

Agouti-related peptide–expressing neurons are mandatory for feeding

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Multiple hormones controlling energy homeostasis regulate the expression of neuropeptide Y (NPY) and agouti-related peptide (AgRP) in the arcuate nucleus of the hypothalamus. Nevertheless, inactivation of the genes encoding NPY and/or AgRP has no impact on food intake in mice. Here we demonstrate that induced selective ablation of AgRP-expressing neurons in adult mice results in acute reduction of feeding, demonstrating direct evidence for a critical role of these neurons in the regulation of energy homeostasis.

A distinct subpopulation of hypothalamic neurons coexpressing AgRP and NPY is the target of various peripheral hormonal signals such as leptin, insulin and ghrelin^{1–3}. Intracerebroventricular injection of AgRP and NPY leads to an increase in food intake, characterizing them as so-called orexigenic peptides^{4,5}. AgRP- and NPY-expressing neurons counteract another arcuate nucleus cell population, the pro-opiomelanocortin (POMC)-expressing neurons, which are considered anorexigenic. The interaction of these two systems seems to be the primary driving force in the regulation of energy homeostasis^{6,7}. However, disruption of the AgRP and NPY genes either alone or in combination does not alter energy homeostasis to the extent that is predicted from the proposed role of these cells⁸.

To directly address the role of AgRP-expressing neurons, we ablated these cells in an inducible manner in adult mice by cell type-specific expression of a diphtheria toxin receptor (DTR) and the administration of diphtheria toxin (for details, see **Supplementary Methods** online). Mice in which expression of DTR from a ubiquitously active promoter is prevented by a *loxP*-flanked stop cassette (inducible DTR (iDTR) mice)⁹ were crossed with transgenic mice expressing Cre recombinase under control of the AgRP promoter, thus leading to DTR expression selectively in AgRP neurons (AgRP^{DTR}; **Supplementary Fig. 1**). To create a specific control population, the same strategy was applied to selectively express the DTR in POMC neurons (POMC^{DTR};

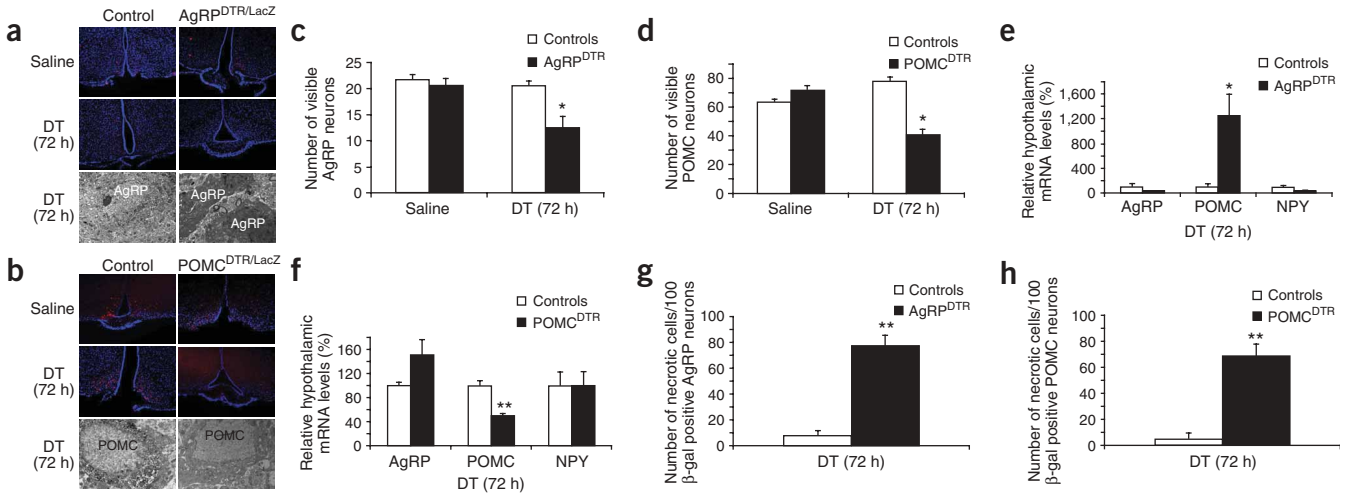
Supplementary Fig. 1). Specificity of Cre-mediated recombination was tested by crossing AgRP^{DTR} and POMC^{DTR} mice with a reporter mouse strain in which Cre recombination activates transcription of the β -galactosidase gene (AgRP^{DTR/LacZ} and POMC^{DTR/LacZ}; **Supplementary Fig. 1**). In this reporter system, we observed a β -galactosidase expression pattern reflecting that of endogenously expressed AgRP and POMC (**Fig. 1a,b**).

To induce ablation of AgRP and POMC neurons, we injected AgRP^{DTR}, POMC^{DTR} and control mice (iDTR/Cre⁻ or wild-type littermates) intraperitoneally (i.p.) with 40 ng/g (body weight) of diphtheria toxin on two consecutive days. To determine the consequence of diphtheria toxin injection on neuronal survival, AgRP^{DTR/LacZ}, POMC^{DTR/LacZ} and control littermates underwent the same treatment and were analyzed for β -galactosidase expression by immunohistochemistry before and 72 h after the start of diphtheria toxin treatment. This analysis showed a reduction in β -galactosidase-positive AgRP and POMC neurons by approximately 50% as early as 72 h after the first diphtheria toxin treatment (**Fig. 1a–d**). Simultaneously, we found a corresponding 50% reduction of AgRP and POMC mRNA levels in the treated AgRP^{DTR} and POMC^{DTR} mice (**Fig. 1e,f**). To investigate the neuronal morphology of AgRP^{DTR/LacZ} and POMC^{DTR/LacZ} mice, we performed electron microscopy on brain sections after β -galactosidase immunostaining. This analysis confirmed the previously observed amount of cell death and demonstrated that the majority of remaining β -galactosidase-positive neurons showed clear signs of degeneration (AgRP^{DTR/LacZ}, 78/100; POMC^{DTR/LacZ}, 69/100; **Fig. 1g,h**). These data indicate that more than 85% of AgRP or POMC neurons were either necrotic or degenerated 72 h after starting diphtheria toxin treatment. On the other hand, the number of POMC-expressing cells in the arcuate nucleus of diphtheria toxin-treated AgRP^{DTR} and control mice as assessed by immunohistochemistry was indistinguishable, indicating a high degree of specificity for AgRP neuron ablation in our approach (**Supplementary Fig. 2**). Thus, intraperitoneal administration of diphtheria toxin in mice with targeted expression of DTR in AgRP- or POMC-expressing neurons resulted in rapid, efficient and selective death of these cells.

To determine the physiological consequence of acute AgRP or POMC neuron ablation in adult mice, AgRP^{DTR}, POMC^{DTR} and controls first received two sham i.p. injections with saline, followed by 2 d of i.p. diphtheria toxin administration. Within 48 h of the first diphtheria toxin injection, we observed a rapid and significant reduction of body weight in AgRP^{DTR} mice, whereas diphtheria toxin treatment did not affect body weight in control mice (**Fig. 2a**). Notably, we found a significant reduction of food intake in AgRP^{DTR} mice as early as 24 h after the first diphtheria toxin injection (**Fig. 2b**). In

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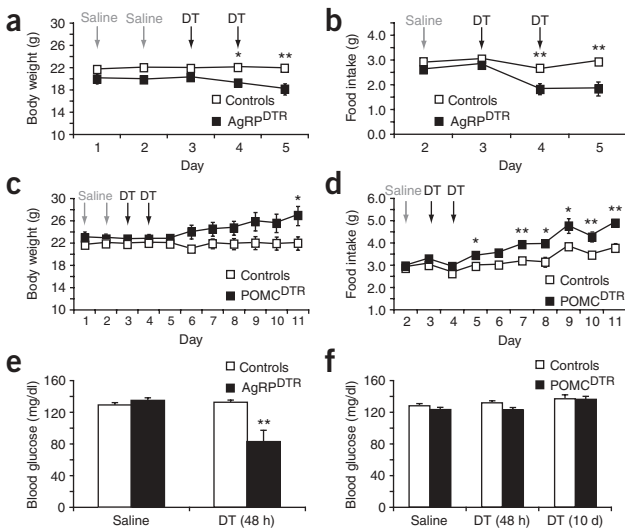
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contrast, food intake and body weight of $POMC^{DTR}$ mice started to increase gradually 2 d after diphtheria toxin treatment (Fig. 2c,d). Consistent with the observed starvation, diphtheria toxin-treated $AgRP^{DTR}$ mice showed a marked reduction in blood glucose (Fig. 2e) as well as plasma insulin and leptin concentrations (data not shown). Corticosterone concentration 48 h after the first diphtheria toxin treatment remained unchanged (Supplementary Fig. 3). This result rules out the possibility that ablation of AgRP-expressing cells in the adrenal gland might contribute significantly to the observed phenotype. Moreover, consistent with unaltered glucocorticoid con-

centrations, we did not detect Cre-mediated recombination in the adrenal gland of $AgRP^{DTR/LacZ}$ mice (Supplementary Fig. 3). In contrast, we found a significant gradual reduction ($P < 0.01$) of plasma corticosterone concentrations in $POMC^{DTR}$ mice arising as a result of POMC cell ablation in the pituitary gland (Supplementary Fig. 3). Nevertheless, blood glucose concentrations remained unaltered in $POMC^{DTR}$ mice (Fig. 2f). These data demonstrate that selective ablation of AgRP-expressing neurons in adult mice results in immediate onset of anorexia, whereas induced POMC cell ablation leads to a delayed onset of hyperphagia, consecutive obesity and hypocortisolism.

In summary, the present results provide direct experimental evidence that neurons producing AgRP and POMC are critical for the regulation of feeding in adult mammals. The phenotype of mice with induced ablation of POMC-expressing cells is consistent with the phenotype of mice lacking $POMC^{10}$, whereas the acute ablation of AgRP-expressing cells produces a phenotype opposite to the phenotype of mice lacking AgRP and/or NPY⁸. This difference is likely to arise from the fact that



AgRP- and NPY-deficient mice lack expression of these neuropeptides but retain the neurons that produce them, engaging alternative mechanisms to regulate feeding. Recently reported experiments aiming to ablate AgRP neurons by transgenic expression of a neurotoxin provided evidence for a role of AgRP-expressing cells in the regulation of energy homeostasis¹¹. As the experimental procedure in this case ablated only 20–47% of AgRP neurons in a prolonged manner, it did not exclude the possibility for compensatory changes to contribute to the phenotype. Our acute and efficient AgRP cell ablation and the resulting phenotype directly prove the critical role of the targeted neurons. Moreover, the opposite phenotypes of AgRP^{DTR} and POMC^{DTR} mice rule out that the AgRP^{DTR} phenotype arises from unspecific cell death occurring in the arcuate nucleus. As AgRP/NPY neurons are GABAergic cells¹², and GABA has been shown to have a major role in the regulation of feeding¹³, GABA release from AgRP-expressing neurons rather than release of AgRP or NPY may control food intake. Although acute disinhibition of POMC neurons upon death of GABAergic AgRP neurons might result in robust activation of the melanocortin pathway leading to anorexia, it is equally reasonable to suggest that the AgRP/GABA system acts independently from the POMC neurons for the minute-by-minute regulation of feeding. Nevertheless, the clear increase in POMC expression upon AgRP cell ablation indicates the presence of an AgRP neuron-dependent mechanism of POMC regulation, which deserves further study (Fig. 1e). Alternatively, NPY and AgRP action might be required for proper control of feeding, but compensatory changes resulting from the lack of these neuropeptides in conventional knockout mice mask this function. Acute inactivation of NPY function by viral overexpression of antisense cRNAs has indeed provided evidence for a role of these neuropeptides in the regulation of feeding¹⁴. Finally, although unlikely and not detectable with the methods used in this study, a different population of AgRP-expressing cells besides the arcuate nucleus neurons might have a role in energy homeostasis. Further analysis of the mechanisms

resulting in acute anorexia upon ablation of cells expressing AgRP may help to validate new targets for the treatment of obesity.

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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1. Schwartz, M.W., Seeley, R.J., Campfield, L.A., Burn, P. & Baskin, D.G. *J. Clin. Invest.* **98**, 1101–1106 (1996).
2. Schwartz, M.W. *et al. Endocrinology* **130**, 3608–3616 (1992).
3. Chen, H.Y. *et al. Endocrinology* **145**, 2607–2612 (2004).
4. Ollmann, M.M. *et al. Science* **278**, 135–138 (1997).
5. Pierroz, D.D., Catzeflis, C., Aebi, A.C., Rivier, J.E. & Aubert, M.L. *Endocrinology* **137**, 3–12 (1996).
6. Cone, R.D. *Trends Endocrinol. Metab.* **10**, 211–216 (1999).
7. Huszar, D. *et al. Cell* **88**, 131–141 (1997).
8. Qian, S. *Mol. Cell. Biol.* **22**, 5027–5035 (2002).
9. Buch, T. *et al. Nat. Methods* **2**, 419–426 (2005).
10. Yaswen, L., Diehl, N., Brennan, M.B. & Hochgeschwender, U. *Nat. Med.* **5**, 1066–1070 (1999).
11. Bewick, G.A. *et al. FASEB J.* published online 11 August 2005 (doi:10.1096/fj.04-3434fje).
12. Horvath, T.L., Bechmann, I., Naftolin, F., Kalra, S.P. & Leranah, C. *Brain Res.* **756**, 283–286 (1997).
13. Cowley, M.A. *et al. Nature* **411**, 480–484 (2001).
14. Gardiner, J.V. *et al. Biochem. Biophys. Res. Commun.* **327**, 1088–1093 (2005).