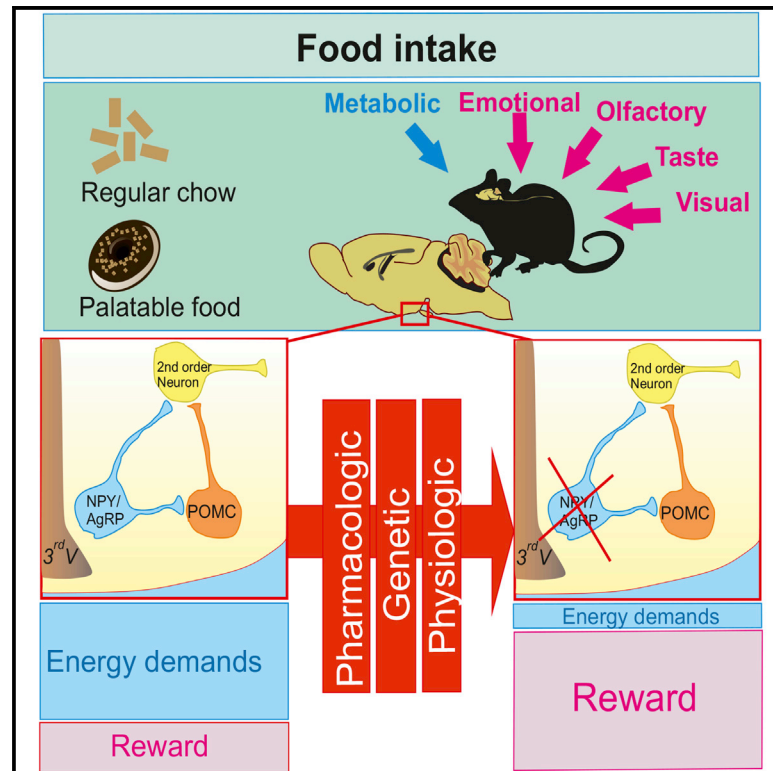


Cell Metabolism

Palatability Can Drive Feeding Independent of AgRP Neurons

Graphical Abstract



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In Brief

Denis et al. show that AgRP neurons drive feeding behavior on normal chow, although, when a highly palatable diet is substituted, AgRP neurons become dispensable, and the hedonic circuitry operates feeding on its own. AgRP-ablated mice consume excess palatable food and are hypersensitive to stress-induced anorexia, which are hallmarks of comfort feeding.

Highlights

- AgRP neurons are important to drive feeding when food is not palatable
- AgRP neurons are dispensable when food is highly palatable
- Animals with compromised AgRP neuron activity are a model of comfort feeding
- Inhibition of AgRP neurons promotes reward feeding



Palatability Can Drive Feeding Independent of AgRP Neurons

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SUMMARY

Feeding behavior is exquisitely regulated by homeostatic and hedonic neural substrates that integrate energy demand as well as the reinforcing and rewarding aspects of food. Understanding the net contribution of homeostatic and reward-driven feeding has become critical because of the ubiquitous source of energy-dense foods and the consequent obesity epidemic. Hypothalamic agouti-related peptide-secreting neurons (AgRP neurons) provide the primary orexigenic drive of homeostatic feeding. Using models of neuronal inhibition or ablation, we demonstrate that the feeding response to a fast ghrelin or serotonin receptor agonist relies on AgRP neurons. However, when palatable food is provided, AgRP neurons are dispensable for an appropriate feeding response. In addition, AgRP-ablated mice present exacerbated stress-induced anorexia and palatable food intake—a hallmark of comfort feeding. These results suggest that, when AgRP neuron activity is impaired, neural circuits sensitive to emotion and stress are engaged and modulated by food palatability and dopamine signaling.

INTRODUCTION

The last several decades have witnessed a pandemic expansion of pathologies related to high-fat and high-carbohydrate diets, including obesity, diabetes, dyslipidemia, and cardiovascular diseases—collectively referred to as metabolic syndrome (Eckel et al., 2005; Reaven, 2006). Globally, there has been both an increased intake of energy-dense foods that are high in fat, sugar, and salt and a concomitant decrease in physical activity (Eckel et al., 2005; Reaven, 2006). Despite the increasing prevalence and burden of metabolic syndrome, effective and safe treatments to reverse the epidemic remain limited.

Appropriate energy homeostasis results from the exquisite balance between energy intake and energy expenditure. Several determinants of feeding behavior have been studied extensively and encompass the homeostatic regulation of nutrient intake, generally attributed to hypothalamic brainstem circuitry (Saper et al., 2002). Non-homeostatic feeding is, in part, a consequence of the potent reinforcing and motivational properties of food tied closely to the release of dopamine in limbic brain regions, which is particularly stimulated by high-fat and high-sugar (HFHS) foods (Di Chiara and Imperato, 1988; Dallman et al., 2005; Narayanan et al., 2010). Although the presence of high-fat, nutrient-rich food is essentially ubiquitous in developed countries, not all individuals with access to these foods consume them in excess or become obese. It is therefore likely that certain individuals become more vulnerable to the hedonic or reinforcing effects of calorie-rich food and, ultimately, form eating habits

that become disassociated from the homeostatic mechanisms that normally maintain energy balance (Berthoud, 2004). Understanding the functional connection between reward-driven nutrient intake and homeostatic control of energy balance is a key step toward a clinical breakthrough in the treatment of hyperphagia and obesity.

The arcuate nucleus (ARC) of the hypothalamus is regarded as a primary location for the integration of circulating signals of hunger and satiety. “First-order neurons” located in the ARC are the first to respond to the circulating signals of hunger and satiety. Neurons that make pro-opiomelanocortin (POMC) produce melanocortin peptides, including α -melanocyte-stimulating hormones (α -MSH). Binding of α -MSHs to G protein-coupled melanocortin receptors 3 and 4 (MCR3 and MCR4) leads to decreased food intake and increased energy expenditure. Conversely, energy deprivation triggers increased activity of the neighboring neurons that make neuropeptide Y (NPY), agouti-related peptide (AgRP), and γ -aminobutyric acid (GABA) (AgRP neurons), resulting in increased inhibitory tone onto POMC neurons and competitive inhibition of melanocortin signaling at post-synaptic neurons located in many brain regions (Morton et al., 2006). Serotonin (5-hydroxytryptamine, 5-HT) is known to have anorexic properties, and serotonergic drugs have been used widely as anti-obesity treatments (Lam and Heisler, 2007). Serotonergic innervation of ARC neurons mediates the satiety action of serotonin through the concerted activation of POMC neurons and inhibition of AgRP neurons (Heisler et al., 2006). Administration of ghrelin, a stomach-derived 28-amino-acid peptide, in both humans and rodents has been shown to increase food intake through binding to the growth hormone secretagogue receptor 1 (GHS-R) (Kojima et al., 1999). We and others have shown that AgRP neuron integrity is crucial for the ghrelin-induced feeding response (Chen et al., 2004; Luquet et al., 2007).

Receptors for 5-HT, leptin, or ghrelin are also found in extra-arcuate (ARC) targets, including pontine, brainstem, and midbrain structures. Although the direct access of circulating hormones such as ghrelin or leptin to these structures lying away from the blood brain barrier (BBB) entry point is still a matter of debate, injection of these hormones into the ventral tegmental area (VTA) or parabrachial nucleus (PBN) can inhibit feeding (Abizaid et al., 2006; Figlewicz et al., 2003; Fulton et al., 2006; Hommel et al., 2006; Jerlhag et al., 2007; Lee et al., 1998; Naleid et al., 2005; Quarta et al., 2009; Zigman et al., 2006). These observations raise the question of the specific contribution of ARC versus extra-ARC neurons in the balance between reward-driven or energy-driven nutrient intake. This question becomes crucial when sugar- and fat-rich diets are readily available and may contribute to addictive-like feeding behavior (DiLeone et al., 2012).

Mouse models with transient or chronic loss of AgRP neuron activity provide an ideal tool to dissect the role of homeostatic versus non-homeostatic regulation of energy intake. Taking advantage of these models, we demonstrate that feeding behavior on a normal chow diet relies on AgRP neurons. However, when a highly palatable diet is substituted, the AgRP neurons are no longer necessary. We also show that AgRP-ablated mice consume excess palatable food and are hypersensitive to stress-induced anorexia. We propose that activation of the

hedonic circuitry overrides the homeostatic circuitry in the chronic inhibition of AgRP neurons.

RESULTS

AgRP-Ablated Mice Have Impaired Hunger-Associated Feeding Responses on a Standard Diet

Activation of hypothalamic AgRP neurons promotes robust feeding (Aponte et al., 2011; Krashes et al., 2011). To decipher the contribution of AgRP neurons in hedonic versus non-hedonic regulation of feeding, we used a model in which AgRP neurons can be ablated selectively (Joly-Amado et al., 2012; Luquet et al., 2005, 2007). Briefly, mice with the human diphtheria toxin (DT) receptor under the control of *AgRP* regulatory elements (*AgRP^{DT^R}*) are injected with DT during the first week after birth (AgRP-ablated mice), and control mice of the same genotype are injected with saline (control mice). We reproduced our previous observation that neonatal ablation of AgRP neurons does not affect daily chow intake (Figure S1A). However, feeding responses to peripherally or centrally injected ghrelin were abolished (Figures S1B and S1C; Joly-Amado et al., 2012; Luquet et al., 2005, 2007). Peripheral injection of bio-active, fluorescently labeled ghrelin (Leyris et al., 2011; Schaeffer et al., 2013) resulted in robust accumulation of fluorescence in the ARC, whereas this signal was virtually absent in mice lacking AgRP neurons (Figure 1A). In addition, ghrelin induced Fos in ARC neurons following intra-cerebroventricular (ICV) injection, which was lost in AgRP-ablated mice (Figure 1B). These observations establish that AgRP neurons are important for ghrelin binding and action.

Food Palatability Restores the Feeding Response to Ghrelin in Mice Lacking AgRP Neurons

We have observed previously that feeding after a fast can be restored according to the degree of palatability of the food presented for refeeding (Luquet et al., 2005, 2007). Provision of a highly palatable HFHS diet allowed a normal feeding response after a fast by AgRP-ablated mice (Figure 1C). This observation raises the possibility that the palatability or reward value of food might compensate for defective feeding in AgRP-ablated mice. The feeding response to centrally injected ghrelin was assessed in control or AgRP-ablated mice fed either regular chow or an HFHS diet on several consecutive days (Figure 1D). ICV injection of ghrelin produced a significant increase in food intake by both groups of mice on the HFHS diet (Figure 1E), supporting the hypothesis that ghrelin-induced feeding in the absence of AgRP neurons is dependent on diet palatability. Body weight measurements confirmed that food intake rather than spillage had occurred during the 4 hr following the injection (Figure S1D). The ghrelin-induced increase in locomotor activity was identical between control and AgRP-ablated mice, indicating that AgRP neurons are not essential for ghrelin-induced locomotor activity (Figure S1E). We also assessed the feeding response to peripherally administered ghrelin in the same groups of animals with chow diet versus 2 or 10 weeks on an HFHS diet. Although ghrelin injected intraperitoneally (i.p.) did not trigger chow intake by mice lacking AgRP neurons, it significantly increased consumption after being on the HFHS diet for 2 or 10 weeks (Figure 1F). Although control animals exhibited a more robust increase in

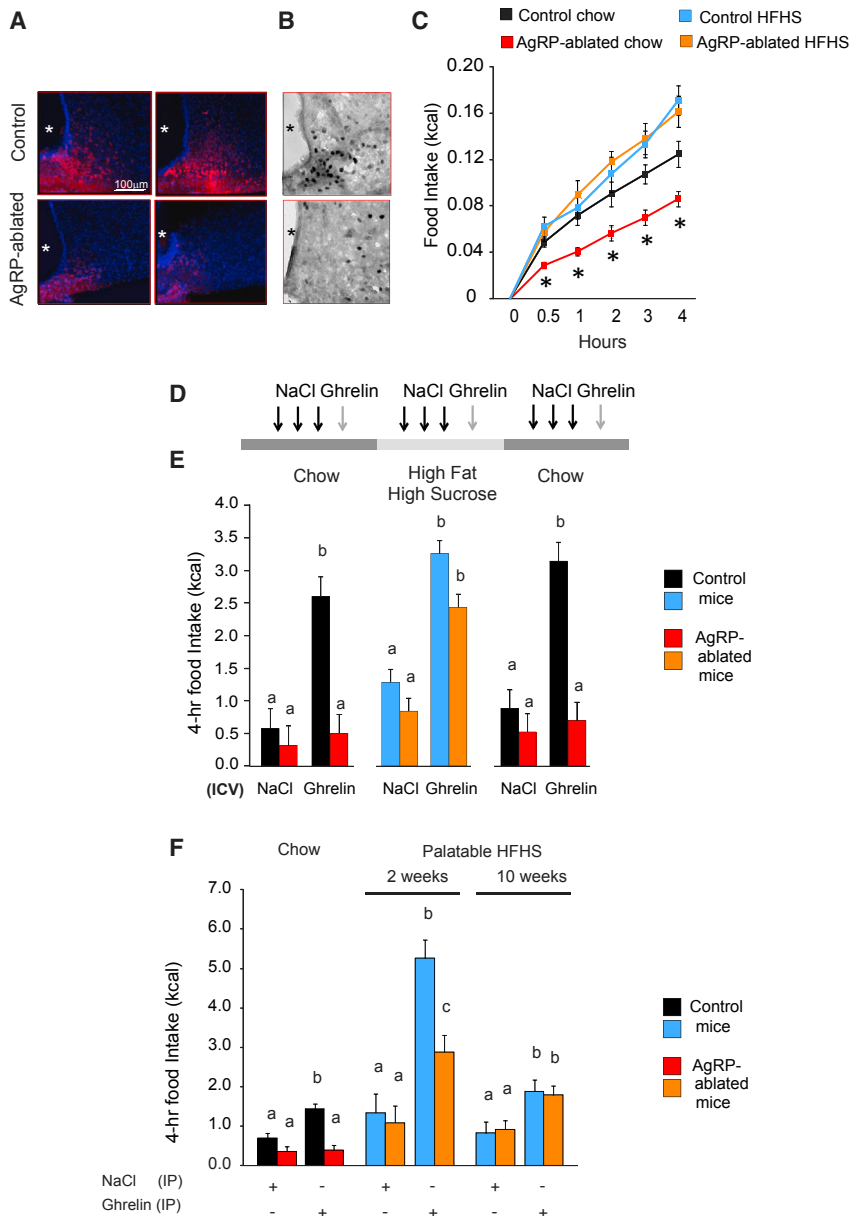


Figure 1. Food Palatability Restores Ghrelin-Induced Feeding in AgRP-Ablated Mice

(A and B) Representative confocal images of brain sections after injection (i.v.) of active fluorescent ghrelin (A) and immunohistochemistry for *c-fos* after a 24 hr fast (B) of AgRP-ablated (bottom) and control (top) mice. Blue, Hoechst; red, incorporated fluorescent ghrelin (A); dark, Fos immunostaining (B). asterisks, third ventricle.

(C) 4-hr caloric response to fasting in AgRP-ablated (red and orange) or control (black and blue) mice presented with a chow (black and red) or HFHS diet (blue and orange).

(D) Representative time bar of three (daily) ICV injections of vehicle and octanoyl ghrelin on the fourth day in control (black and blue) and AgRP-ablated (red and orange) mice presented consecutively with a chow diet, HFHS diet, and then chow diet. Three days of acclimation were allowed with the new diet before the next series of injection.

(E) 4-hr cumulative caloric intake in 4-month-old control (black and blue) and AgRP-ablated mice (red and orange) after ICV injection of NaCl or octanoyl ghrelin injection.

(F) 4-hr cumulative caloric intake after i.p. injection of octanoyl ghrelin in 4-month-old control (black and blue) and AgRP-ablated mice (red and orange) fed with a chow diet or 2 or 10 weeks of HFHS diet. $n = 6$ minimum in each group.

* $p < 0.05$, control versus AgRP-ablated mice. a, b, and c differ significantly ($p < 0.05$). Data are expressed as mean \pm SEM. See also Figure S1.

feeding response to ghrelin after 2 weeks of HFHS diet exposure, the responses of both groups were identical after 10 weeks of HFHS diet exposure. This result is consistent with the observation that diet-induced obesity leads to ghrelin resistance (Briggs et al., 2010, 2014) and suggests that diet-induced obesity promotes a phenotype similar to that achieved with loss of AgRP neurons.

Feeding Relies on Food Palatability and Dopamine Tone when the Activity of AgRP Neurons Is Compromised

We reasoned that, in the absence of AgRP neurons, the orexigenic drive might rely on dopamine tone because the ghrelin receptor is expressed in the VTA (Mani et al., 2014), where its activation has been shown to trigger feeding (Abizaid et al., 2006; Egecioglu et al., 2010; Jerlhag et al., 2007; Naleid et al.,

2005). Control and AgRP-ablated mice were implanted with ICV and intra-VTA cannulas, and the feeding responses to ghrelin were monitored on chow or an HFHS diet in the presence or absence of [D-Lys3]-GHRP-6, a selective antagonist of the ghrelin receptor (Traebert et al., 2002) that is known to reduce food intake (Ishii et al., 2002). Ghrelin (ICV) induced a significant increase in both chow and HFHS diet intake in control mice, but this effect was restricted to the HFHS diet in AgRP-ablated mice (Figures 2A and 2B).

Concomitant administration of the ghrelin receptor antagonist in the VTA reduced the orexigenic action observed in both control and AgRP-ablated mice (Figures 2A and 2B). These data support the hypothesis that VTA ghrelin is required for the orexigenic action of ghrelin in AgRP-ablated mice fed a palatable diet.

To further implicate the importance of dopamine signaling, we used a dopamine re-uptake inhibitor to determine if that would enhance chow consumption after a fast by AgRP-ablated mice. Fasted mice were tested on chow or an HFHS diet in conjunction with peripheral injection of saline or GBR 12935 dihydrochloride (GBR) (Billes and Cowley, 2007). We found that pre-treatment with GBR selectively restored 4-hr chow intake in response to a fast in AgRP-ablated mice without affecting control mice (Figures 2C and 2D). Importantly, the same treatment did not change feeding after a fast in control or AgRP-ablated

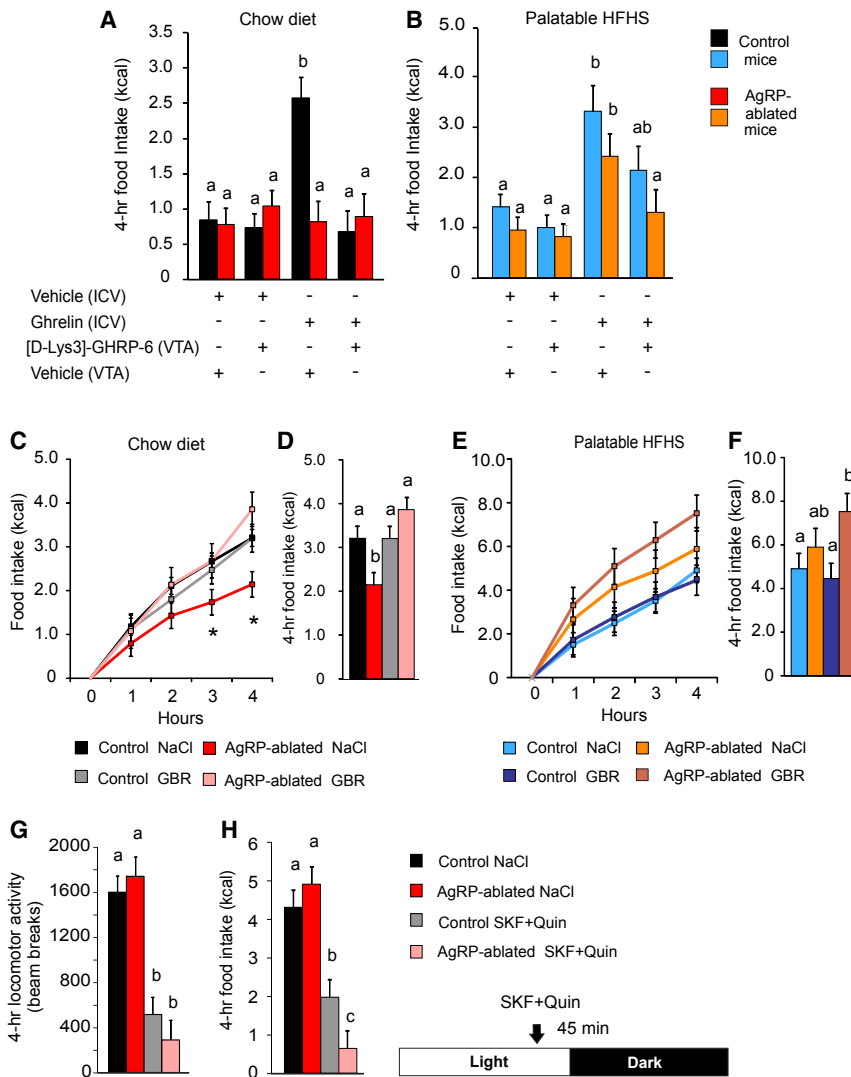


Figure 2. Feeding Responses Rely on Dopamine Tone in AgRP-Ablated Mice

(A and B) 4-hr cumulative caloric intake of AgRP-ablated (red and blue) or control (black and orange) mice fed a chow (A) or HFHS diet (B) after a concomitant ICV and intra-VTA injection of vehicle or octanoyled ghrelin and [D-lys3-GHRP-6] or vehicle, respectively.

(C–F) 4-hr caloric response (C and E) and cumulative caloric intake (D and F) after a fast in AgRP-ablated (red, rose, orange, and brown) or control (black, gray, blue, and navy blue) mice presented with a chow (black, red, gray, and rose) or HFHS diet (blue, orange, navy blue, and brown) and injected i.p. with NaCl or a selective dopamine reuptake inhibitor (GBR).

(G and H) Spontaneous locomotor activity (G) and caloric intake (H) of 6-month-old lean control animals (black and gray) and AgRP-ablated (red and rose) at the onset of the dark period after an i.p. injection (45 min before the dark period) of the selective D₁R agonist SKF 38393 hydrobromide and the selective D₂R agonist quinpirole hydrochloride (SKF + Quin).

n = 6 minimum in each group. a, b, and c differ significantly (p < 0.05). Data are expressed as mean ± SEM. See also Figure S2.

mice on the HFHS diet when assessed (Figures 2E and 2F). GBR injection also selectively increased locomotor activity in AgRP-ablated mice when refed on chow (Figure S2A).

We also took the reciprocal approach by exploring the anorectic action of dopamine receptor agonists in AgRP-ablated mice. Control and AgRP-ablated mice were injected with a mixture of selective dopamine D₁ and D₂ receptor agonists (D₁/D₂ mixture) that produce anorexia (Cooper and Al-Naser, 2006; Kuo, 2002; Romero-Picó et al., 2013). Animals were injected with saline or D₁/D₂ mixture 45 min before the onset of the dark period. AgRP-ablated mice displayed a similar decrease in spontaneous locomotor activity as control mice (Figure 2G) but showed enhanced sensitivity to the anorectic action of the agonists (Figure 2H). Monoamine quantification in various brain regions did not reveal any compensatory changes in dopamine content between control and AgRP-ablated mice (Figure S3). Likewise, the abundance of various dopamine-related mRNAs was unaffected (Figure S4). These results support our hypothesis that feeding by mice lacking AgRP neurons relies on dopaminergic reward circuitry.

Because compensatory mechanisms in mice lacking AgRP neurons from birth cannot be ruled out completely, we also tested whether enhancing food palatability and dopamine neuron activity could prevent the starvation that follows ablation of AgRP neurons in adult animals. AgRP^{DTR} mice were put on a genetic background in which activation of dopamine neurons is mediated through the restricted expression of Trpv1, the capsaicin-receptor, in dopamine neurons (referred to as AgRP^{DTR}; DAT^{TRPV1} mice; Güler et al., 2012). Adult AgRP^{DTR}; DAT^{TRPV1} mice were presented with a palatable high-fat diet, and the consequence of AgRP neuron ablation was evaluated in the presence or absence of capsaicin in the drinking water (Figures 3C and 3D). AgRP neuron ablation led to rapid body weight loss and starvation (Figures 3B–3D). However, mild activation of dopamine neurons with capsaicin in the water prevented body weight (BW) loss and starvation in AgRP^{DTR}; DAT^{TRPV1} mice provided with a palatable diet (Figures 3C and 3D). When these mice were on a chow diet, capsaicin treatment was unable to prevent starvation (data not shown).

We also tested the effect of transient inhibition of AgRP neurons in adult mice using a designer receptor exclusively activated by designer drug (DREADD) approach. Male mice expressing Cre specifically in AgRP neurons (*AgRP^{ires-Cre}*) received a bilateral stereotactic injection of the Cre-dependent AAV9-hSyn-DIO-HA-KOR expressing the newly designed Gi-coupled DREADD using the κ opioid receptor (KORD) activated by salvinorin B (SalB), a pharmacologically inert ligand (Vardy et al.,

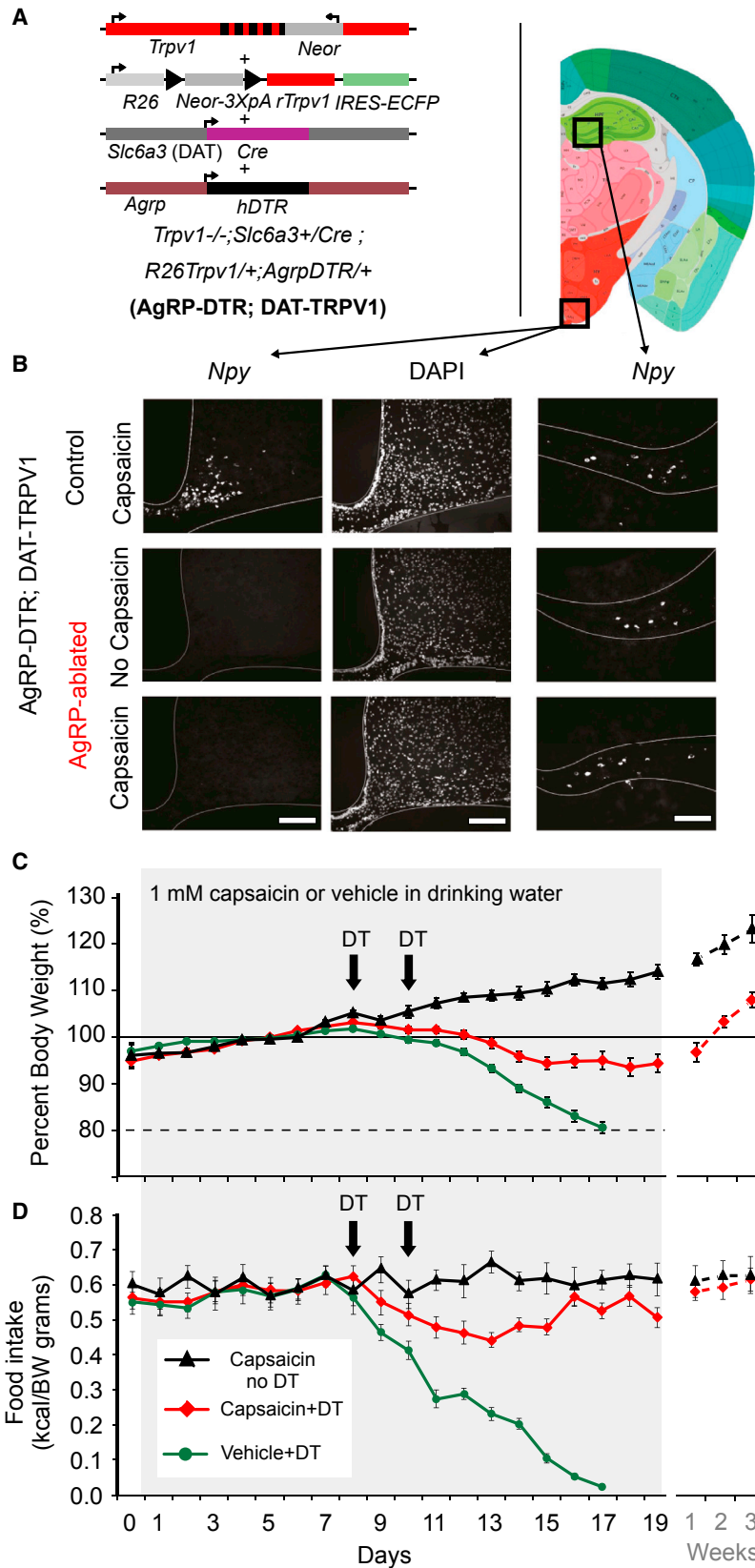


Figure 3. Mild Activation of Dopamine Neurons Prevents Starvation following Acute Ablation of AgRP Neurons in Adult Mice

(A) *Trpv1*^{-/-}; *Slc6a3*^{+/*Cre*}; *R26*^{Trpv1/+} mice (DAT^{TRPV1}; Güler et al., 2012) were crossed to *Agrp*^{DTR/+} mice (Luquet et al., 2005) on the C57Bl/6 background to generate AgRP^{DTR} mice. The resulting triple transgenic mice conditionally express the rat *Trpv1* (*rTrpv1*) gene from the *Gt(ROSA)26Sor* (*R26*) locus only in dopamine neurons expressing CRE recombinase under the dopamine transporter (*Slc6a3* or *DAT*) promoter on the *Trpv1* knockout background (*Trpv1*^{-/-}) *Neor*, neomycin resistance gene; IRES-ECFP, internal ribosome entry site-enhanced cyan fluorescent protein.

(B) Fluorescent in situ hybridization for NPY (left and right) and 4',6-diamidino-2-phenylindole (DAPI) counterstaining (center) in the arcuate (left) and hippocampus (right) of control (top) and AgRP^{DTR} mice (bottom) injected with diphtheria toxin. Scale bars, 200 μ m.

(C and D) Body weight change (C, percent) and food intake (D, kilocalories per gram of BW) of AgRP^{DTR}; DAT^{TRPV1} mice with capsaicin (1 mM) in the drinking water (black triangle, n = 6 mice) or injected twice with DT with capsaicin (red diamond, n = 14 mice) or vehicle (green circle, n = 15 mice) in the drinking water. Following AgRP neuron ablation, all AgRP^{DTR}; DAT^{TRPV1} mice with no access to capsaicin treatment had a concomitant decrease in food intake and body weight within 7 days. Mice at 80% of baseline body weight were not rescued and sacrificed.

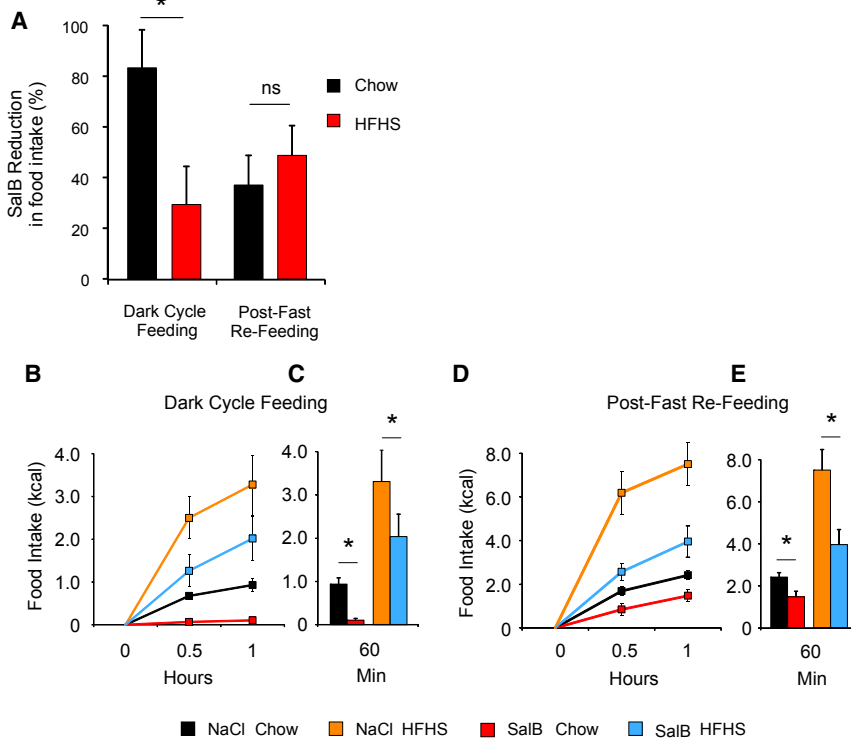


Figure 4. Acute Silencing of AgRP Neurons Differentially Impact Chow and Palatable Food Intake

(A) SalB-mediated inhibition of food intake in percentage over saline injection inhibition of food intake initiated for each animal. Comparison between groups was performed using ANOVA for each paradigm. Values are expressed as mean \pm SEM issue of the ANOVA. * $p < 0.05$; ns, non-significant.

(B–E) 2-hr food intake (kilocalories) after onset of the dark period (B and C) or after a fast (D and E). Values are expressed as mean \pm SEM of animals treated with saline or SalB under a standard and an HFHS diet. Food intake was recorded at 0.5 and 1 hr. The values in (C) and (E) are expressed as mean \pm SEM at 1 hr. Comparison was performed between saline and SalB treatment using a nonparametric Wilcoxon-Mann-Whitney test. * $p < 0.05$.

2015). SalB-mediated AgRP neuron silencing was assessed during spontaneous feeding at the onset of the dark period and in response to a fast on both chow and an HFHS diet. Acute silencing of AgRP neurons resulted in an $\sim 80\%$ decrease in spontaneous chow intake compared with saline injection, whereas the palatable diet intake was inhibited by only $\sim 30\%$ (Figures 4A–4C). Following a fast, SalB-mediated inhibition of AgRP neurons resulted in an $\sim 50\%$ decrease in food intake regardless of diet palatability (Figures 4A–4E). These results show that, depending on metabolic status, the contribution of metabolic demands and reward contribute differentially to overall nutrient intake.

To determine whether palatability or nutrient content could explain the difference in feeding response observed in mice lacking AgRP neurons, control and AgRP-ablated mice were compared for preference of either caloric (sucrose) or non-caloric (saccharin) solution versus water in a two-bottle paradigm. Both groups had similar preferences for sucrose or saccharin, with a greater preference for sucrose over saccharin (Figure S5A), as well as a similar shift in preference under conditions of increasing sucrose concentration in both 2-hr and overnight consumption tests (Figures S5B–S5D). These data indicate that control and AgRP-ablated mice have similar responses to sweeteners, suggesting that fat in the HFHS diet is more likely to affect the palatability preference displayed by AgRP-ablated mice.

Ablation of AgRP Neurons Reveals a Functional Dichotomy in the Anorectic Action of Serotonin According to Food Palatability

5-HT innervation of ARC neurons mediates satiety by the concerted activation of both the Gi-coupled 5-HT_{1B} receptors

on AgRP neurons and Gq-coupled 5-HT_{2C} receptor activation on POMC neurons (Heisler et al., 2006). This arrangement predicts that AgRP-ablated mice should manifest reduced sensitivity to the anorectic effects of 5HT_{1B}R compared with the 5HT_{2C}R agonist. The feeding response after a fast on a chow diet was monitored following administration of the selective 5-HT_{1B}R agonist CP94253 (CP) and the 5-HT_{2C}R agonist WAY161503 (WAY), using doses that produce a significant reduction in refeeding (Heisler et al., 2006). Although pretreatment with CP 45 min prior to refeeding produced an $\sim 50\%$ decrease in chow intake in control animals, this treatment was ineffective compared with saline in mice lacking AgRP neurons (Figures 5A and 5B). WAY injection produced a greater anorectic response in AgRP-ablated mice than in control mice (Figures 5C and 5D). These results provide evidence that, despite the fact that several other brain regions express 5-HT_{1B} receptors, AgRP neurons are a primary mediator in the anorectic action of the 5-HT_{1B}R agonist. Having established that AgRP neurons are a principal mediator of both ghrelin and 5-HT_{1B}R agonist responses, we predicted that transient inhibition of AgRP neurons with one agonist should reduce feeding by the other agonist in wild-type animals. Feeding responses to peripherally injected ghrelin (500 $\mu\text{g}/\text{kg}$, i.p.) alone or in association with a pretreatment with CP (8 mg/kg, i.p.) were measured for 2 hr in wild-type mice. CP inhibited ghrelin-induced feeding as predicted (Figures 5E and 5F). Quantification of serotonin content in various brain areas did not reveal any adaptive change as a consequence of AgRP neuron ablation (Figure S3).

Based on the radical change in ghrelin response that we observed by manipulating diet palatability, we also assessed the feeding response to 5-HT receptor agonists when mice were provided with a palatable diet, using the same experimental design as in Figures 5A–5D. With substitution of an HFHS diet, both saline-treated control and AgRP-ablated mice displayed a similar feeding response and had similar anorectic responses to both 5-HT_{2C}R and 5-HT_{1B}R agonists (Figures 5G–5J),

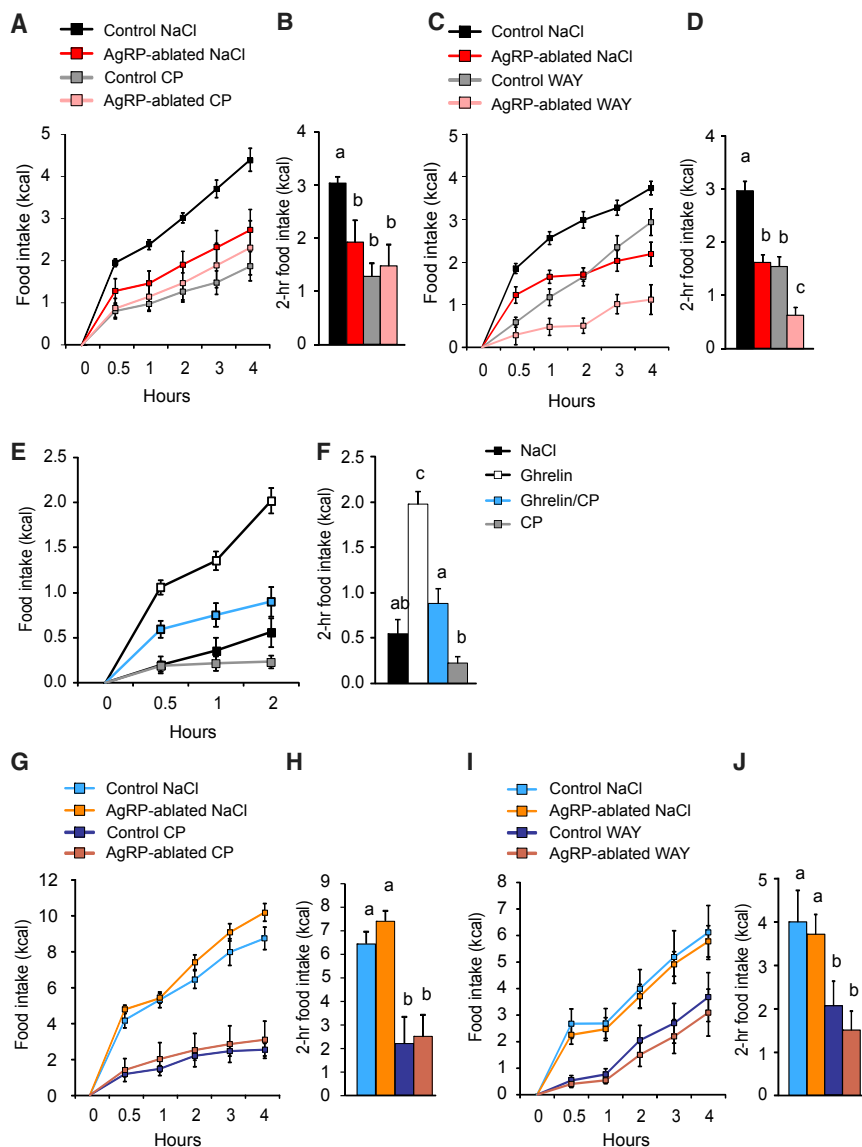


Figure 5. Satiety Action of the 5-HT Agonist Requires AgRP Neuron Integrity on a Chow Diet but Not on a Palatable Diet

(A–D) 4-hr caloric response to fasting (A and C) and 2-hr cumulative food intake (B and D) of control (black and gray) and AgRP-ablated mice (red and rose) fed a chow diet and injected i.p., 45 min prior to refeeding with NaCl (black and red), the 5-HT_{1B}R agonist CP (A and C; gray and rose), or the 5-HT_{2C}R agonist WAY (B and D; gray and rose). (E and F) 2-hr caloric intake (E) and cumulative intake (F) after an injection (i.p.) of NaCl (black) and ghrelin (white box) associated with CP (blue box) and CP alone (gray box).

(G–J) 4-hr feeding (G and I) and cumulative caloric intake (H and J) in response to fasting in AgRP-ablated (orange and brown) or control (blue and navy blue) mice fed an HFHS diet and injected 45 min prior to refeeding with NaCl (blue and orange), CP (G and H; navy blue and brown), or WAY (I and J; navy blue and brown).

n = 6–10 mice in each group. a, b, and c, differ significantly ($p < 0.05$). Data are expressed as mean \pm SEM.

sustained overfeeding (Figure 6A) and body weight gain (Figure 6B).

Reciprocally, social isolation achieved by singly housing control or AgRP-ablated mice was used to quantify the action of a mild stress on food intake (Hao et al., 2001). Two-month-old control and AgRP-ablated mice with the same body weight were housed singly and given normal chow. Body weight loss was significantly greater in AgRP-ablated mice during the 2 weeks following social isolation (Figure 6C). When access to a palatable liquid diet was provided, the body weight of the AgRP-ablated mice was regained rapidly (Figure 6C). Furthermore, although control animals main-

sustained overfeeding (Figure 6A) and body weight gain (Figure 6B).

Feeding Behavior in the Absence of AgRP Neurons: A Model of Comfort Feeding

Our data suggest that ingestion of a palatable diet is relatively independent of AgRP neurons. We predict, therefore, that mice lacking AgRP neurons represent a good model of “comfort feeding” in which feeding in response to energy demands is crippled because of the lack of a hypothalamic orexigenic drive and relies essentially on reward. We evaluated the daily caloric intake over a period of 4 months, during which animals were exposed to chow for 2 months after weaning and then shifted to an HFHS diet for another 2-month period. Food intake was measured weekly by group-housed mice. Although chow intake was similar between the two groups before and after shifting diets, shifting AgRP-ablated mice onto the HFHS diet resulted in

tained caloric intake, the AgRP-ablated mice consumed more calories (Figure 6D). Our finding that AgRP-ablated mice exhibit exaggerated hyperphagia on a palatable diet and greater anorexia during stress is consistent with the hypothesis that, in the absence of AgRP neurons, feeding is less motivated by energy demand and more sensitive to changes in stress and reward value (Chuang et al., 2011).

AgRP-Ablated Mice Display Impaired Feeding but Abnormal Anticipation on a Food-Restricted Schedule

Restricting feeding to a short time window is a paradigm used to assess food-seeking behavior in response to scheduled meals. Animals with limited access to food learn to anticipate food availability by increasing locomotion (food anticipatory activity [FAA]) and optimize their consummatory behavior during that period. Ghrelin signaling is critical for FAA and consumption (Blum et al., 2009; Verhagen et al., 2011). When assessed on a chow diet, we found that the total locomotor activity by AgRP-ablated

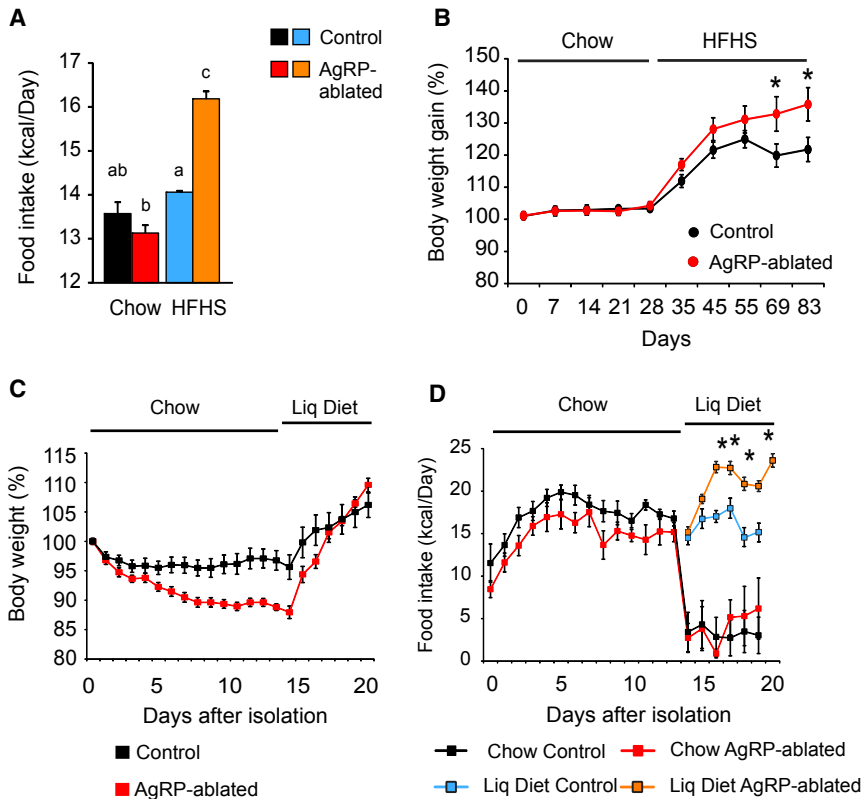


Figure 6. Ablation of AgRP Neurons Creates a Context Favorable for Comfort Feeding

(A) Weekly averaged caloric intake over 2 months by control (black and blue) and AgRP-ablated (red and orange) mice presented with a chow (black and red) and then an HFHS diet (blue and orange). (B) Body weight gain of control (black) and AgRP-ablated (red) mice monitored during the shift to the HFHS diet.

(C and D) Evaluation of body weight change (C) and caloric intake (D) by AgRP-ablated (red and orange) or control (black and blue) mice over 20 days after social isolation fed with a chow diet (black and red) and presented with a liquid (Liq) diet on the five remaining days (blue and orange).

n = 6–10 mice in each group. *p < 0.05, control versus AgRP-ablated mice. a, b, and c differ significantly (p < 0.05). Data are expressed as mean ± SEM.

mice was initially decreased relative to control mice but recovered by day 5. When the mice were on an HFHS diet, the AgRP-ablated group recovered normal locomotion faster (Figure 7A). AgRP-ablated mice on a chow diet had decreased FAA that reached significance only on day 4 after the start of restriction (Figure 7B). No deficit FAA was observed by AgRP-ablated mice on the HFHS diet.

Spontaneous activity during the 2 hr before the meal and the 2 hr of meal availability were the same for all four groups (Figure 7C). Chow consumption was blunted significantly in AgRP-ablated mice compared with control mice (Figure 7D) but more so by AgRP-ablated mice on a chow diet than on an HFHS diet (Figures S6A and S7A). These results agree with a study showing that partial ablation of AgRP neurons leads to defective adaptation of feeding and FAA (Tan et al., 2014; Figures 7B and 7D; Figure S7A). The observation that FAA behavior is intact even when ghrelin action on AgRP neurons is not possible suggests that ghrelin-dependent anticipatory behavior is mediated by other neural circuits.

DISCUSSION

The mammalian brain has evolved several very efficient and redundant interconnected neural circuits that encode both metabolic needs and reward in the complex sequence of feeding behavior. Although the hedonic drive to eat energy-dense food has the evolutionary advantage of promoting a strong motivational response to seek and consume nutrients and ensure survival in times of scarcity, it has become a challenge because of the radical change in feeding habits in modern society character-

ized by an almost ubiquitous source of calorie-dense foods (Saper et al., 2002). Understanding the net contribution of homeostatic energy-driven and non-homeostatic reward-driven feeding has become critical and requires a deeper understanding of the neuronal mechanism by which energy needs and reward participate in food intake.

AgRP neurons represent a critical component of the homeostatic circuitry that respond to decreased nutrient availability by engaging the full sequence of feeding behaviors (Aponte et al., 2011; Carter et al., 2013; Cowley et al., 1999, 2003; Krashes et al., 2011). Furthermore, ablation of AgRP neurons in adult mice results in profound anorexia (Bewick et al., 2005; Gropp et al., 2005; Luquet et al., 2005; Xu et al., 2005). Therefore, these neurons represent a critical component of the homeostatic circuitry.

To address the contribution of AgRP neurons in reward- and non-reward-driven feeding, we used various models in which AgRP neuron activity is compromised. We found that, although AgRP neuron integrity is required to mediate normal feeding after a fast, administration of ghrelin or a 5-HT_{1B}R agonist, or during a meal-restricted schedule, AgRP neurons are dispensable when feeding is assessed on an HFHS diet. Ghrelin-induced feeding by AgRP-ablated mice required the integrity of the ghrelin signaling pathway in the VTA, and enhancing dopamine tone or food palatability selectively restored feeding to either post-natal or adult AgRP neuron ablation. Reciprocally, dopamine receptor agonists had a more pronounced satiety action in AgRP-ablated mice compared with controls. Importantly, long-term exposure to an HFHS diet, which results in ghrelin resistance in AgRP neurons (Briggs et al., 2010), produced a behavioral response to ghrelin similar to that observed in AgRP-ablated mice. Mice lacking AgRP neurons are also more susceptible to both stress-induced anorexia and palatable-diet hyperphagia. The palatability effect is observed after neonatal ablation, after adult ablation, or with transient inactivation of AgRP neurons. Therefore, this phenomenon is not entirely due to developmental effects.

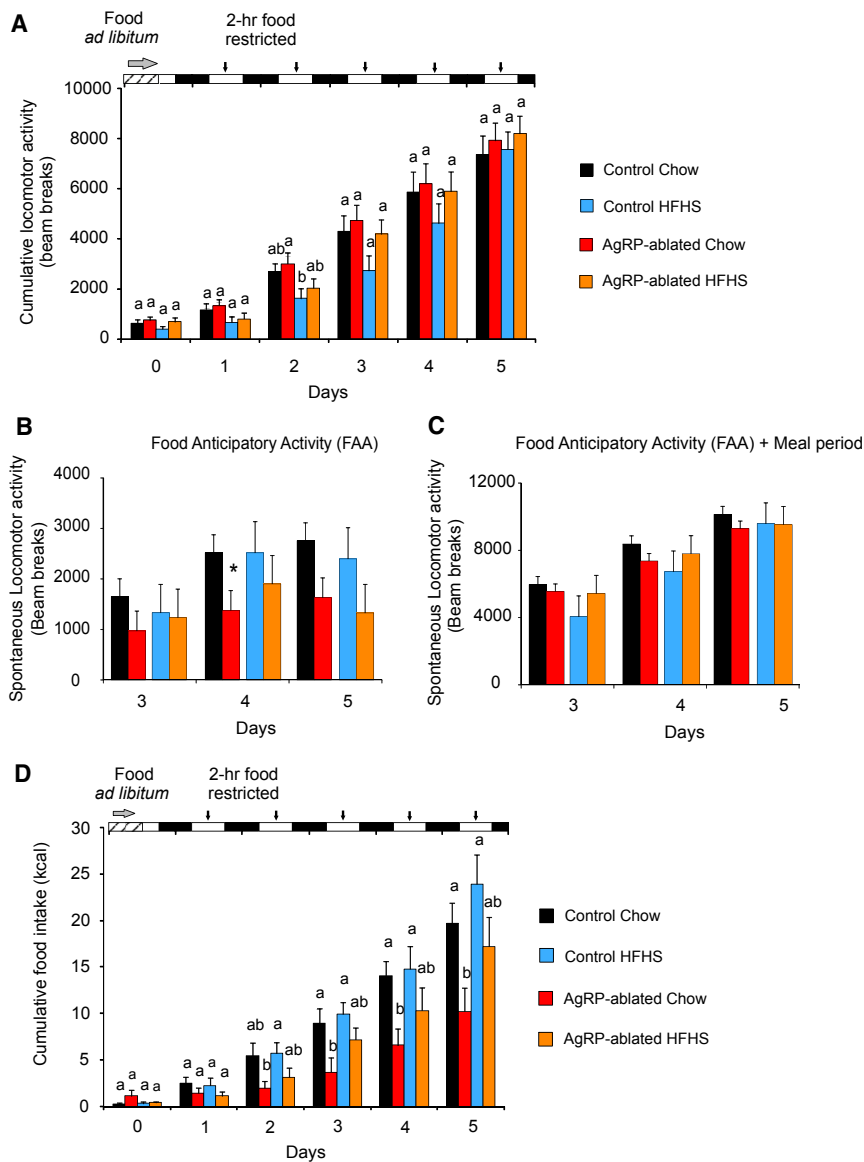


Figure 7. Food Palatability Restores the Feeding Deficit of AgRP-Ablated Mice on a Feeding-Restricted Schedule

(A and D) Five-day cumulative locomotor activity (A) and cumulative caloric intake (D) of control (black and blue) and AgRP-ablated (red and orange) mice with 2-hr restricted access to chow (black and red) or an HFHS diet (blue and orange). $n = 6/\text{group}$. a, b, and c, differ significantly ($p < 0.05$). Time bars: food *ad libitum* (striped), daylight (white), and night (black). Arrows indicate 2-hr food access.

(B and C) Spontaneous locomotor activity of control (black and blue) and AgRP-ablated (red and orange) mice fed with a chow (black and red) or HFHS diet (blue and orange) on days 3, 4, and 5 of the restricted schedule, recorded during the 2 hr before food was available (B) and during the 2 hr before food access (FAA) and 2 hr of food availability (Meal) (C). ANOVA with groups, diet, and days and their interactions as factors showed a significance-diminished locomotor activity referred as FAA in mice lacking AgRP neurons only when fed a chow diet (ANOVA, $2,307 \pm 206.3$ versus $1,327 \pm 225.9$ under a chow diet, $p < 0.01$; $2,082 \pm 346.1$ versus $1,489 \pm 325.1$ under HFHS conditions, ns).

$n = 6/\text{group}$. * $p < 0.05$, control versus AgRP-ablated mice. Data are expressed as mean \pm SEM. See also Figures S6 and S7.

Our results suggest that, when AgRP neuron activity is impaired, neural circuits sensitive to emotion and stress are engaged and modulated by food palatability and dopamine signaling.

Our data demonstrate that peripherally injected ghrelin can increase consumption of palatable food in a VTA-dependent manner when AgRP neurons are absent. This result is reminiscent of a recent study showing that ghrelin action on dopaminergic neurons is sufficient to drive food reward in a context of chronic stress, referred to as comfort feeding (Chuang et al., 2011). Our observations are also in line with a study showing that loss of AgRP neuron function promotes greater excitability of VTA dopamine neurons (Dietrich et al., 2012). We show that dopamine neuron activity and food palatability are both important contributors to food intake when AgRP neuron activity is chronically compromised.

From an evolutionary standpoint, animals have evolved mechanisms to cope with the ever-present potential for starvation. AgRP neurons are most active during starvation conditions,

and they send axonal projections to many brain regions (Broberger et al., 1998), presumably to orchestrate adaptive responses that facilitate energy conservation (e.g., by inhibiting metabolism, growth, reproduction, and satiation), while promoting foraging and feeding (Betley et al., 2013). Therefore, in the absence of AgRP neurons, we predict that suppression of energy-demanding activities would be impaired and that feeding would depend on alternative neural circuitry. Chronic desensitization of AgRP neurons because of long-term exposure to an energy-rich diet could result in the progressive replacement of metabolic demand by reward seeking as the main driver of food intake. Reciprocally, consuming highly palatable food may provide stronger taste signals as well as increased dopamine release by the VTA and, ultimately, adaptive change in the reward circuitry (Lockie and Andrews, 2013). During chronic exposure to a palatable diet, orosensory inputs relaying food reward might gradually prevail on AgRP-mediated inputs relaying metabolic needs. The overall consequence would be to minor the implication of AgRP neuron activity onto nutrient intake. Under these conditions, the contribution of AgRP neurons could be largely bypassed by extrinsic hedonic neural circuitry.

In addition, any attempt to chronically decrease AgRP neuron activity as a pharmacological intervention might have the adverse consequence of enhancing neural circuits that are more susceptible to emotion (stress or anxiety) and food reward—hallmarks of comfort feeding (Dallman et al., 2005).

EXPERIMENTAL PROCEDURES

Animals

All animal experiments were performed with approval of the Animal Care Committee of the University Paris Diderot-Paris 7. Mice (8-9 months old or as otherwise stated in the text) were housed individually in stainless steel cages in a room maintained at $22.5 \pm 1^\circ\text{C}$ with lights scheduled from 7 am to 7 pm. Animals had access to water and food *ad libitum* unless specified otherwise. Details are provided in the [Supplemental Experimental Procedures](#).

Drugs and Peptides

Drugs or peptides were dissolved according to the manufacturer in vehicle solution: saline for i.p. injection and artificial cerebrospinal fluid (Alzet) for central injection. Acetylated ghrelin (Polypeptide) was either administered i.p. (150 nmol/kg BW) or centrally (1 nmol/mouse at a rate of 0.4 $\mu\text{l}/\text{min}$ in a final volume of 0.8 μl). Antagonist (D-[Lys3]-GHRP-6 (Tocris Bioscience) of the ghrelin receptor (GHS-R1a) was administered centrally (1 nmol/mouse at a rate of 0.4 $\mu\text{l}/\text{min}$ in a final volume of 0.8 μl). Selective serotonin receptor agonists (Tocris) were administered i.p. at 10 $\mu\text{l}/\text{g}$ of BW: the 5-HT_{1B}R agonist CP (8 mg/kg) and the 5-HT_{2C}R agonist WAY (7 mg/kg). Dopamine receptor agonists and an inhibitor of dopamine re-uptake (Tocris) were administered i.p. at 10 $\mu\text{l}/\text{g}$ of BW. Final concentrations were the selective dopamine receptor 1 agonist (D1R) SKF 38393 hydrobromide (10 mg/kg), the selective dopamine receptor 2 (D2R) agonist quinpirole hydrochloride (0.2 mg/kg), and the inhibitor of dopamine uptake GBR (7.5 mg/kg). Capsaicin (97% by high-pressure liquid chromatography [HPLC], Sigma) containing drinking water was prepared as a 1 M stock solution in ethanol and diluted 1,000-fold in 1% Tween 80 (1 mM final concentration). The control solution contained 0.1% ethanol and 1% Tween 80. DT (50 $\mu\text{g}/\text{kg}$, List Biological Laboratories) or vehicle injection was administered via subcutaneous or intramuscular routes in neonates or adult animals, respectively. (SalB was dissolved in DMSO and injected i.p. at 10 mg/kg as described by [Vardy et al. \(2015\)](#)).

Fluorescent Ghrelin Synthesis and Binding In Vivo

Bioactive fluorescent ghrelin was obtained as described previously ([Schaeffer et al., 2013](#)). Briefly, active fluorescent ghrelin (25 nmol in 100 μl) was injected intravenously (i.v.) into the tail vein in both control and AgRP-ablated mice fed with either a chow diet or an HFHS diet. Terminally anesthetized mice at 5–10 min post-injection were perfused via the heart with 10 ml of potassium phosphate buffer 0.1 M (PBS). Brains were collected and immersed for 24 hr in 4% paraformaldehyde solution (PFA), subsequently sliced using a Leica vibratome (50- μm coronal slices), and prepared for confocal imaging as described in the [Supplemental Experimental Procedures \(Schaeffer et al., 2013\)](#).

Spontaneous Activity and Feeding

Spontaneous feeding and locomotor activity (beam breaks/hour) were measured using automated feeding and activity recording in Labmaster (TSE Systems). Each cage was equipped with a set of highly sensitive feeding and drinking sensors for automated online measurement and embedded in a frame with an infrared light beam-based locomotion monitoring system. The sensors for detection of movement operate efficiently under both light and dark phases, allowing continuous recording. Mice were housed individually and acclimated to the chambers for 48 hr before experimental measurements.

Acute Feeding after a Fast

Feeding after a fast was carried out as follows. The cage bedding was changed, and food was removed at 10 am until the next day at 10 am, when carefully weighed food was replaced and weighed again 4 hr later. Body weight was measured before and after the fasting period and 4 hr after refeeding. When appropriate, 30 min prior to replacing the food, animals received an i.p. injection of vehicle or selected compounds.

Time-Restricted Feeding Schedule

Mice were subjected to a time-restricted feeding protocol. Six control and six AgRP-ablated mice were housed individually in an activity recording system with automated food and liquid access control (Labmaster, TSE

Systems). Following 72 hr of adaptation to the new environment with food and drink *ad libitum*, mice were temporally restricted access to food from 12 pm to 2 pm every day for 5 consecutive days. Spontaneous locomotor activity and food and water intake were monitored continuously. When appropriate, food spillage was collected and subtracted from the calculated food intake.

Data Analysis

The results are expressed as mean \pm SEM. Variance equality was analyzed by F test (Microsoft Excel), and comparisons between groups were carried out using Student's t test or nonparametric Mann-Whitney-Wilcoxon's test (Minitab). When appropriate, analyses of variances were performed, followed either by Bonferroni or Tukey's post hoc test with the appropriate parameters and their interaction as factor (Minitab, Paris, France). Unless indicated otherwise in the text, a p value < 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article at <http://dx.doi.org/10.1016/j.cmet.2015.07.011>.

AUTHOR CONTRIBUTIONS

A.J.A. and R.G.P., designed and performed most of the experiments. R.G.P. developed the ghrelin aspect, and A.J.A. collected data on serotonin. J.C., C.C., A.-S.D., and A.L. participated in *in vivo* experiments and indirect calorimetry. C.R. performed HPLC measurements, and N.K. and S.M. performed the molecular analysis. M.S., F.L., and B.D. contributed to the fluorescence ghrelin analysis, and A.L. performed c-fos studies. J.A.F., J.M., and P.V. designed and provided fluorescent ghrelin. R.P., S.P., and A.G. contributed data with TRPV-dependent activation of dopamine neurons. E.W. and M.K. contributed to DREADD inhibition of AgRP neurons with T.H., and C.M. contributed to data analysis and interpretation and also helped with the manuscript. S.L. acquired funding, conceptualized and interpreted the studies, and wrote the manuscript.

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