Ablation of neurons expressing agouti-related protein, but not melanin concentrating hormone, in leptin-deficient mice restores metabolic functions and fertility

Qi Wu, Benjamin B. Whiddon, and Richard D. Palmiter[1]

Howard Hughes Medical Institute and Departments of Biochemistry and Genome Sciences, University of Washington, Seattle, WA 98195

Leptin-deficient ($Lep^{ob/ob}$) mice are obese, diabetic, and infertile. Ablation of neurons that make agouti-related protein (AgRP) in moderately obese adult $Lep^{ob/ob}$ mice caused severe anorexia. The mice stopped eating for 2 wk and then gradually recovered. Their body weight fell to within a normal range for WT mice, at which point food intake and glucose tolerance were restored to that of WT mice. Remarkably, both male and female $Lep^{ob/ob}$ mice became fertile. Ablation of neurons that express melanin-concentrating hormone (MCH) in adult $Lep^{ob/ob}$ mice had no effect on food intake, body weight, or fertility, but resulted in improved glucose tolerance. We conclude that AgRP-expressing neurons play a critical role in mediating the metabolic syndrome and infertility of $Lep^{ob/ob}$ mice, whereas MCH-expressing neurons have only a minor role.

diphtheria toxin | norepinephrine | neuropeptide Y | gonadotropin releasing protein | kisspeptin

The spontaneous nonsense mutation in the leptin gene that generated $Lep^{ob/ob}$ mice results in obesity, hyperphagia, diabetes, and infertility (1, 2). These phenotypes can be reversed by chronic delivery of recombinant leptin (3, 4). The neural circuitry underlying these phenotypes has been the subject of intense investigation (5–7). Mice lacking leptin have elevated levels of neuropeptide Y (NPY) and melanin-concentrating hormone (MCH) in the hypothalamus (8, 9), and genetic experiments have demonstrated that $Lep^{ob/ob}$ mice lacking either of these peptides have an improved metabolic phenotype (10, 11).

Because hypothalamic NPY-expressing neurons send projections to the vicinity of MCH-expressing neurons (12), which have electrophysiological responses to NPY (13), the two neuronal populations may be part of the same circuitry mediating the phenotype of $Lep^{ob/ob}$ mice. NPY-expressing neurons in the arcuate nucleus also express agouti-related protein (AgRP), which exerts the same orexigenic effects as NPY but by a different mechanism (14).

We take advantage of the restricted expression of AgRP and MCH to hypothalamic neurons by targeting the human diphtheria toxin (DT) receptor to the genes encoding those neuropeptides, thereby allowing their selective ablation by administration of DT (15). Ablation of AgRP-expressing neurons (AgRP neurons) in adult mice results in severe anorexia and death in ~6 d (15, 16). In this study, we applied this same methodology to MCH-expressing neurons (MCH neurons) and asked whether ablation of either of these neuronal populations in adult $Lep^{ob/ob}$ mice is beneficial (as predicted from the constitutive loss of the neuropeptides) or lethal (as predicted from the loss of AgRP neurons in a WT background).

Results

Young $Lep^{ob/ob}$ Mice Do Not Survive Ablation of AgRP Neurons. $Lep^{ob/+}$; $Agrp^{DTR/+}$ mice were bred with $Lep^{ob/+}$ mice to generate $Lep^{ob/ob}$; $Agrp^{DTR/+}$ mice, and $Lep^{ob/ob}$;$Agrp^{+/+}$ or $Lep^{ob/+}$;$Agrp^{DTR/+}$ controls. After treatment of young male and female mice weighing ~27 g with DT, all of the mice carrying the $Agrp^{DTR}$ allele (whether $Lep^{ob/ob}$ or $Lep^{ob/+}$) gradually stopped feeding and lost 20% of their body weight during the next 6 d and would have succumbed, whereas mice without the $Agrp^{DTR}$ allele maintained

their feeding and body weight (Fig. 1). Statistical analyses of our results are reported in the figure legends. Analysis of the brains of DT-treated mice at the end of the experiment revealed a virtual absence of AgRP neurons in $Agrp^{DTR/+}$ mice, as measured by immunocytochemistry or quantitative PCR for $Agrp$ transcripts (Fig. S1). We conclude that the absence of leptin does not prevent ablation of AgRP neurons by DT and does not protect these young mice from severe anorexia.

Young $Lep^{ob/ob}$ Mice Survive Ablation of MCH Neurons. Mice with the DT receptor targeted to the $Pmch$ locus, which encodes MCH, were produced as described in *Materials and Methods*. Young $Lep^{ob/ob}$; $Pmch^{DTR/+}$ mice and $Lep^{ob/+}$;$Pmch^{DTR/+}$ controls were treated with DT or saline. DT treatment did not alter food intake of $Lep^{ob/ob}$; $Pmch^{DTR/+}$ mice, but it slightly reduced the rate at which male (but not female) $Lep^{ob/ob}$;$Pmch^{DTR/+}$ mice gained weight (Fig. 2). Analysis of the brains from $Lep^{ob/ob}$;$Pmch^{DTR/+}$ mice and controls at the end of the experiment revealed that in both groups of mice, >95% of the MCH neurons were gone, as measured by immunocytochemistry or quantitative PCR for $Pmch$ transcripts (Fig. S2). DT treatment of similarly aged control mice lacking the DT receptor had no effect on body weight, food intake, or glucose tolerance. Older, more obese (35–45 g body weight) $Lep^{ob/ob}$;$Pmch^{DTR/+}$ mice also survived DT treatment, with a modest effect on body weight and improvement in glucose tolerance. This experiment reaffirms that DT-mediated ablation of neurons with the attendant gliosis does not promote anorexia; thus, the effects of AgRP neuron ablation are likely specific to that neuronal population.

Moderately Obese $Lep^{ob/ob}$;$Agrp^{DTR/+}$ Mice Survive AgRP Neuron Ablation. We previously demonstrated that mice can adapt to the loss of AgRP neurons, but this adaptation takes ~10 d (16). Thus, we tested the hypothesis that older, more obese $Lep^{ob/ob}$ mice also might be able to adapt by using their greater energy reserves. Moderately obese (35–40 g body weight) $Lep^{ob/ob}$;$Agrp^{+/+}$ and lean (20–25 g) $Lep^{ob/+}$;$Agrp^{DTR/+}$ controls were treated with DT. The lean control mice gradually stopped eating, lost 20% of their body weight, and became moribund over the subsequent 6 d, in agreement with previous results. However, both male and female $Lep^{ob/ob}$; $Agrp^{DTR/+}$ mice weighing 35–40 g ceased eating within 6 d, and did not eat over the next 12 d. During that time, their body weight fell to ~22–25 g. They then began to eat again at a rate similar to that of WT mice, and their body weight gradually increased but remained within the normal range (Fig. 3). The obese mice ($Lep^{ob/ob}$;$Agrp^{+/+}$)
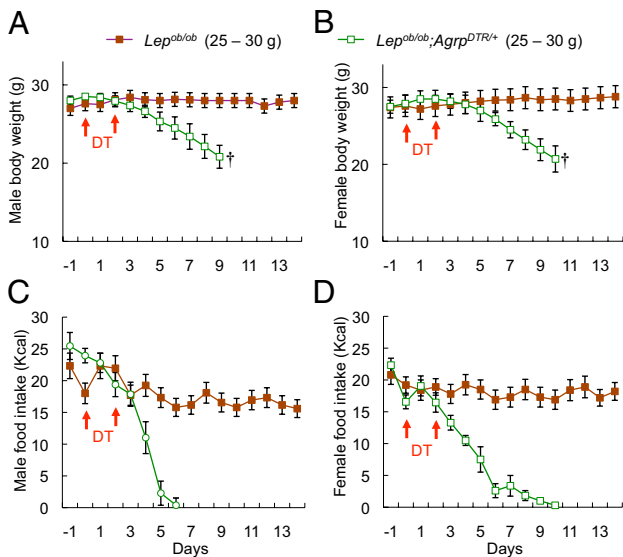
Fig. 1. Ablation of AgRP neurons in young, leptin-deficient mice leads to anorexia. (*A* and *B*) Body weight of DT-treated male (*A*) and female (*B*) $Lep^{ob/ob}$ mice (~6 wk old, 25–30 g) and $Lep^{ob/ob};Agrp^{DTR/+}$ mice (~6 wk old, 25–30 g). (*C* and *D*) Daily food intake by the mice described in *A* and *B*. All mice were raised on standard chow pellets. Error bars represent SEM. $P < 0.01$, ANOVA on body weight and food intake between the two groups (days 5–9). [†]Mice removed from the study because they became moribund.

that did not express the DT receptor were unaffected by DT administration (Fig. 3).

Improved Glucose Homeostasis After Ablation of AgRP or MCH Neurons.

$Lep^{ob/ob}$ mice become diabetic as they gain weight, exhibiting hyperglycemia, hyperinsulinemia, and glucose intolerance (17).

By 26 d after ablation of AgRP neurons in female and male $Lep^{ob/ob};Agrp^{DTR/+}$ mice weighing 35–40 g, fasting blood glucose levels had returned to normal. Their response to glucose tolerance testing was not statistically different from that of $Lep^{ob/+}$ controls and significantly better than that of saline-treated $Lep^{ob/ob};Agrp^{DTR/+}$ mice (Fig. 4 *A* and *B*). Ablation of MCH neurons in the $Lep^{ob/ob};Pmch^{DTR/+}$ mice also improved glucose tolerance in both females and males compared with saline-treated obese controls (Fig. 4 *C* and *D*). This improvement was independent of a decrease in body weight, unlike that occurring after ablation of AgRP neurons.

Restoration of Fertility to $Lep^{ob/ob}$ Mice After Ablation of AgRP but Not MCH Neurons.

Male $Lep^{ob/ob}$ mice occasionally sire a litter when they are young, but female $Lep^{ob/ob}$ mice are infertile (3, 6). The fertility of DT-treated mice was tested by placing individual males and females with WT mice of the opposite sex and recording the birth and size of litters. Both male (5/5) and female (6/6) $Lep^{ob/ob};Agrp^{DTR/+}$ mice weighing 35–40 g became fertile after ablation of AgRP neurons; some of the males sired several litters, and some females bore more than one litter within 4 mo after DT treatment (Table 1). Based on these data, we calculate that the hypothalamic-pituitary-gonadal axis becomes active within 10–16 d after the first DT injection in female leptin-deficient mice and 11–23 d in male leptin-deficient mice (*Discussion*). Table 1 also shows that the $Lep^{ob/ob};Agrp^{DTR/+}$ males used in these breeding experiments had a mean body weight of 31.4 g, compared with ~55 g in untreated male $Lep^{ob/ob};Agrp^{DTR/+}$ mice of the same age. Thus, the ablated mice remained relatively lean for several months.

The fertility of $Lep^{ob/ob};Pmch^{DTR/+}$ mice that had been treated with DT was tested as well. Only one of nine males sired a litter, but none of the six females became pregnant. Therefore, ablation of MCH neurons did not improve the fertility of $Lep^{ob/ob};Pmch^{DTR/+}$ mice. Ablation of MCH neurons in $Lep^{ob/+};Pmch^{DTR/+}$ controls did not disrupt fertility.
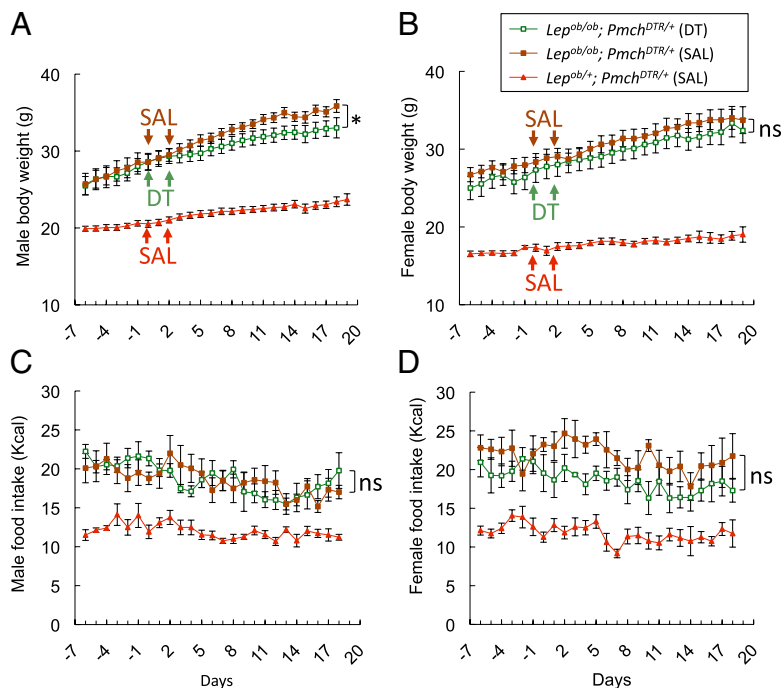

Fig. 2. Body weight and food intake after ablation of MCH neurons in $Lep^{ob/ob}$ mice. (*A* and *B*) Body weight was measured in obese male (*A*) and female (*B*) $Lep^{ob/ob};Pmch^{DTR/+}$ and lean $Lep^{ob/+};Pmch^{DTR/+}$ mice that had been injected with either saline (SAL) or DT on the days indicated by arrows. (*C* and *D*) Daily food intake by the mice described in *A* and *B*. Error bars represent SEM ($n = 9–11$ per group). *$P = 0.03$ for the interaction of time and DT treatment in two-way repeated-measures ANOVA. NS, not significant. $P > 0.05$.
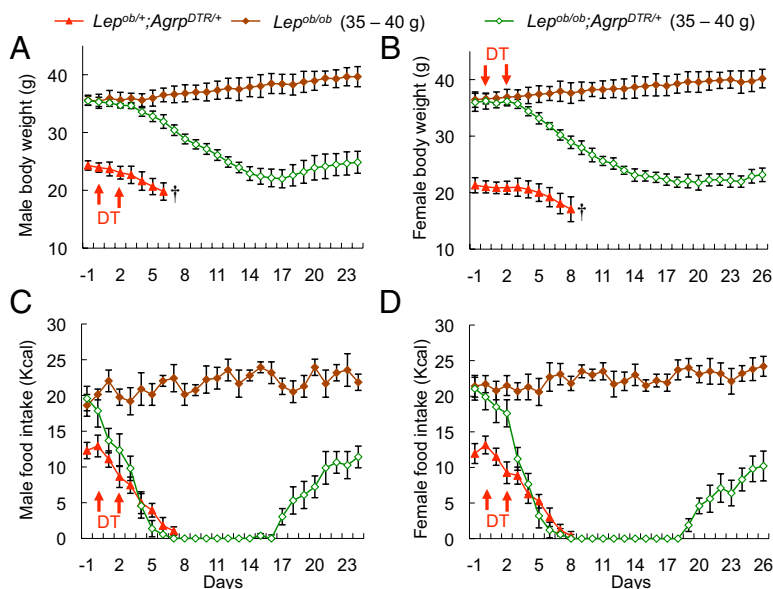
Fig. 3. Moderately obese leptin-deficient mice survived acute ablation of AgRP neurons and exhibited normal body weight with significantly reduced food intake. (*A* and *B*) Body weight of DT-treated male (*A*) and female (*B*) mice from three groups (1) $Lep^{ob/+};Agrp^{DTR/+}$ mice (~8 wk old, 20–25 g); (2) $Lep^{ob/ob}$ mice (~8 wk old, 35–40 g); and (3) $Lep^{ob/ob};Agrp^{DTR/+}$ mice (~8 wk old, 35–40 g). (*C* and *D*) Daily food intake by the mice described in *A* and *B*. All mice were raised on standard chow pellets. Error bars represent SEM. $P < 0.01$, ANOVA on body weight and food intake between group 2 and group 3 (days 5–24). [†]Mice removed from study because they became moribund.

Severely Obese $Lep^{ob/ob}$ Mice Do Not Survive Ablation of AgRP Neurons. We also ablated AgRP neurons in $Lep^{ob/ob}$ mice that weighed > 40 g; however, unlike the moderately obese mice, these >40 g mice became moribund despite their greater energy reserves. Like the lighter male and female $Lep^{ob/ob};Agrp^{DTR/+}$ mice, they stopped eating after ~7 d and began to lose weight; however, they appeared to be unhealthy, and thus the experiment was terminated (Fig. 5 *A–D*). One distinguishing feature of these older, more obese mice is that their body temperature plummeted to slightly above ambient temperature as they became moribund; in comparison, the body temperature of the $Lep^{ob/ob};Agrp^{DTR/+}$ mice weighing <40 g dropped slightly but then returned to that of the $Lep^{ob/ob}$ mice (Fig. S3). $Lep^{ob/ob};Agrp^{DTR/+}$ mice weighing >40 g and that were pair-fed with the group that received DT survived and did not exhibit a drop in body temperature. Thus, the drop in body temperature in the severely obese mice appeared to be a consequence of AgRP neuron ablation, not of reduced food intake.

We suspected that decreased output of the sympathetic nervous system (SNS) might contribute to the mortality of these severely obese mice. Thus, before ablation of AgRP neurons in $Lep^{ob/ob};Agrp^{DTR/+}$ mice weighing ~55 g, we implanted osmotic minipumps to deliver norepinephrine, one of the principle neurotransmitters of the SNS, to potentially promote thermogenesis by brown adipose tissue, stimulate vasoconstriction to conserve heat, and enhance lipolysis (18). This norepinephrine treatment prevented the severe drop in body temperature (Fig. 5*F*) and prolonged the survival of the obese mice after AgRP neuron ablation (Fig. 5*E*) compared with control obese mice that received only the vehicle.

Discussion

Loss of leptin or its receptor results in a severe, early-onset obesity phenotype associated with hyperphagia, diabetes, hypothermia, and infertility in rodents and humans (2, 19). Genetic experiments have established that the lack of leptin signaling in several different neuronal populations in the hypothalamus and elsewhere in the brain contribute to various aspects of the metabolic phenotype (5–7). AgRP neurons express leptin receptors, but their selective removal from AgRP neurons has minimal effects on body weight

or metabolism and does not perturb fertility (20). The dramatic reversal of the metabolic phenotype of $Lep^{ob/ob}$ mice and the restoration of fertility described here suggest that these deleterious effects of leptin deficiency are mediated to a large extent by AgRP neurons. $Lep^{ob/ob}$ mice constitutively lacking MCH also have an
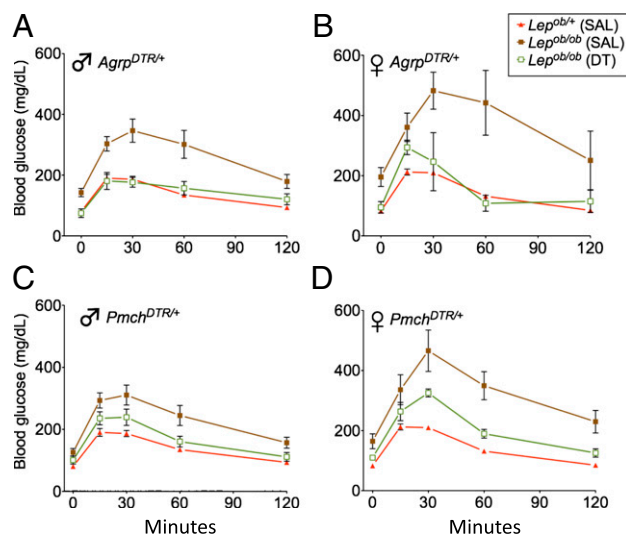

Fig. 4. Glucose tolerance tests after ablation of AgRP or MCH neurons. (*A* and *B*) Obese $Lep^{ob/ob}$ and lean $Lep^{ob/+}$ mice carrying the $Agrp^{DTR}$ allele were injected with either saline (SAL) or DT at 2 wk before glucose tolerance testing. Both males (*A*) and females (*B*) were tested. Error bars represent mean ± SEM (*n* = 5–11 per group). Area under the curve (AUC) measurements revealed significantly lower values in mice that received DT compared with obese $Lep^{ob/ob}$ controls ($P < 0.002$, one-way ANOVA with Dunnett's posttest), but not significantly greater values than those in leptin-replete, lean mice. (*C* and *D*) Glucose tolerance tests were performed as in *A* and *B*, but with the $Pmch^{DTR}$ allele instead of the $Agrp^{DTR}$ allele present. Error bars represent mean ± SEM (*n* = 3–8 per group). $P < 0.004$ for AUC analyses.

Table 1. Restoration of fertility in *Lep^ob/ob^;Agrp^DTR/+^* mice after ablation of AgRP neurons

Sex	DT treatment starts		Mating starts		When first litter born			When second litter born		
	Day	Body weight, g	Day	Body weight, g	Day	Body weight, g	Litter size	Day	Body weight, g	Litter size
Male	1	38.6	43	24.4	78	28.1	5	106	32.8	6
	1	37.4	43	24.7	66	27.5	3	88	30.8	7
	1	35.4	43	26	73	28.5	4	99	32.2	7
	1	35.5	39	22.3	76	25.6	6	103	28.7	7
	1	35.1	39	23.9	66	28.1	6	93	32.5	7
	Average	36.4		24.3	71.8	27.6	4.8	97.8	31.4	6.8
	SD	±1.5		±1.3	±5.6	±1.2	±1.3	±7.3	±1.7	±0.4
Female	1	35.4	39	25.1	64	ND	3	90	ND	5
	1	37.2	39	28.6	68	ND	3	94	ND	5
	1	35.9	39	27.4	70	ND	4	93	ND	6
	Average	36.2		27.0	67.3		3.3	92.3		5.3
	SD	±0.9		±1.8	±3.1		±0.6	±2.1		±0.6

Results shown are for those mice (35–40 g; males and females) where a complete record of body weight and breeding record were obtained. Three additional females were fertile. After a litter was born, the original mates and pups were removed, and fresh mates were provided to score continued fertility for a total of 120 d after DT treatment. One of the males sired a third litter within 120 d.

improved metabolic phenotype (11), similar to that of mice lacking NPY (10). Thus, we anticipated that ablating MCH neurons in adult *Lep^ob/ob^* mice also would attenuate the metabolic phenotype and provide evidence that AgRP and MCH neurons could be part of the same circuitry mediating the deleterious effects of leptin deficiency. However, ablation of MCH neurons had no effect on feeding or fertility, but did slightly improve body weight in male mice and glucose tolerance in both sexes, in agreement with previous results (11). Thus, the beneficial effects of ablating AgRP neurons appear to be largely independent of their potential regulation of MCH neurons.

We initially assumed that *Lep^ob/ob^* mice weighing 35–40 g survived AgRP neuron ablation because these mice could live off their energy reserves, thereby allowing sufficient time for adaptation to occur. However, *Lep^ob/ob^* mice with greater energy reserves (weight >40 g) succumbed. We noticed that the heavier *Lep^ob/ob^* mice demonstrated a dramatic drop in body temperature at ~1 wk after ablation that was not observed in the leaner mice. Perhaps the phenotype of *Lep^ob/ob^* mice becomes more severe with increasing age or body weight and eventually becomes irreversible. The output of the sympathetic nervous system is important for stimulating lipolysis and regulation of body temperature (21, 22) and deteriorates with leptin deficiency (21). Thus, we hypothesized that the inability to mobilize energy reserves and escape from torpor when AgRP neurons are ablated in older *Lep^ob/ob^* mice might account for their morbidity. Consistent with this hypothesis, chronic infusion of norepinephrine during the ablation period prevented the severe loss of body temperature and allowed the more obese *Lep^ob/ob^* mice to survive longer. Body temperature was maintained in the obese *Lep^ob/ob^* mice that were pair-fed with the AgRP neuron-ablated group and survived, indicating that the loss of AgRP neurons exacerbates the dysregulation of sympathetic nervous system with severe obesity. The loss of AgRP, NPY, and/or GABA signaling by AgRP neurons may lead to excessive activity of postsynaptic cells in the paraventricular nucleus or other upstream neurons that regulate sympathetic output (23, 24).

The restoration of fertility in *Lep^ob/ob^* mice after AgRP ablation is remarkable. We focused on females because they are completely infertile, whereas young male *Lep^ob/ob^* mice manifest limited fertility, especially on a calorie-restricted diet (3). Leptin replacement (3) or treatment with gonadotropins and progesterone (25) can partially restore fertility to female *Lep^ob/ob^* mice. We anticipated seeing some effects of AgRP neuron ablation based on a previous observation of improved glucose regulation and fertility in *Lep^ob/ob^* mice lacking NPY (10); however, because NPY is widely expressed, we could not attribute these effects to loss of NPY signaling from

AgRP neurons. Diet-induced obesity impairs fertility (26); thus, the loss of body weight induced by AgRP neuron ablation might help restore fertility in *Lep^ob/ob^* mice (26). However, other explanations are possible as well, given that fertility can be maintained in obese *Lep^ob/ob^* mice without reducing body weight by impairing STAT3 signaling through the leptin receptor (27).

Fertility depends on the pulsatile release of gonadotropin-releasing hormone (GnRH) into the portal system to stimulate follicle-stimulating hormone and luteinizing hormone production and release by the pituitary, resulting in the stimulation of oocyte maturation and ovulation (28). The neuropeptide kisspeptin stimulates GnRH release (29), and loss of kisspeptin signaling impairs fertility (30; but see ref. 31). Infertility of *Lep^ob/ob^* mice is associated with low expression of kisspeptins, low circulating gonadotropins, and dysregulation of GnRH release, but leptin does not appear to act directly on the neurons that produce kisspeptin or GnRH (32–34). Thus, the ability of leptin to hasten puberty and restore fertility to *Lep^ob/ob^* mice might be mediated by its actions on neurons that regulate kisspeptin- and/or GnRH-releasing neurons. AgRP neurons are not necessary for fertility (35); nevertheless, their hyperactivity might prevent fertility, as occurs in *Lep^ob/ob^* mice (36–38). Elevated release of AgRP, NPY, and/or GABA by AgRP neurons may be responsible for suppression of the gonadotropic axis (10, 16).

Murine gestation takes ~20 d, and it takes at least 14 d for folliculogenesis to give rise to oocytes ready to ovulate (39); therefore, adequate gonadotropin production in females probably commences ~34 d earlier than the birth of pups. Production of mature sperm starting from spermatogonial stem cells takes ~35 d (40); thus, adequate gonadotropin production in males probably occurs ~55 d before the birth of pups. Therefore, we estimate that hypothalamic-pituitary-gonadal axis activation occurs within 10–16 d after the first DT injection in female leptin-deficient mice and within 11–23 d in male leptin-deficient mice.

Ablation of AgRP neurons in adult mice leads to severe anorexia, resulting in starvation within 1 wk (15). Our previous experiments demonstrated that this lethal phenotype results from the loss of GABA signaling in the parabrachial nucleus and resulting hyperactivity of neurons in this brain region (16), rather than from the resulting activation of melanocortin signaling (41). Adult mice can adapt to the loss of AgRP neurons and resume feeding if the hyperactivity within the parabrachial nucleus is prevented either pharmacologically or genetically during a critical period of ~10 d (16). Ablation of AgRP neurons before they become functionally mature also allows adaptation (15). Here we demonstrate another paradigm that allows adaptation. Mice with sufficient energy reserves and the ability to mobilize these reserves can survive on
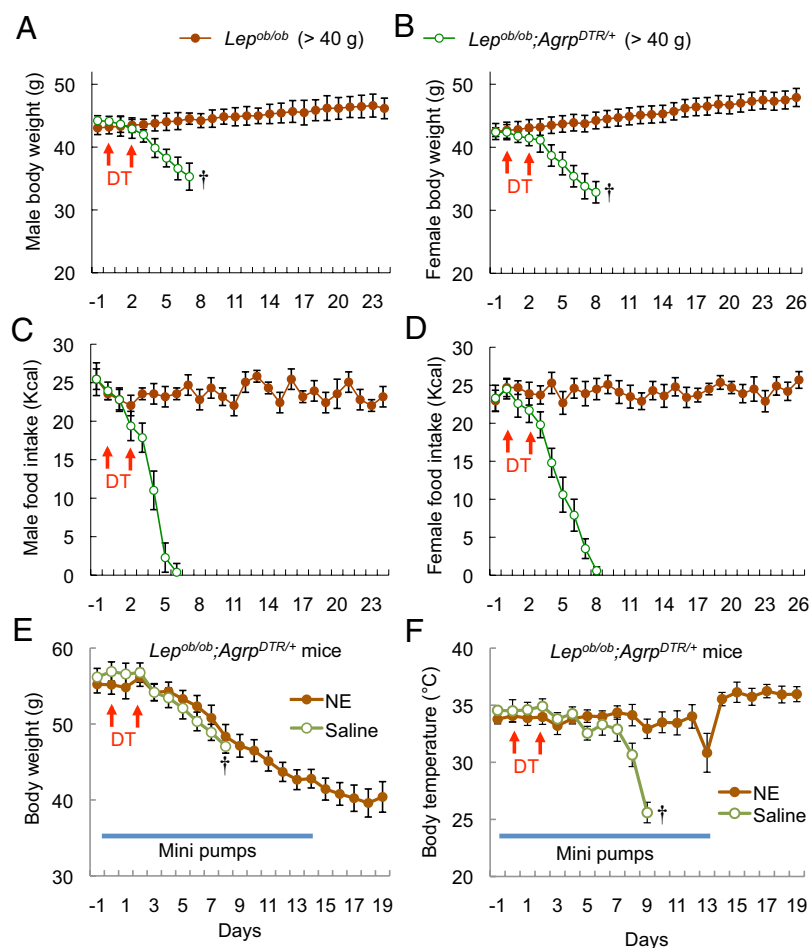
Fig. 5. Severely obese leptin-deficient mice do not survive ablation of AgRP neurons due to hypothermia that can be prevented by chronic treatment with norepinephrine. (*A* and *B*) Body weight of DT-treated male (*A*) and female (*B*) $Lep^{ob/ob}$ mice (~10 wk old, >40 g) and $Lep^{ob/ob};Agrp^{DTR/+}$ mice (~10 wk old, >40 g). (*C* and *D*) Daily food intake by the mice described in *A* and *B*. All mice were raised on standard chow pellets. Error bars represent SEM. $P < 0.01$, ANOVA on body weight and food intake between the two groups (days 4–8). (*E*) Body weight of DT-treated $Lep^{ob/ob};Agrp^{DTR/+}$ mice (~14 wk old, ~55 g) subjected to chronic administration of norepinephrine (1.5 mg/kg/d) through a 14-d osmotic pump implanted s.c. (*F*) Body temperature of the mice described in *E*. [†]Mice removed from the study because they became moribund.

them while the adaptation process occurs. Because AgRP neurons are hyperactive in $Lep^{ob/ob}$ mice, we suspect that loss of GABA signaling after their ablation also leads to hyperactivity within the parabrachial nucleus, but the mice can endure the complete anorexia of 12-d duration by mobilizing their adipose reserves. After the $Lep^{ob/ob}$ mice adapt to the loss of AgRP neurons, their phenotype resembles that of normal mice, as reflected by their feeding, body weight, glucose metabolism, and fertility.

Materials and Methods

Animals and DT Treatment. All animal experiments were approved by the University of Washington's Animal Care and Use Committee. $Lep^{ob/+}$ mice on a C57BL/6 background were obtained from Jackson Laboratories. $Agrp^{DTR}$ mice were produced and bred onto a C57BL/6 genetic background as described previously (15). $Pmch^{DTR}$ mice were generated by PCR amplifying a 360-bp region 5′ of the initiation codon using primers that introduced a unique Kpn1 site at the initiation codon. This fragment was cloned into a targeting vector that contained $PgkDTa$ and HSV-TK genes for negative selection and frt-flanked $SvNeo$ for positive selection. The 5′ arm was extended to ~9 kb by insertion of an XbaI fragment, after which a 4-kb 3′ arm was added. After linearization, the targeting construct was electroporated into G4 ES cells. Two positive colonies (out of 44) were identified by Southern blot analysis of ES cell DNA cut with Kpn1 using a probe just outside of the 5′ arm. One clone that was injected in C57BL/6 hosts gave germline transmission. These mice were bred with FLper mice to remove the frt-flanked $SvNeo$ gene. Mice carrying the $Pmch^{DTR}$ allele were subsequently identified by PCR using one primer in $Pmch$ promoter and

one primer in $hDTR$. These mice were backcrossed to C57BL/6 mice for more than six generations before being bred with $Lep^{ob/+}$ mice. $Lep^{ob/ob};Agrp^{DTR/+}$ and $Lep^{ob/ob};Pmch^{DTR/+}$ mice were generated by double-heterozygote crosses.

Before behavioral experiments, mice were maintained on standard laboratory chow (5053; Lab Diet) that was available ad libitum. When the $Lep^{ob/ob};Agrp^{DTR/+}$ and $Lep^{ob/ob};Pmch^{DTR/+}$ mice were ~6 wk old (weighing 20–25 g), ~8 wk old (weighing 35–40 g), or ~10 wk old (weighing 40–45 g), they were individually housed and switched to another standard chow diet (D12450B; Research Diets) for 7 d before being injected with DT. To ablate AgRP or MCH neurons, DT was injected twice per mouse (i.m., 2 d apart; List Biologicals). Body weight and food intake were recorded daily for several weeks. The DT dose was determined based on body weight at the time of injection, with 50 μg/kg for mice weighing <40 g and 40 μg/kg for mice weighing >40 g. For some experiments, temperature sensor chips (IPTT-300; Bio Medic Data Systems) were inserted s.c. to allow daily recording of body temperature. Experiments were terminated if the body weight of leptin-deficient mice fell to 80% of that of age-matched $Lep^{ob/+}$ littermates or if the leptin-deficient mice appeared moribund.

Fertility was assessed by housing each male DT-treated mouse with two C57BL/6 females (2 mo old) and each DT-treated female with a WT C57BL/6 male (2 mo old). The dates on which litters were born, litter sizes, and body weights of the parents were recorded. Original C57BL/6 mates and litters were removed before fresh mates were provided to score continued fertility.

Minipump Delivery of Norepinephrine. At 3 d before DT administration, osmotic pumps (Alzet model 1002; Durect) were loaded with norepinephrine (30 mM; Sigma-Aldrich) and ascorbate (2%; Sigma-Aldrich) to minimize oxidation and implanted under the skin along with a temperature-monitoring chip (IPTT-300;

Bio Medic Data Systems). Norepinephrine was delivered continuously at a rate of 1.5 mg/kg/day for 14 d until the pumps were depleted.

Glucose Tolerance Tests. At various times before and after DT treatment, glucose tolerance testing was performed by fasting mice overnight for 16 h, then sampling blood from the tail vein at 0, 15, 30, 60, and 120 min after the i.p. injection of D-glucose (1 g/kg). Blood glucose was measured with a FreeStyle Lite glucometer (Abbott Laboratories).

Determining the Extent of AgRP or MCH Neuron Ablation. At the end of the experiment, mice were perfused transcardially with 4% paraformaldehyde and brains were collected to evaluate the extent of AgRP or MCH neuron ablation by one of two methods. In one method, frozen sections (25 μm thick) containing appropriate hypothalamic regions were immunostained with antibodies against AgRP (rabbit anti-AgRP, 1:2,000 dilution; Phoenix Pharmaceuticals), NPY (rabbit anti-NPY, 1:1,000 dilution; Peninsula Laboratories), or MCH (rabbit anti-MCH, 1:1,000 dilution; Phoenix Pharmaceuticals),

followed by Cy2- or Cy3-conjugated secondary antibodies (1:300 dilution; Jackson ImmunoResearch). Then the number of positive cells per section (five sections per mouse) was compared with that in control mice. Alternatively, the hypothalamus was dissected from fixed brain, RNA was isolated using a High Pure FFPE RNA Micro Kit (Roche) and reverse-transcribed with a Brilliant II One-Step SYBR Green QRT-PCR Kit (Stratagene) according to the manufacturer's protocol, and quantitative real-time PCR (Mx3000P; Stratagene) was performed using primers specific for *Agrp* or *Pmch* mRNA, with *Actb* as an internal control. The abundance of *Agrp* or *Pmch* mRNA was calculated relative to that of *Actb* and compared with that in untreated control mice.

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