Short Article

Rapid versus Delayed Stimulation of Feeding by the Endogenously Released AgRP Neuron Mediators GABA, NPY, and AgRP

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SUMMARY

Agouti-related peptide (AgRP) neurons of the hypothalamus release a fast transmitter (GABA) in addition to neuropeptides (neuropeptide Y [NPY] and Agouti-related peptide [AgRP]). This raises questions as to their respective functions. The acute activation of AgRP neurons robustly promotes food intake, while central injections of AgRP, NPY, or GABA agonist results in the marked escalation of food consumption with temporal variance. Given the orexigenic capability of all three of these neuroactive substances in conjunction with their coexpression in AgRP neurons, we looked to unravel their relative temporal role in driving food intake. After the acute stimulation of AgRP neurons with DREADD technology, we found that either GABA or NPY is required for the rapid stimulation of feeding, and the neuropeptide AgRP, through action on MC4 receptors, is sufficient to induce feeding over a delayed yet prolonged period. These studies help to elucidate the neurochemical mechanisms of AgRP neurons in controlling temporally distinct phases of eating.

INTRODUCTION

As it becomes more and more apparent that many neurons containing fast-acting neurotransmitters corelease slower neuromodulator peptides (van den Pol, 2012), new methodology is necessary in order to interpret their relative roles and contributions on downstream circuits. Here, we aimed to dissect the functional contributions of the neurotransmitter and neuromodulators (collectively termed "neuromediators") released by Agouti-related peptide (AgRP) neurons, a small subset of hypothalamic neurons located in the arcuate nucleus (ARC). AgRP neurons release three known neuroactive chemicals, the amino acid transmitter GABA, and the neuropeptides neuropeptide Y (NPY) and AgRP, from which their name is derived.

Many lines of evidence strongly support a critical role for AgRP neurons in driving food intake, as either optogenetic (Aponte et al., 2011) or pharmacogenetic (Krashes et al., 2011) AgRP neuronal stimulation evokes rapid feeding, and the initial hour of feeding during optogenetic AgRP photoactivation is independent of the melanocortin pathway (Aponte et al., 2011). Conversely, pharmacogenetic inhibition of AgRP neurons blunts food consumption (Krashes et al., 2011), and the acute ablation of AgRP neurons in adult animals results in the cessation of feeding and, ultimately, starvation (Gropp et al., 2005; Luquet et al., 2005), demonstrating their necessity in regulating appetite.

The pharmacological administration of either NPY or AgRP into the hypothalamus induces a robust hyperphagic response in rodents (Clark et al., 1984; Rossi et al., 1998; Semjonous et al., 2009) with distinct temporal dynamics; NPY results in immediate feeding, whereas AgRP increases food intake over a delayed, longer time scale (Semjonous et al., 2009). Moreover, the genetic overexpression of the *AgRP* gene encoding the neuropeptide, which acts as an antagonist and inverse agonist on downstream melanocortin 4 receptors (MC4R), promotes food intake (Ollmann et al., 1997). Furthermore, GABA receptor agonists administered to the nucleus accumbens shell elicit intense feeding (Stratford and Kelley, 1997). Additionally, GABAergic signaling onto the parabrachial nucleus (PBN), a downstream target of the AgRP circuit, restores appetite after acute AgRP neuron ablation (Wu et al., 2009). Thus, each of these neuromediators has been shown to play a part in enhancing food consumption.

Despite these findings, the targeted deletion of *Agrp*, *Npy*, and/or *Slc32a1* (vesicular GABA transporter [VGAT]; required for GABA release) all have minimal effects on feeding (Erickson et al., 1996; Qian et al., 2002; Tong et al., 2008). To circumvent these inconsistencies, we employed stimulatory designer receptors exclusively activated by designer drugs (DREADD) technology (Alexander et al., 2009; Krashes et al., 2011) in order to acutely and explicitly activate AgRP neurons in genetic mouse models that have had the release of GABA, NPY, or MC4R signaling disrupted. Using this approach, we are able to test for both the necessity and sufficiency of these individual neuromediators in regulating short-term and long-term food intake (Table S1 available online).
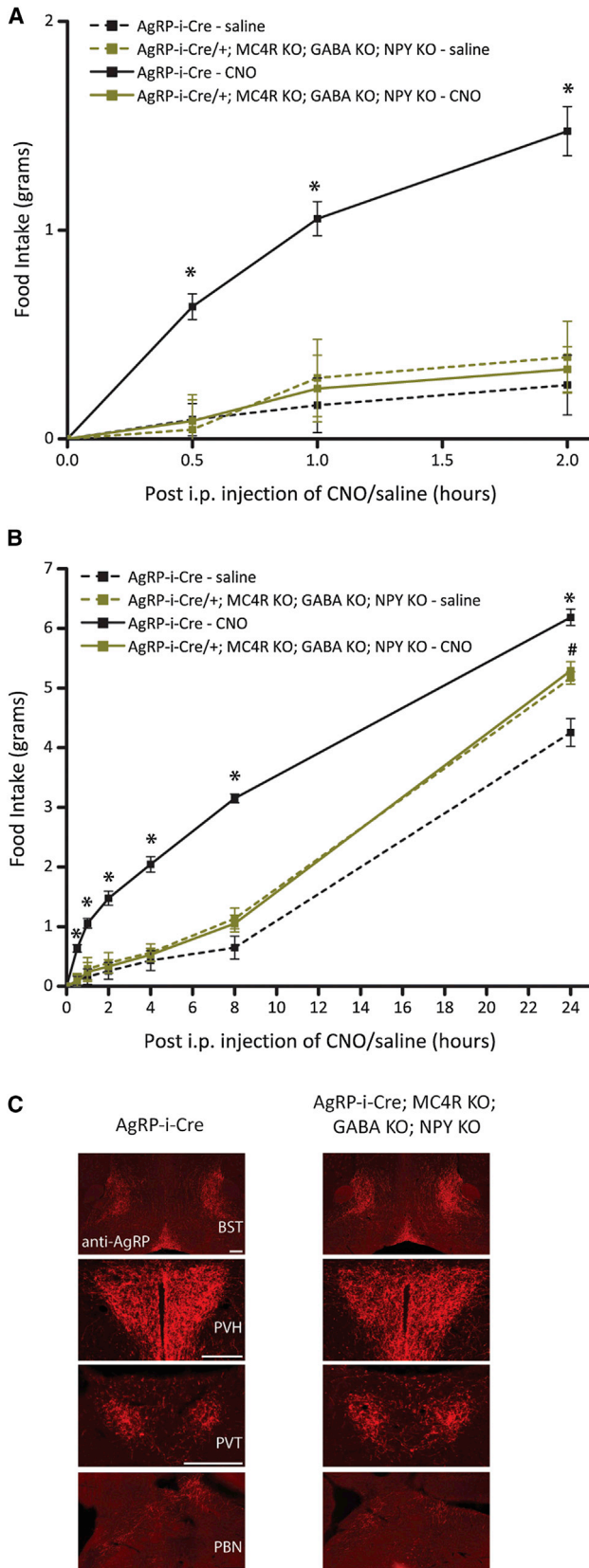
Figure 1. Acute Pharmacogenetic Activation of AgRP Neurons in Mice without the Release of GABA, NPY, and AgRP Signaling via MC4Rs, Collectively, Lack DREADD-Mediated Increases in Food Intake

Data were pooled across multiple trials. All mice in these studies were bilaterally injected with AAV8-DIO-hM3Dq-mCherry in the ARC.

(A and B) The activation of AgRP neurons increases food intake in *AgRP-ires-Cre* mice (black line) but has no effect on food intake in *AgRP-ires-Cre; Mc4r$^{-/-}$; Vgat$^{flox/flox}$; Npy$^{-/-}$* triple KO mice (light green line). CNO (solid line; 0.3 mg/kg of body weight, i.p.) or saline (dotted line) was injected 3 hr after the start of the lights on cycle, and food intake was assessed 1 and 2 (A) and 4, 8, and 24 hr (B) postinjection (PI) over three trials of each treatment. Data shown are from male mice (error bars indicate mean ± SEM, n = 8 *AgRP-ires-Cre* mice; n = 5 *AgRP-ires-Cre; Mc4r$^{-/-}$; Vgat$^{flox/flox}$; Npy$^{-/-}$* mice; *p < 0.05 *AgRP-ires-Cre* CNO group versus all other groups; #p < 0.05 *AgRP-ires-Cre; Mc4r$^{-/-}$; Vgat$^{flox/flox}$; Npy$^{-/-}$* groups versus *AgRP-ires-Cre* saline group).

(C) Immunohistochemical analysis of AgRP projections in *AgRP-ires-Cre* mice (left) and *AgRP-ires-Cre; Mc4r$^{-/-}$; Vgat$^{flox/flox}$; Npy$^{-/-}$* triple KO mice (right) to (top-bottom) the bed nucleus of the stria terminalis (BST), paraventricular hypothalamus (PVH), paraventricular thalamus (PVT), and lateral parabrachial nucleus (PBNl) reveals no gross differences in morphology and/or density (200 μm). See also Figures S1 and S4 and Table S1.

RESULTS

DREADD-Mediated AgRP Stimulation in Triple KO Mice Abrogates Feeding Response

As proof of principle, we targeted a Cre-dependent adeno-associated virus (AAV) expressing the hM3Dq excitatory DREADD (Krashes et al., 2011), specifically to AgRP neurons with either *AgRP*-ires-*Cre* control or *AgRP-ires-Cre; Mc4r$^{-/-}$; Vgat$^{flox/flox}$; Npy$^{-/-}$* triple knockout (KO) mice lacking GABA release from AgRP neurons as well as ubiquitous deletion of NPY and MC4R, the downstream target of the AgRP neuropeptide (Balthasar et al., 2005; Erickson et al., 1996; Marsh et al., 1999b; Tong et al., 2008). It should be noted that the *Mc4r$^{-/-}$* mice are obese because of hyperphagia and reduced energy expenditure (Balthasar et al., 2005); however, the studies presented here evaluate feeding behavior after acute AgRP neural activation, making it possible to evaluate the acute role of the AgRP neuropeptide.

Food intake was first measured near the beginning of the light cycle, a time when mice normally refrain from eating, and continually monitored throughout the day (food intake was assessed at 0.5, 1, 2, 4, 8, and 24 hr postinjection). Each trial consisted of saline administration on day 1, CNO administration on day 2, and no injection on day 3 and was repeated over three trials to demonstrate the robustness and stability of the response (for each series of studies, the pooled data from all trials are shown in the main figures, and the data from the first trial are shown in the corresponding Supplemental Figures). Importantly, the results from the pooled trials, in all cases, were similar to the results from the first trial. Even in this calorically replete state, the acute activation of AgRP neurons, via clozapine-N-oxide (CNO; 0.3 mg/kg) administration, resulted in voracious feeding in *AgRP-ires-Cre* mice over the first 2 hr (Figures 1A and S1A) and across a 24 hr window (Figures 1B and S1B) in comparison to the same mice after a saline injection (Krashes et al., 2011) but failed to evoke food intake in triple KO mice, demonstrating that the release of either GABA, NPY, and/or AgRP action onto MC4Rs is required for increases in food intake after direct AgRP neuronal stimulation (a post hoc Tukey test showed that CNO-treated *AgRP-ires-Cre* mice exhibited significantly
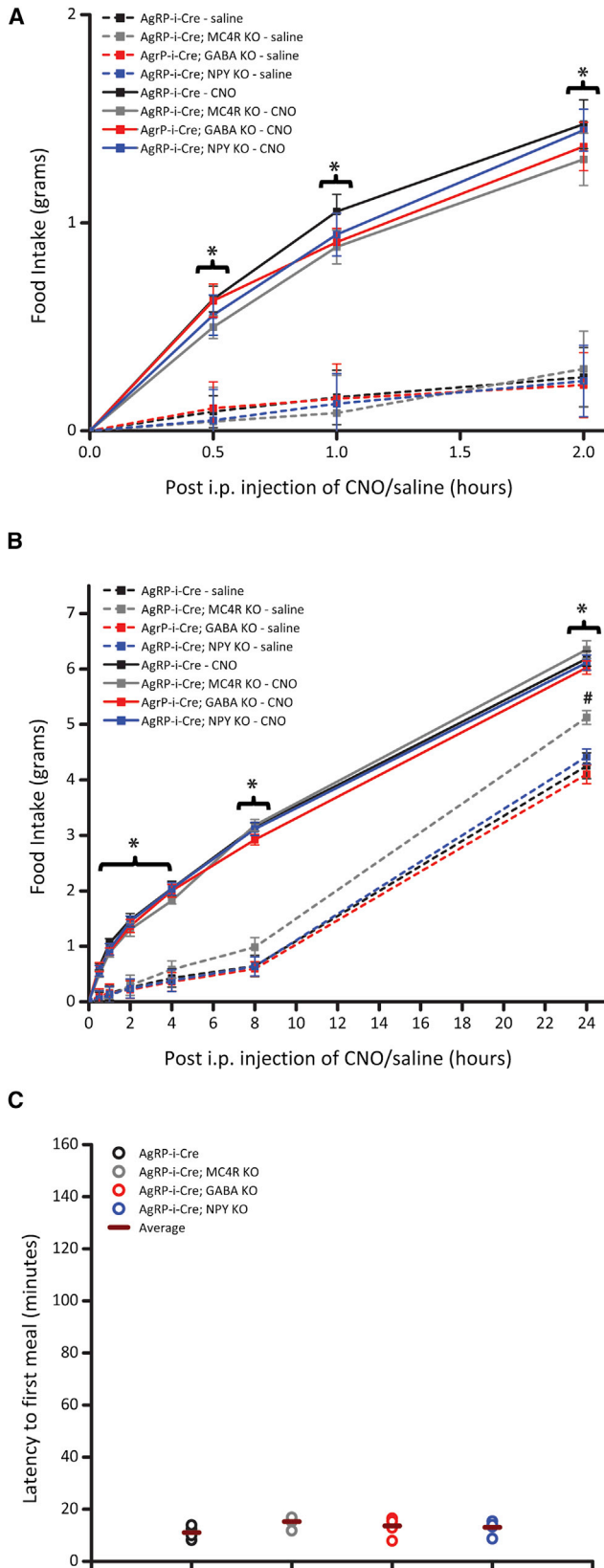
Figure 2. Acute Pharmacogenetic Activation of AgRP Neurons in Mice without the Release of GABA, NPY, or AgRP Signaling via MC4Rs, Individually, Display Intact DREADD-Mediated Increases in Food Intake

Data were pooled across multiple trials. All mice in these studies were bilaterally injected with AAV8-DIO-hM3Dq-mCherry in the ARC.

(A–C) The activation of AgRP neurons increases comparable levels of food intake and latency to first meal in *AgRP-ires-Cre* mice (black line), *AgRP-ires-Cre; Mc4r$^{-/-}$* KO mice (gray line), *AgRP-ires-Cre; Vgat$^{flox/flox}$* KO mice (red line), and *AgRP-ires-Cre; Npy$^{-/-}$* KO mice (blue line). CNO (solid line; 0.3 mg/kg of body weight, i.p.) or saline (dotted line) was injected 3 hr after the start of the lights on cycle, and food intake was assessed 1 and 2 (A) and 4, 8, and 24 hr (B) PI over three trials of each treatment. Data shown are from male mice (Error bars indicate mean ± SEM, n = 8 *AgRP-ires-Cre* mice; n = 5 *AgRP-ires-Cre; Mc4r$^{-/-}$* mice; n = 7 *AgRP-ires-Cre; Vgat$^{flox/flox}$* mice; n = 8 *AgRP-ires-Cre; Npy$^{-/-}$* mice; *p < 0.05 CNO groups versus all saline groups; #p < 0.05 *AgRP-ires-Cre; Mc4r$^{-/-}$* saline group versus all other saline groups).

(C) Latency to first meal after acute pharmacogenetic activation of AgRP neurons. Each circle represents the average of two trials for each mouse; the horizontal bar represents the average of all mice. Data shown are from male mice (mean ± SEM, n = 7 *AgRP-ires-Cre* mice; n = 5 *AgRP-ires-Cre; Mc4r$^{-/-}$* mice; n = 5 *AgRP-ires-Cre; Vgat$^{flox/flox}$* mice; n = 5 *AgRP-ires-Cre; Npy$^{-/-}$* KO mice).

See also Figure S2 and Table S1.

elevated levels of food intake in comparison to saline-treated *AgRP-ires-Cre* and triple KO and CNO-treated triple KO mice at all time points, p < 0.05. Although mice in a *Mc4r$^{-/-}$* KO background consume elevated daily levels of food (Figure 1B), DREADD-mediated AgRP activation in these triple KO mice resulted in comparable food intake observed in the same mice after saline injections.

Mice lacking leptin (*Lep$^{ob/ob}$*) or leptin receptor (*Lepr$^{db/db}$*) have severe morphological alterations in their AgRP axonal projections (Bouret et al., 2012). To analyze projection fields of AgRP neurons in both *AgRP-ires-Cre* and triple KO mice, we performed immunohistochemistry for AgRP protein. Significantly, we detected no gross anatomical differences in either AgRP neural innervation patterns or terminal field projections to different downstream brain regions, such as the bed nucleus of the stria terminalis (BST), paraventricular hypothalamus (PVH), paraventricular thalamus, and lateral parabrachial nucleus; Figure 1C). Therefore, the absence of increased food intake after CNO-mediated AgRP activation in triple KO mice is not due to obvious axonal projection perturbations.

Activation of AgRP Neurons in Single KO Mice Induces Feeding

To decipher the relevant neuromediator necessary for the robust increase of food intake after AgRP stimulation, we acutely activated AgRP neurons in mice that could not either release GABA (*AgRP-ires-Cre; Vgat$^{flox/flox}$*) or NPY (*AgRP-ires-Cre; Npy$^{-/-}$*) or engage MC4Rs (*AgRP-ires-Cre; Mc4r$^{-/-}$*). Surprisingly, we found that the disruption of each of these individually had no impediment on the CNO-mediated induction of feeding over both the short (Figures 2A and S2A) and long term (Figures 2B and S2B), given that the levels of food intake were comparable in saline- versus CNO-injected single KO mice (a post hoc Tukey test showed that CNO-treated *AgRP-ires-Cre* and all single KO mice exhibited significantly elevated levels of food intake in comparison to saline-treated *AgRP-ires-Cre* and single KO mice at all time points, p < 0.05). Importantly, we show that
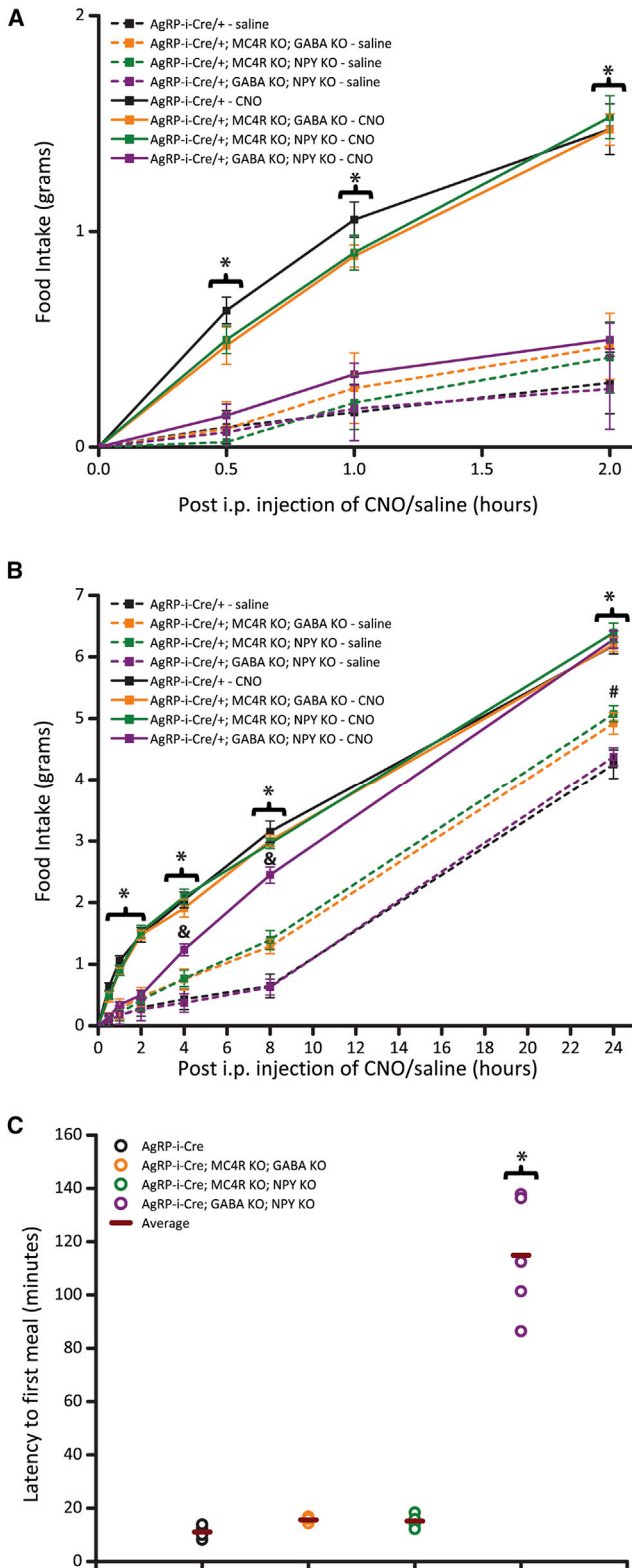
A

B

C

Figure 3. Acute Pharmacogenetic Activation of AgRP Neurons in Mice without the Release of GABA and NPY, collectively, Display Delayed DREADD-Mediated Increases in Food Intake

Data were pooled across multiple trials. All mice in these studies were bilaterally injected with AAV8-DIO-hM3Dq-mCherry in the ARC.

the acute stimulation of AgRP neurons in $Mc4r^{-/-}$ mice drives feeding beyond the elevated levels normally observed in this strain.

The behavior elicited by AgRP neuronal activation is highly stereotyped with mice engaging in meals, as defined by continuous feeding for >3 min shortly after CNO injection. These episodes are characterized by mice approaching the food hopper, initiating feeding behavior, and continuing to visibly eat for an extended period of time exceeding 3 min (Krashes et al., 2011). We quantified this latency to the first meal after DREADD-mediated AgRP activation by recording how long it took mice to commence in their first meal after a CNO injection. It is important to note that, in the absence of CNO stimulation, mice near the beginning of the light cycle (9–11:40 am) fail to engage in >3 min feeding bouts (latency data are not shown, but see Figure 2A). Notably, each group of single KO mice began eating shortly after CNO administration (~15 min; Figures 2C and S2C), comparable to *AgRP-ires-Cre* mice. Thus, each of these individual neuromediators alone is dispensable for increased food intake after AgRP activation.

GABA or NPY Are Required for the Rapid Onset of Food Intake, whereas AgRP Is Sufficient for Chronic Food Intake

To address both the necessity of transmitter pairs and the sufficiency of each individual neuromediator, we generated each possible double KO combination and assessed the effects on feeding behavior after acute AgRP neuronal activation. Stimulating AgRP neurons in mice with no NPY release and no downstream AgRP signaling via MC4Rs (*AgRP-ires-Cre; $Mc4r^{-/-}$; $Npy^{-/-}$*) or with both disrupted GABA release and downstream AgRP signaling via MC4Rs (*AgRP-ires-Cre; $Mc4r^{-/-}$; $Vgat^{flox/flox}$*) had no consequence on the DREADD-mediated increase in food intake over both the short (Figures 3A and S3A) and long term (Figures 3B and S3B; a posthoc Tukey test

(A–C) The activation of AgRP neurons increases comparable levels of food intake (A and B) and latency to first meal (C) in *AgRP-ires-Cre* mice (black line), *AgRP-ires-Cre; $Mc4r^{-/-}$; $Vgat^{flox/flox}$* double KO mice (orange line), and *AgRP-ires-Cre; $Mc4r^{-/-}$; $Npy^{-/-}$* double KO mice (green line). In contrast, the activation of AgRP neurons in *AgRP-ires-Cre; $Vgat^{flox/flox}$; $Npy^{-/-}$* double KO mice (purple line) resulted in highly attenuated short-term feeding (A), increased latency to first meal (C), and late-onset hyperphagia (B). CNO (solid line; 0.3 mg/kg of body weight, i.p.) or saline (dotted line) was injected 3 hr after the start of the lights on cycle and food intake was assessed 1 and 2 (A) and 4, 8, and 24 hr (B) PI over three trials of each treatment. Data shown are from male mice (error bars indicate mean ± SEM, n = 8 *AgRP-ires-Cre* mice; n = 5 *AgRP-ires-Cre; $Mc4r^{-/-}$; $Vgat^{flox/flox}$* mice; n = 5 *AgRP-ires-Cre; $Mc4r^{-/-}$; $Npy^{-/-}$* mice; n = 8 *AgRP-ires-Cre; $Vgat^{flox/flox}$; $Npy^{-/-}$* mice; *p < 0.05 *AgRP-ires-Cre*, *AgRP-ires-Cre; $Mc4r^{-/-}$; $Vgat^{flox/flox}$* and *AgRP-ires-Cre; $Mc4r^{-/-}$; $Npy^{-/-}$* CNO groups versus *AgRP-ires-Cre; $Vgat^{flox/flox}$; $Npy^{-/-}$* CNO and all saline groups; #p < 0.05 *AgRP-ires-Cre; $Mc4r^{-/-}$; $Vgat^{flox/flox}$* and *AgRP-ires-Cre; $Mc4r^{-/-}$; $Npy^{-/-}$* saline groups versus all other saline groups; &p < 0.05 *AgRP-ires-Cre; $Vgat^{flox/flox}$; $Npy^{-/-}$* CNO group versus all saline groups).

(C) Latency to first meal after acute pharmacogenetic activation of AgRP neurons. Each circle represents the average of two trials for each mouse; the horizontal bar represents the average of all mice. Data shown are from male mice (mean ± SEM, n = 7 *AgRP-ires-Cre* mice; n = 5 *AgRP-ires-Cre; $Mc4r^{-/-}$; $Vgat^{flox/flox}$* mice; n = 5 *AgRP-ires-Cre; $Mc4r^{-/-}$; $Npy^{-/-}$* mice; n = 5 *AgRP-ires-Cre; $Vgat^{flox/flox}$; $Npy^{-/-}$* mice; *p < 0.05 *AgRP-ires-Cre; $Vgat^{flox/flox}$; $Npy^{-/-}$* group versus all other groups).

See also Figure S3 and Table S1.

showed that CNO-treated *AgRP-ires-Cre* and double KO mice exhibited significantly elevated levels of food intake in comparison to saline-treated *AgRP-ires-Cre* and double KO mice at all time points, p < 0.05. This result demonstrates that AgRP neural release of either NPY or GABA alone is sufficient to drive feeding post-AgRP activation, given that these mice began eating soon after CNO injection (∼15 min; Figures 3C and S3C).

In stark contrast, we found that DREADD-mediated AgRP activation in mice with perturbed GABA and NPY release (*AgRP-ires-Cre; Vgat^flox/flox; Npy^−/−*) abrogated short-term feeding (Figures 3A and S3A; a post hoc Tukey test showed that CNO-treated *AgRP-ires-Cre* mice exhibited significantly elevated levels of food intake in comparison to saline-treated *AgRP-ires-Cre* and *AgRP-ires-Cre; Vgat^flox/flox; Npy^−/−* mice at all time points, p < 0.05, and CNO-treated *AgRP-ires-Cre; Vgat^flox/flox; Npy^−/−* mice at time points 0.5, 1, 4, and 8, p < 0.05). These mice initiated food intake ∼2 hr after CNO injection (Figures 3C and S3C). This suggests that either GABA or NPY are required for the acute feeding phase and the neuropeptide AgRP is insufficient to mediate this early response. Remarkably, these double KO mice exhibited late-onset hyperphagia over 24 hr, ultimately reaching similar quantitative levels of food intake as all the other mice tested (Figures 3B and S3B). Importantly, although actions of AgRP neuropeptide independent of the MC4R have been described (Fu and van den Pol, 2008; Marsh et al., 1999a), we show that the rise in food consumption subsequent to acute AgRP activation is dependent on AgRP peptide signaling through MC4Rs because it is absent in the triple KO mice (Figure 1).

Compromising GABA and NPY Release from AgRP Neurons Delays Physiological Food Intake

The above studies employed DREADD technology in order to artificially activate AgRP neurons, but, to investigate this biphasic feeding due to differential AgRP neuromediator release in a physiological state, we assessed dark cycle food intake, the active feeding period in rodents, in both *AgRP-ires-Cre* and *AgRP-ires-Cre; Vgat^flox/flox; Npy^−/−* double KO mice. Importantly, using *Npy-hrGFP* mice to visualize AgRP neurons (Liu et al., 2012), we demonstrate enhanced AgRP neuron activity during the dark cycle in comparison to basal levels near the beginning of the light cycle (Figure 4A). Membrane potential and firing rate of AgRP neurons are significantly elevated during the dark cycle (calorically deficient state) in comparison to the onset of the light cycle (calorically replete state) (Figure 4A), consistent with a recent finding that AgRP neurons receive approximately double the frequency of spontaneous excitatory postsynaptic currents at the beginning of the dark period in comparison to the fed state (Yang et al., 2011).

In vivo feeding measurements revealed that the double KO mice exhibited decreased food consumption during the first 2 and 4 hr window of the dark cycle in comparison to littermate controls (Figure 4B; a post hoc Tukey test showed that *AgRP-ires-Cre* exhibited significantly elevated levels of food intake in comparison to double KO mice at 2 and 4 hr, p < 0.05). Despite this early temporal reduction in food intake in the double KO mice, both groups ate comparable amounts of food over the entire 12 hr dark cycle and 24 hr (Figure 4C). Assuming that hunger signaling flows through AgRP neural output, and given the increased activity of these neurons during the dark cycle (Fig-

ure 4A), these results would suggest that the AgRP neuropeptide is sufficient to drive comparable levels of feeding over a delayed, prolonged temporal scale in a physiological condition.

DISCUSSION

Given the discrepancies between pharmacological and genetic deletion experiments, we took an alternative approach for assigning prandial function to the distinct neuromediators released by AgRP neurons. By employing DREADD technology to rapidly and remotely stimulate AgRP neural activity with genetic mouse lines harboring gene manipulations that perturb GABA, NPY, and/or AgRP signaling, our findings suggest the following conclusions. First, the complete absence of CNO-stimulated feeding in triple KO mice indicates that at least one of these neuroactive chemicals is necessary to drive AgRP-mediated feeding behavior. Second, it demonstrates that there are no other functionally relevant mediators released by AgRP neurons that promote food intake in our assay.

Third, the absence of any impairment in single KO mice indicates that there is redundancy in the effects of these three mediators. However, our study does not resolve whether this redundancy is due to similar roles played by one or both of the remaining mediators or to compensation caused by chronic genetic KO of a given mediator. Interestingly, compensation has been described following specific AgRP neural manipulations, as adaptive mechanisms were observed after both cell-specific ablation (Gropp et al., 2005; Luquet et al., 2005) in neonatal animals and slow progressive loss of AgRP neurons (Xu et al., 2005). Also, it was recently suggested that, on the basis of optogenetic assessment of connectivity, *Npy^−/−* mice show strengthened AgRP neuron → PVH neuron GABAergic transmission (Atasoy et al., 2012). Such compensation could explain how GABA substitutes for the absence of NPY in rapid stimulation of feeding. On the other hand, it is presently unknown how NPY substitutes for the absence of GABAergic transmission.

Fourth, the near complete absence of feeding during the first 2 hr of stimulation of double KO mice lacking NPY and GABA signaling indicates that (1) AgRP by itself is unable to activate feeding quickly but is able to do so chronically, consistent with the delayed kinetics of MC4R signaling following pro-opiomelanocortin (POMC) neural manipulations (Aponte et al., 2011; Atasoy et al., 2012; Zhan et al., 2013) and (2) either GABA or NPY, both of which are inhibitory transmitters, are required for rapid feeding and are able to compensate for each other in these constitutive genetic deletion backgrounds. This ability of GABA and NPY to compensate for each other with regard to rapid feeding is surprising, given that one is a synaptic fasting-acting neurotransmitter, whereas the other is a neuropeptide. Interestingly, acute brain slice electrophysiology has demonstrated the fast temporal dynamics of NPY application onto downstream POMC neurons (Cowley et al., 2001). Additionally, it was recently shown that NPY puncta in the axons of hypothalamic neurons colocalized with the sites of GABA transmitter release (Ramamoorthy et al., 2011), implying that NPY may be released synaptically, as suggested by immunohistochemistry (Broberger et al., 1998) and in vivo manipulations (Atasoy et al., 2012). In agreement with this observation, electron microscopy verified the co-expression of NPY and GABA in axon terminals and revealed that
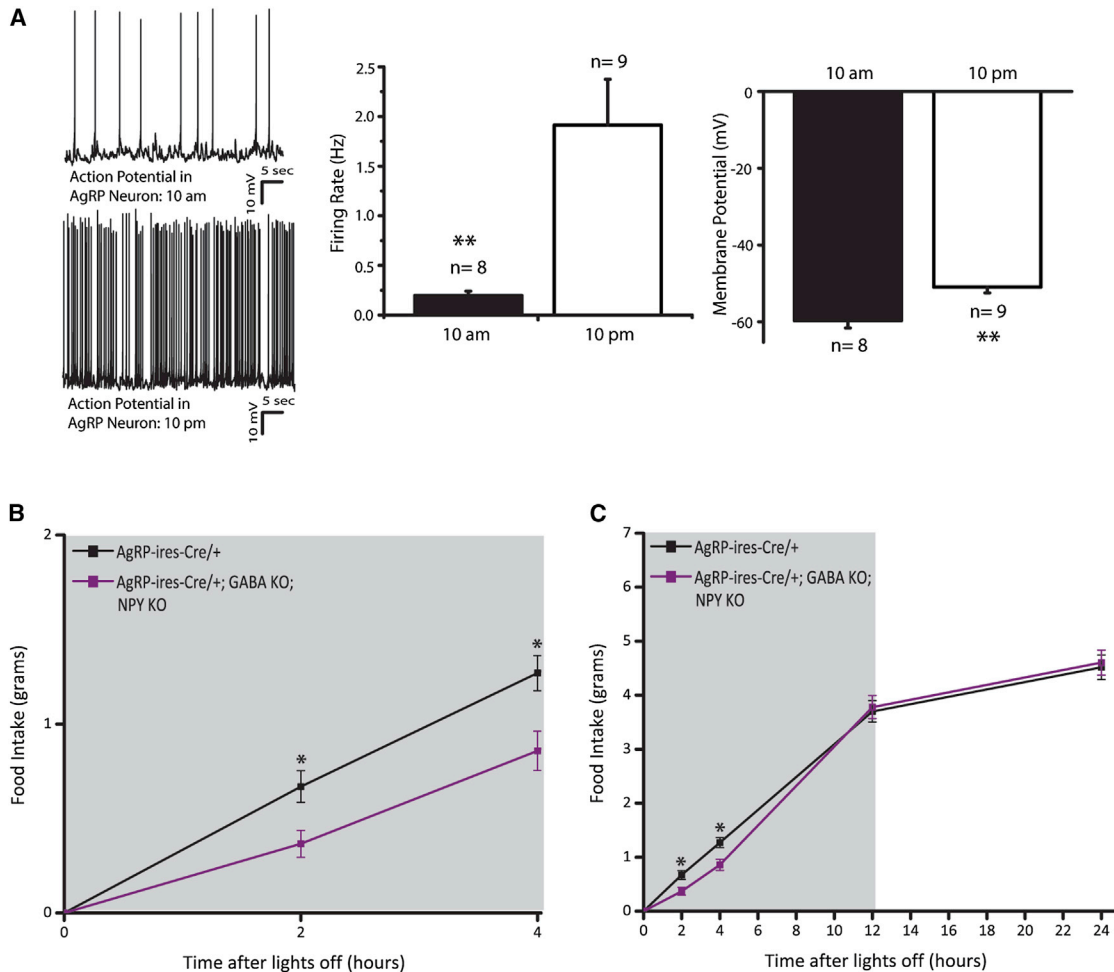
Figure 4. Mice with the Compromised Release of Both GABA and NPY from AgRP Neurons Display a Delayed Physiological Increase in Dark Cycle Food Intake

(A) Light cycle (10 a.m.; n = 8) versus dark cycle (10 p.m.; n = 9) electrophysiological properties of AgRP neurons (error bars indicate mean + SEM; *p < 0.05). Top, representative trace of AgRP neuron in the light cycle. Bottom, representative trace of AgRP neuron in the dark cycle. Right, quantitative analyses of firing rate and membrane potential of AgRP neurons in the light cycle (black bar) versus dark cycle (white bar).

(B and C) Dark cycle food intake measurements assessed at 2 and 4 (B) and 12 and 24 hr (C) after lights are shut off. Grey background indicates the dark cycle period. Data shown are from male mice (error bars indicate mean ± SEM, n = 9 AgRP-ires-Cre mice; n = 9 AgRP-ires-Cre; Vgat$^{flox/flox}$; Npy$^{-/-}$ mice; *p < 0.05).

these boutons formed synapses onto POMC perikarya (Cowley et al., 2001).

To assess the physiological function of these neuroactive substances, we monitored food intake during the dark cycle, a time when mice normally consume most of their food. Notably, the impaired feeding response in *AgRP-ires-Cre; Vgat*$^{flox/flox}$; *Npy*$^{-/-}$ double KO mice over the first few hours of dark cycle feeding, when the basal firing rate of AgRP neurons is high, suggest that their role in rapidly activating feeding is physiologically relevant and not just restricted to the pharmacogenetic activation of AgRP neurons. Furthermore, although double KO mice show postponed feeding behavior during the first 2 and 4 hr of the dark cycle, food intake levels were comparable to those of control mice by the end of the entire 12 hr dark period, suggesting either that the neuropeptide AgRP is sufficient to evoke chronic feeding or that the induction of food intake can occur via AgRP neural-independent circuit mechanisms.

Finally, the absence of any stimulation of feeding in triple KO mice combined with the late onset, but the robust stimulation, of feeding in GABA and NPY double KO mice (which retain MC4R signaling) strongly suggests that the hyperphagic response caused by released AgRP is due to the inhibition of MC4Rs and not MC3Rs or other nonmelanocortin receptor-mediated effects. This delayed yet dynamic role for AgRP in promoting chronic food intake in comparison to the rapid effects of NPY aligns with the temporal differences of drug-elicited feeding episodes observed after pharmacological injection studies (Semjonous et al., 2009).

EXPERIMENTAL PROCEDURES

Animals

All animal care and experimental procedures were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

AgRP-ires-Cre (Tong et al., 2008), *Vgat^flox/flox* (Tong et al., 2008), *Npy^−/−* (Erickson et al., 1996), and *Mc4r^−/−* (Balthasar et al., 2005) mice were previously described. KO mice were all bred to the homozygous state for experiments.

Stereotaxic Injections
Mice were injected as previously described (Krashes et al., 2011). In brief, 200 nL bilateral injections of AAV8-DIO-hM3Dq-mCherry (titer 1.2×10^{12} genomes copies per ml) were made in the ARC. All injected mice were positive for bilateral hits (Figure S4).

Food Intake Studies
Food intake studies on chow were performed as previously described (Krashes et al., 2011). For light cycle DREADD-mediated AgRP activation studies, 10- to 12-week-old male mice were intraperitoneal (i.p.) injected with either saline or CNO (0.3 mg/kg) at 9 a.m., and food intake was monitored at 0.5, 1, 2, 4, 8, and 24 hr after injection. Studies were run similar to the within-subject design, and each trial consisted of a cycle of saline administration on day 1, CNO administration on day 2, and no injection on day 3 and was repeated over three trials. An averaged consumption value across trials for each animal for each time point was obtained by averaging the three values for that time point from each of the three trials. Then, this averaged value was used in conjunction with similarly obtained values from the other animals within the same treatment and genotype group to create a mean ± SEM and statistically analyzed. For latency studies, a lab timer (Control Company) was used to record the time it took mice to engage in a continuous 3 min meal immediately after CNO injection. For dark cycle physiological studies, food intake was monitored at 2, 4, 12, and 24 hr after lights were turned off (6 p.m.) in 10- to 12-week-old male mice.

Immunohistochemistry
This procedure was performed as previously described (Bouret et al., 2012). Rabbit anti-AgRP (1:2,000; Phoenix Pharmaceuticals) primary antibody and Alexa Fluor 594 donkey anti-mouse Ig (H+L; Invitrogen; 1:200) secondary antibody were used to analyze AgRP projections. Fluorescent images were captured and processed under identical parameters with an Olympus VS120 slide scanner microscope.

Electrophysiology
The protocols of slice preparation and whole-cell recording were previously described (Krashes et al., 2011; Liu et al., 2012). We used 5- to 7-week-old *Npy-hrGFP* mice to visualize AgRP neurons.

Statistics
Statistical analyses were performed with KaleidaGraph (Synergy Software). DREADD-mediated feeding studies were run as a within-subject design, and a final consumption value for each animal was obtained from an average of three trials. Data were analyzed with a two-way repeated measures ANOVA, and the interaction of genotype and treatment was studied. A p value of < 0.05 (*, #, and $) was considered significant in these studies. Error bars indicate mean ± SEM. We analyzed all the data with a repeated measures ANOVA with two between-subject factors (genotype and treatment) and one repeated factor (time point). We found the main effects of genotype ($F_{5,82} = 6.929$, $p < 0.05$), treatment ($F_{1,86} = 403.216$, $p < 0.05$), and time point ($F_{5,82} = 2907.265$, $p < 0.05$) and a three-way interaction between these factors ($F_{35,347} = 3.169$, $p < 0.05$).

For dark cycle physiological feeding studies, two genotypes were assessed for food intake across four time points. Data were analyzed with a repeated measures ANOVA with one between-subject factor (genotype) and one repeated measure (time point). We found a main effect of time point ($F_{4,30} = 171.813$, $p < 0.05$), no significant effect of genotype ($F_{1,33} = 1.210$, $p = 0.28$), and a significant interaction between genotype and time point ($F_{4,30} = 11.866$, $p < 0.05$).

SUPPLEMENTAL INFORMATION
Supplemental Information contains Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2013.09.009.

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