kb *Bgl*II fragment containing exons 3–5 from the *Ucp* gene. Linearized vector was electroporated into D3 embryonic stem cells[11] and G418 resistant clones analysed by Southern blot analysis. Correctly targeted clones were microinjected into the blastocysts of C57BL/6J embryos to generate chimaeras that were bred to 129/SvPas mice.

RNA analysis. Total RNA (10 μg), isolated from the brown fat of 35-day-old mice using the guanidinium thiocyanate method[23], was analysed by northern blots[24]. Hybridization probes were prepared with [^{32}P]dCTP as described[25]. Blots were hybridized with probes for the mRNAs of *Ucp*[12], subunit Vb of cytochrome oxidase[26], aP2[27], cytoplasmic and mitochondrial glycerol-3-phosphate dehydrogenase[18,28] and *Ucp2*. The sequence of an EST (accession number W89820) that encoded a protein with 58% identity to the brown fat specific UCP was submitted to Genbank by the IMAGE Consortium. This cDNA clone, Ucp2, was obtained from the American Type Culture Collection and used as a probe to isolate a full length cDNA clones from a brown adipose tissue library[18]. Comparisons of the secondary structure of brown fat specific UCP to UCP2 were made with the protein prediction programs of Kyte and Doolittle[29] available in GeneWorks.

Immunoblots. Immunoblots of UCP were carried out on mitochondrial fractions separated on SDS–PAGE as described[8] except that an antirabbit IgG horse radish peroxidase–ECL detection kit (Amersham) was used.

Physiological measurements. Oxygen consumption was measured with computerized equipment including a 1-litre chamber maintained at 28 °C, and air flow of 500 ml per min (regulated with a mass flowmeter; Brooks Instrument Division, Emerson Electric) and an oxygen analyser (Beckman Industrial Oxygen Analyzer, model 755)[30]. Mice were awake and unrestrained for the study. Resting rate of oxygen consumption was determined when the mouse was curled up and still, about 1.5 h after being placed in the chamber. The β3 agonist, CL 316,243, was dissolved in sterile saline and injected subcutaneously (1 μg per g body weight). Postinjection assessment was made ~1.0 h afterwards when the mouse was still and usually stretched out and panting.

The body temperature of mice was first measured at room temperature with an electronic thermistor equipped with a rectal probe of 1 mm diameter. Mice were then transferred to a cold room maintained at 5 °C where the body temperature was measured every hour until the rectal temperature dropped to 27 °C or for the duration of 24 h if mice were resistant to the cold.

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Thermoregulatory and metabolic phenotypes of mice lacking noradrenaline and adrenaline

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Adrenaline and noradrenaline, the main effectors of the sympathetic nervous system and adrenal medulla, respectively, are thought to control adiposity and energy balance through several mechanisms. They promote catabolism of triglycerides and glycogen[1], stimulate food intake when injected into the central nervous system[2], activate thermogenesis in brown adipose tissue[3,4], and regulate heat loss through modulation of peripheral vasoconstriction and piloerection[1]. Thermogenesis in brown adipose tissue occurs in response to cold and overeating (diet induced)[5–7], and there is an inverse relationship between diet-induced thermogenesis and obesity both in humans[8] and in animal models[9–12]. As a potential model for obesity, we generated mice that cannot synthesize noradrenaline or adrenaline by inactivating the gene that encodes dopamine β-hydroxylase. These mice are cold intolerant because they have impaired peripheral vasoconstriction and are unable to induce thermogenesis in brown adipose tissue through uncoupling protein (UCP1). The mutants have increased food intake but do not become obese because their basal metabolic rate is also elevated. The unexpected increase in basal metabolic rate is not due to hyperthyroidism, compensation by the widely expressed uncoupling protein UCP2, or shivering.

Most mice lacking the gene for dopamine β-hydroxylase ($dbh^{-/-}$ mice) die *in utero* but can be rescued by supplying the precursor L-threo-3,4-dihydroxyphenylserine (DOPS) in the maternal drinking water from embryonic day 9.5 until birth[13]. Most $dbh^{-/-}$ mice become viable adults without further treatment. There is a delay of 1–2 weeks in the rapid adolescent growth phase of the mutant mice[13], resulting in slightly lower body weight relative to controls (89%, $P = 0.003$; Fig. 1a) at 4–5 months of age, when all studies reported here were performed. The reduction in body weight is distributed equally among the major organs except for the spleen, which was ~10% larger ($P = 0.04$), and brown adipose tissue, which was over twice the weight of controls ($P = 2 \times 10^{-6}$; Fig. 1a). White adipose tissue did not differ between genotypes on a per-animal or per-body weight basis ($P = 0.36$ and $P = 0.85$, respectively; Fig. 1a). In addition, plasma leptin levels of $dbh^{-/-}$ mice ($6.43 \pm 1.43\ ng\ ml^{-1}$, $n = 10$) were similar to those of $dbh^{+/-}$ mice ($6.64 \pm 1.37\ ng\ ml^{-1}$, $n = 10$). The increase in brown adipose tissue was not due to an increase in cell number, as measured by DNA content, and the RNA/DNA ratio was normal (Fig. 1a). Histologically, the lipid vacuoles in the brown adipocytes of mutant mice were markedly enlarged compared with those of controls (Fig. 1b), suggesting that the tissue was hypoactive.

The thermogenic activity of brown adipose tissue depends largely on UCP1, which generates heat by dissipating the mitochondrial proton gradient[3,4]. The expression of UCP1 is regulated transcriptionally by β-adrenergic and thyroid-hormone stimulation[14]. There was a large increase in UCP1 mRNA in control mice at room temperature (22 °C) relative to thermoneutrality (30 °C), and placing them at 4 °C for 2 h did not increase UCP1 mRNA further (Fig. 2a), although at longer times it would (data not shown). In contrast, $dbh^{-/-}$ mice exhibited low UCP1 mRNA levels under all three conditions (Fig. 2a). Those $dbh^{-/-}$ mice with noradrenaline restored by prior administration of DOPS had normal levels of UCP1 mRNA after 2 h at 4 °C. Adrenaline is not restored, and the elevated levels of dopamine persist with the regimen of DOPS used here. A single injection of DOPS normalizes UCP1 mRNA levels

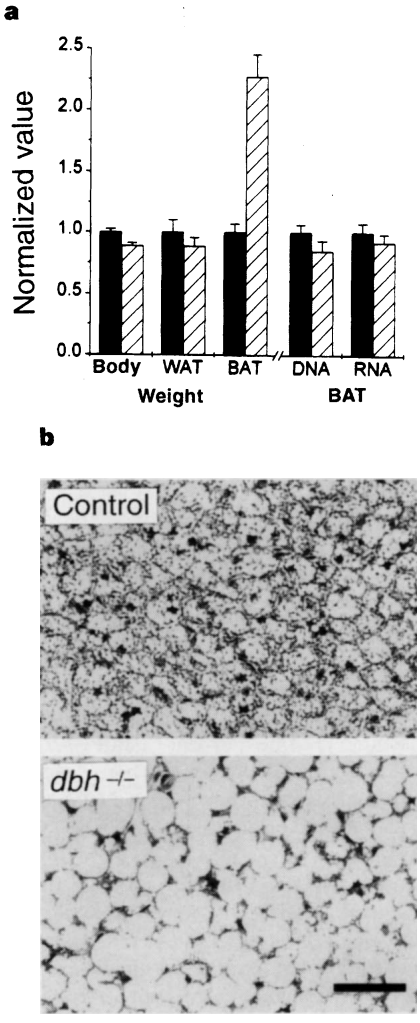

Figure 1 Hypertrophy of brown adipose tissue (BAT) in $dbh^{-/-}$ mice (hatched bars) relative to controls (black bars). **a**, BAT weight was selectively increased in the mutant mice despite having normal DNA content (DNA) and RNA/DNA (RNA). Control values normalized to 1 ($n = 11$ for each genotype) were: 31.2 g (body), 599 mg (WAT), 55 mg (BAT), 90 μg (DNA), 1.66 μg (RNA/μg DNA). **b**, Lipid vacuoles were enlarged in the BAT of the mutant mice. Scale bar, 50 μm.