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 41. We thank J. K. Elmquist, C. A. Walsh, V. L. Sheen and R. H. Friedel for critical reading of the manuscript and helpful comments; A. C. Budde for help with the gold thioglucose experiment; and K.-F. Storch for many valuable discussions during the course of this work. Confocal microscope analyses were carried out at the Harvard Center for Neurodegeneration and Repair. This work was supported by grant DKR3728082 from the NIH (J.S.F.).

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Material and Methods

Figs. S1 to S7

References

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NPY/AgRP Neurons Are Essential for Feeding in Adult Mice but Can Be Ablated in Neonates

Serge Luquet, Francisco A. Perez, Thomas S. Hnasko, Richard D. Palmiter*

Hypothalamic neurons that express neuropeptide Y (NPY) and agouti-related protein (AgRP) are thought to be critical regulators of feeding behavior and body weight. To determine whether NPY/AgRP neurons are essential in mice, we targeted the human diphtheria toxin receptor to the *Agrp* locus, which allows temporally controlled ablation of NPY/AgRP neurons to occur after an injection of diphtheria toxin. Neonatal ablation of NPY/AgRP neurons had minimal effects on feeding, whereas their ablation in adults caused rapid starvation. These results suggest that network-based compensatory mechanisms can develop after the ablation of NPY/AgRP neurons in neonates but do not readily occur when these neurons become essential in adults.

The arcuate nucleus (ARC) of the hypothalamus is a site of convergence of central and peripheral signals of energy stores, and it contains at least two distinct populations of neurons that are critically involved in the regulation of body weight (1–3). Orexigenic neuropeptide Y/agouti-related protein (NPY/AgRP) neurons and anorexigenic pro-opiomelanocortin (POMC) neurons respond to circulating satiety and hunger signals, including glucose, leptin, insulin, ghrelin, and peptide YY (4, 5). Both populations exert an inhibitory tone onto each other, and they also send dense projections to other hypothalamic areas, including the paraventricular nucleus (PVN), zona incerta, perifornical area, and lateral hypothalamic area (6, 7). POMC neurons reduce food intake and increase energy expenditure by releasing α -

melanocyte-stimulating hormone (α MSH), a product of POMC processing, which activates melanocortin-4 receptors (MC4R). NPY/AgRP neurons have the opposite effects, inhibiting POMC neurons and antagonizing the action of α MSH on MC4R-bearing cells via the release of AgRP (a natural antagonist of α MSH) (8). Despite the fact that intracranial injection of either NPY or AgRP stimulates robust feeding in rodents (1–3), mutations that prevent the expression of AgRP, NPY, or various receptors for NPY have little impact on feeding behavior (3, 9–11). In contrast, mutations that prevent production of leptin, leptin receptor, POMC, or MC4R lead to obesity in mice and other species (12–17). These observations raise the question of whether signaling by NPY, AgRP, or any other transmitter made by these cells is important for the regulation of body weight.

To assess whether NPY/AgRP neurons are essential for feeding, we adopted a “toxin receptor–mediated cell knockout” strategy (18) to specifically ablate these neurons in a temporally controlled manner (19). Because *Agrp*

gene expression is restricted to NPY/AgRP neurons in the brain (20, 21), we targeted the expression of the human diphtheria toxin receptor cDNA (*DTR*) to the *Agrp* locus in embryonic stem cells and generated *Agrp*^{DTR/+} mice that express the human *DTR* in NPY/AgRP neurons (fig. S1). In situ hybridization revealed that human *DTR* mRNA was expressed in the ARC of *Agrp*^{DTR/+} mice but not in controls (fig. S2).

Neonatal ablation of NPY/AgRP neurons was performed by injecting 1-day-old *Agrp*^{DTR/+} and control *Agrp*^{+/+} pups (genotype unknown at time of injection) with diphtheria toxin (DT) at 50 μ g of DT per kg mouse (μ g/kg) (subcutaneous), a dose tolerated by controls (18, 21). After 9 weeks, all mice were fasted for 2 days to increase NPY and AgRP expression before they were killed (22). Brains were fixed, sectioned, and analyzed for NPY expression by immunohistochemistry. DT injection reduced the number of NPY-positive cells in the ARC by ~85% (*Agrp*^{DTR/+} mice had 9.7 ± 0.9 neurons per section, $n = 5$ mice; controls had 78 ± 2 neurons per section, $n = 3$, $P < 0.001$) (Fig. 1, A to D). There was a concomitant reduction of NPY fibers in the PVN (Fig. 1, E and F), but NPY-expressing cells outside the ARC were spared (fig. S3). AgRP staining in the ARC and PVN was also reduced after DT treatment in *Agrp*^{DTR/+} mice (fig. S3). The integrity of POMC neurons was demonstrated by using antibodies to adrenocorticotrophic hormone (ACTH), another peptide product of POMC (Fig. 1, G and H). The loss of NPY/AgRP cells and the retention of POMC cells in the ARC was also documented by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) of *Agrp* and *Pomc* mRNA (Fig. 1I).

If NPY/AgRP neurons are critical regulators of energy balance, then their ablation should negatively affect food intake and body weight. However, when newborn pups generated from a cross of *Agrp*^{DTR/+} and *Agrp*^{+/+} mice were injected with DT and their body weights recorded starting at weaning, there

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was only a slight (~11%) reduction in the body weight of *AgRP^{DTR/+}* mice compared with controls (fig. S4). Food consumption by 9-week-old littermates was monitored using “lickometer” cages that dispensed water and liquid food. The number of licks and total food consumption were the same for both groups of mice, either before or after a 12-hour fast (Fig. 2A). At the end of each experiment, the depletion of NPY immunoreactivity in the ARC was verified. Similar results were obtained when either *AgRP^{DTR/+}* or *AgRP^{DTR/DTR}* neonatal mice (up to 8 days old) were injected either once or twice with DT at 50 $\mu\text{g}/\text{kg}$ (i.e., the survival of DT-injected mice to adulthood was independent of genotype, $n > 100$). These results indicate that the majority (approaching 100% in some cases) (fig. S3) of NPY/AgRP cells in the ARC can be ablated in neonatal mice with little impact on food consumption or body weight.

We also examined the effects of administering DT to adult *AgRP^{+/+}*, *AgRP^{DTR/+}*, or *AgRP^{DTR/DTR}* mice (table S1). Adult mice were allowed to acclimate to lickometer cages for several days and then two intraperitoneal (ip) injections of DT (50 $\mu\text{g}/\text{kg}$, 3 days apart) were administered. There was an irreversible arrest of feeding after the second injection of DT into *AgRP^{DTR/DTR}* mice but not into controls (Fig. 2B). All *AgRP^{DTR/DTR}* mice treated this way lost ~20% of their body weight within 2 days of the second injection and were killed for immunohistochemical detection of NPY/AgRP neurons, which were always depleted by >80%. Injection of adult heterozygous *AgRP^{DTR/+}* mice with DT (either once or twice) also terminated feeding (table S1). The loss of AgRP-producing cells was also measured by semiquantitative RT-PCR in *AgRP^{DTR/+}* mice, which revealed a comparable loss of AgRP transcripts in neonatal and adult mice treated with DT. Occasionally, control mice also succumbed from this treatment, probably because of nonspecific toxicity associated with ip administration of DT.

Intramuscular (im) injection of DT in adult mice produced more reliable responses compared with ip injection (18). Littermates (*AgRP^{+/+}* or *AgRP^{DTR/DTR}*) received either one injection of DT (50 $\mu\text{g}/\text{kg}$, im), or two injections, 2 days apart. The consumption of liquid food by DT-treated *AgRP^{DTR/DTR}* mice fell below 20% of normal 7 days after a single injection of DT or within 5 days with two injections (Fig. 3, A and C, and fig. S5). At these time points, all *AgRP^{DTR/DTR}* mice lost ~20% of their body weight and had to be euthanized, whereas control mice maintained body weight and survived (Fig. 3, B and D, and table S1). Water consumption increased in *AgRP^{DTR/DTR}* mice after DT injections, demonstrating that the reduction in food intake was not due to an inability to reach the feeding tubes or to lick. Hand feeding via oral gavage with liquid food could sustain DT-treated mice, confirming

that the lack of feeding was responsible for their loss of body weight. NPY immunostaining of sections through the ARC confirmed that most NPY/AgRP cell bodies were ablated from the ARC of *AgRP^{DTR/DTR}* mice (Fig. 3, E and F). The number of NPY fibers in the PVN was also reduced in *AgRP^{DTR/DTR}* mice compared with controls (Fig. 3, I to L). The number of POMC neurons was normal, but the ACTH staining appeared to be more robust in the *AgRP^{DTR/DTR}* mice treated with DT (Fig. 3, G and H), which is consistent with the loss of NPY/AgRP inhibitory input onto POMC cells.

To demonstrate that loss of feeding is the consequence of central action of DT, the toxin was delivered to the third ventricle of *AgRP^{DTR/DTR}* mice. All injected *AgRP^{DTR/DTR}* mice stopped eating, whereas controls were unaffected (table S1).

AgRP^{DTR/+} or *AgRP^{DTR/DTR}* neonates survived all of the DT treatments that led to starvation in adults, despite comparable ablation of NPY/AgRP neurons, which suggests that some form of compensation occurs in neonates. Perhaps residual neonatal NPY/AgRP neurons can enhance their signaling better than can adult neurons, or DTR-expressing cells may continue to be born after neonatal DT injection, allowing survival. These explanations predict that mice treated neonatally with DT would be susceptible to DT exposure as adults. However, most (5 of 7) mice injected with DT as neonates survived when DT was injected in the third ventricle as adults (fig. S6). Ventricular injection of DT was used to minimize potential immune responses to prior DT exposure; however, in agreement with others (23), neonatal exposure to DT generates minimal neutralizing antibody (fig. S7). In another experiment, most (5 of 8) neonatally treated *AgRP^{DTR/DTR}* mice survived im injection of DT (50 $\mu\text{g}/\text{kg}$) as adults. The fractional survival of doubly exposed *AgRP^{DTR/DTR}* mice suggests alternate modes of compensation (24).

The ablation of NPY/AgRP neurons in neonates is not only tolerated but produces compensatory changes that allow almost normal growth and feeding in the adult. Nevertheless, we predict that hormonal or metabolic signals that depend on NPY/AgRP neurons, e.g., ghrelin (25), may be compromised. The melanocortin signaling pathway, which is important for body-weight regulation in adults, may not be critical for feeding by neonates. Thus, ablating NPY/AgRP neurons before the POMC cells become critical may allow development of a network-based compensatory mechanism. Changes in synapses within the ARC after restoration of leptin to young *Lepr^{ob/ob}* mice illustrate one form of plasticity that can occur in the hypothalamus (26, 27). Presumably, the loss of signaling molecules made by NPY/AgRP neurons initiates the compensatory adaptations in neonates, but the nature of those signals and the identity of the cells that respond

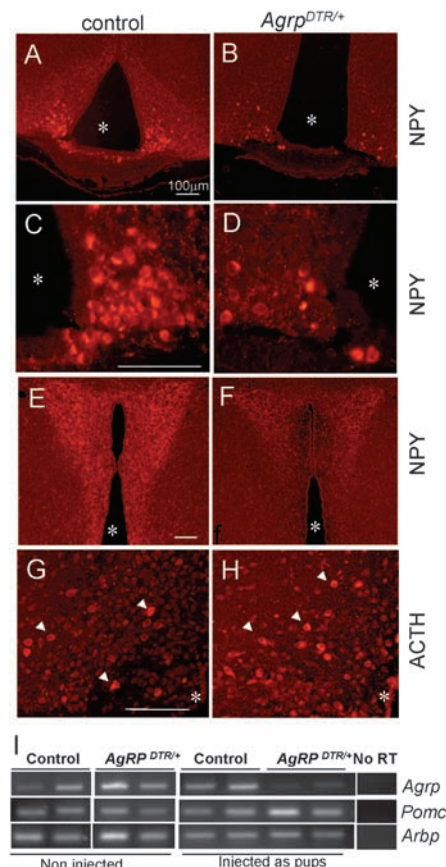


Fig. 1. DT injection in neonates ablates NPY neurons in the arcuate nucleus. Both control and *AgRP^{DTR/+}* mice were injected as pups with DT (50 $\mu\text{g}/\text{kg}$). After 9 weeks, animals were fasted for 2 days to increase NPY signal and killed for brain immunohistochemistry. (A and B) Representative NPY immunostaining of ARC neurons of control (A) and *AgRP^{DTR/+}* mice (B). (C and D) Higher magnifications of ARC region. (E and F) NPY immunostaining of PVN from control (E) and *AgRP^{DTR/+}* mice (F). (G and H) ACTH immunostaining of the ARC from controls (G) and *AgRP^{DTR/+}* mice (H). White arrowheads point to POMC cell bodies. The asterisks indicate third ventricle. Scale bar, 100 μm . (I) Semi-quantitative RT-PCR for *AgRP*, *Pomc* mRNA, and *Arbp* mRNA, as control.

remain to be discovered. This adaptation could explain why conventional inactivation of *Npy* and/or *AgRP* genes has little effect on body-weight regulation (10, 11).

The NPY/AgRP neurons in the ARC become a critical component of the feeding neurocircuitry sometime between 8 and 45 days after birth. By this time, the melanocortin signaling pathway is established; hence, ablation of NPY/AgRP neurons may remove a critical inhibitory tone, leading to excessive melanocortin signaling and starvation. However, NPY/AgRP neurons project widely (19), so their ablation in the adult may perturb other critical signaling pathways, resulting in starvation.

Note added in proof: Two related papers (28, 29) were published online while this Report

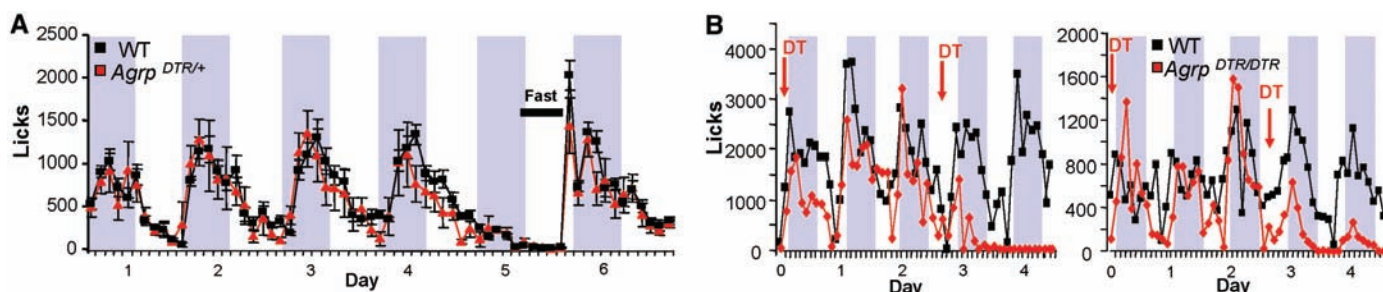


Fig. 2. Ablation of NPY/AgRP neurons in neonatal or adult mice differentially alters feeding behavior. (A) Licking pattern of control (wild type, WT) (black, $n = 4$) and $Agrp^{DTR/+}$ mice (red, $n = 4$) that were injected with DT (50 $\mu\text{g}/\text{kg}$) as neonates and tested at 9 weeks. The graph illustrates 3 days of baseline feeding, followed by a 12-hour fast,

and refeeding response. The total number of licks in 2-hour bins is plotted. (B) Representative licking pattern of an individual control and $Agrp^{DTR/+}$ mouse (left panel, 12 weeks old; right panel, 7 weeks old) in response to two injections (arrows) of DT (50 $\mu\text{g}/\text{kg}$, ip). Shaded areas represent the dark phase. Error bars represent SEM.

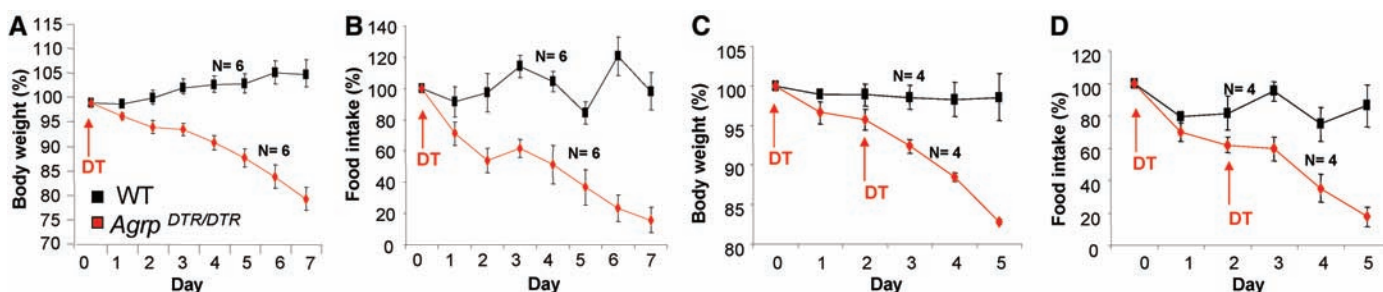
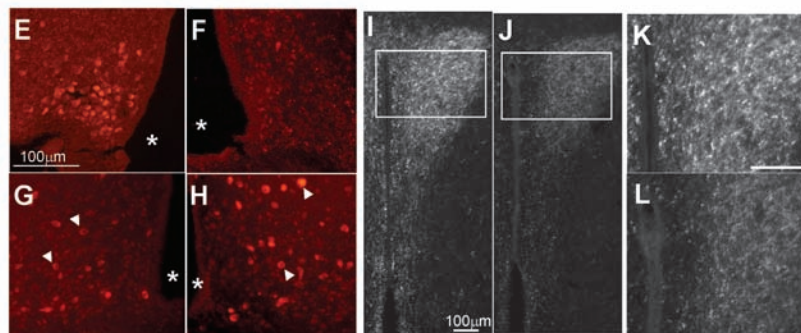


Fig. 3. DT injection induces a dose-dependent arrest of feeding in adult $Agrp^{DTR/DTR}$ mice. (A and B) Body weight (A) and food intake (B) of adult control (black, $n = 6$) and $Agrp^{DTR/DTR}$ mice (red, $n = 6$) injected once (arrow) with DT (50 $\mu\text{g}/\text{kg}$, im). (C and D) Body weight (C) and food intake (D) of adult control (black, $n = 4$) and $Agrp^{DTR/DTR}$ mice (red, $n = 4$) injected twice (arrows) with DT (50 $\mu\text{g}/\text{kg}$, im). Error bars represent SEM. (E and F) Representative NPY immunostaining of control (E) and $Agrp^{DTR/DTR}$ (F) mouse. (G and H) ACTH immunostaining of ARC neurons in control (G) and $Agrp^{DTR/DTR}$ mouse (H) that were injected as adults with DT. Brains were collected for histology when the mice had lost $\sim 20\%$ of body weight; controls were fasted to comparable weight loss. Arrowheads, POMC cell bodies; asterisk, third ventricle. (I and J) NPY-fiber immunostaining in the PVN of the same control (I) and $Agrp^{DTR/DTR}$ mice (J) as above. (K and L) Higher magnification of boxed areas in (I) and (J), respectively. Scale bar, 100 μm .



was under review. In both cases, the authors report that partial ablation of NPY/AgRP neurons results in smaller mice that eat less than controls do. The partial ablation is probably the consequence of gradual ablation or partial penetrance of transgene expression. Neither paper describes the starvation phenotype nor the neonatal compensation reported here.

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24. There may be two different modes of compensation. In one mode, a critical number of NPY/AgRP neurons

survive neonatal exposure to DT and continue to function in the adult; their subsequent ablation results in loss of appetite and rapid loss of weight. In the other mode, most NPY/AgRP neurons are ablated in the neonates, and compensatory mechanisms develop; these mice are unaffected by adult exposure to DT.

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Supporting Online Material

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