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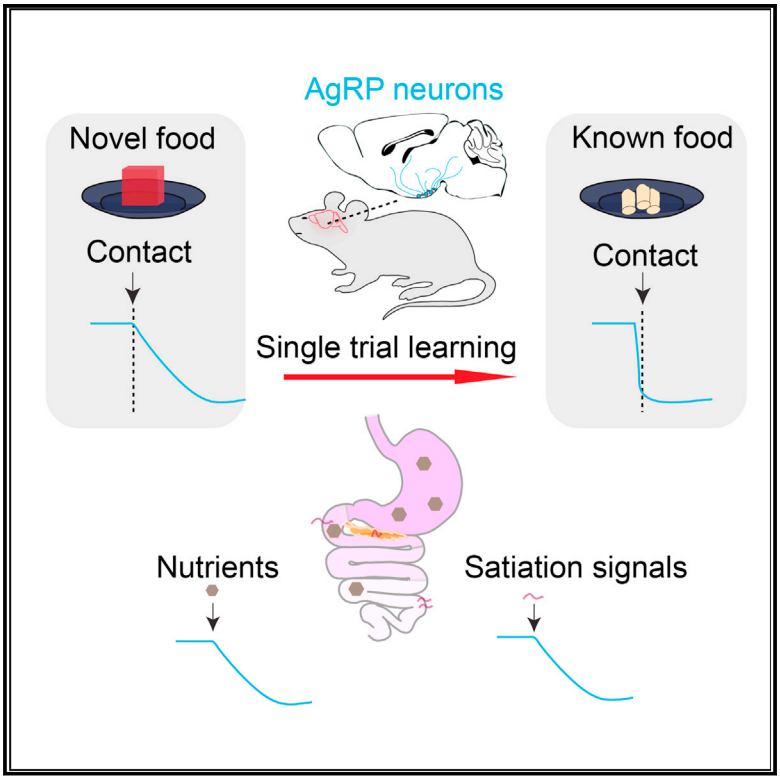
Nutritive, Post-ingestive Signals Are the Primary Regulators of AgRP Neuron Activity

Graphical Abstract



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In Brief

Su et al. demonstrate that nutrient content in the GI tract is rapidly signaled to hypothalamic neurons activated by hunger. This rapid effect is mediated by three satiation signals that synergistically reduce the activity of AgRP neurons. These findings uncover how hunger circuits in the brain are regulated and raise the possibility that hunger can be pharmacologically controlled.

Highlights

- Ingested calories are necessary and sufficient to reduce AgRP neuron activity
- A single exposure to caloric food trains sensory cues to reduce AgRP neuron activity
- Satiation signals synergistically reduce AgRP neuron activity, similar to nutrients

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Nutritive, Post-ingestive Signals Are the Primary Regulators of AgRP Neuron Activity

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SUMMARY

The brain regulates food intake by processing sensory cues and peripheral physiological signals, but the neural basis of this integration remains unclear. Hypothalamic, agouti-related protein (AgRP)-expressing neurons are critical regulators of food intake. AgRP neuron activity is high during hunger and is rapidly reduced by the sight and smell of food. Here, we reveal two distinct components of AgRP neuron activity regulation: a rapid but transient sensory-driven signal and a slower, sustained calorie-dependent signal. We discovered that nutrients are necessary and sufficient for sustained reductions in AgRP neuron activity and that activity reductions are proportional to the calories obtained. This change in activity is recapitulated by exogenous administration of gut-derived satiation signals. Furthermore, we showed that the nutritive value of food trains sensory systems—in a single trial—to drive rapid, anticipatory AgRP neuron activity inhibition. Together, these data demonstrate that nutrients are the primary regulators of AgRP neuron activity.

INTRODUCTION

Food intake is tightly controlled by interactions between the brain and the periphery. Three types of signals can influence feeding behavior: (1) pre-consummatory sensory signals, such as visual and olfactory cues that predict the availability of food (Petrovich et al., 2002; Weingarten, 1983); (2) orosensory signals, such as taste (Booth, 1972; Campbell and Davis, 1974; Lucas and Sclafani, 1989); and (3) physiological signals, such as the detection of nutrients and the release of post-prandial satiation signals (Cummings and Overduin, 2007; McHugh and Moran, 1978; Sobrino Crespo et al., 2014).

Classical studies have demonstrated the ability to associate a novel sensory cue with the presentation of food (Pavlov and Fol'bort, 1926; Weingarten, 1983). Cues predicting food lead to physiological changes before food is available and can even drive food intake in sated individuals, a phenomenon known as cue-potentiated feeding (Petrovich et al., 2002; Weingarten, 1983). More recently, it has been demonstrated that animals can learn to associate nonnutritive orosensory cues with gastric nutrients (Han et al., 2016; Lucas and Sclafani, 1989). These studies suggest that individuals use both pre-consummatory and orosensory food cues to predict energy availability.

Once food is consumed, nutrients are detected in the gastrointestinal (GI) tract. Ingested substances are sensed by mechanoreceptors in the stomach, which are activated by gastric distension. Stomach contents are emptied in proportion to caloric load, and nutrients are detected in the small intestine (Cummings and Overduin, 2007; Powley and Phillips, 2004). The ability for GI preloads to reduce food intake in a calorie-dependent manner (McHugh and Moran, 1978) suggests that gut-derived signals control food intake. Indeed, as nutrients are detected, satiation hormones are released from the GI tract to terminate meals (Cummings and Overduin, 2007; Sobrino Crespo et al., 2014). These effects are mediated, at least in part, by hindbrain circuits (Berthoud et al., 2006; Carter et al., 2013; Hayes et al., 2008). A critical next step is to understand how these signals are transmitted to other brain regions, such as the hypothalamus, that integrate different homeostatic signals to influence feeding behavior.

The activity of hypothalamic, agouti-related protein (AgRP)-expressing neurons is influenced by both energy status and exteroceptive sensory cues, suggesting that this neural population is a node for the integration of these signals. These neurons stimulate voracious feeding when excited (Aponte et al., 2011; Krashes et al., 2011), and when AgRP neurons are ablated, animals stop consuming food and die of starvation (Luquet et al., 2005). AgRP neurons exhibit high levels of activity in nutrient deficit (Mandelblat-Cerf et al., 2015; Takahashi and Cone, 2005), and their activity is reduced following food consumption (Betley et al., 2015; Chen et al., 2015; Mandelblat-Cerf et al., 2015). Recent studies have shown that activity in AgRP neurons rapidly decreases upon presentation of cues that predict food, such as the sight or smell of food (Betley et al., 2015; Chen et al., 2015; Mandelblat-Cerf et al., 2015). These studies have challenged the classical view that AgRP neurons are homeostatically regulated, leading to the current model of AgRP neurons as sensory detectors of food (Betley et al., 2015; Chen et al., 2015; Mandelblat-Cerf et al., 2015). However, consuming food appears necessary to maintain reduced AgRP neuron activity levels, since removing food after a short interval leads to a rapid reversion

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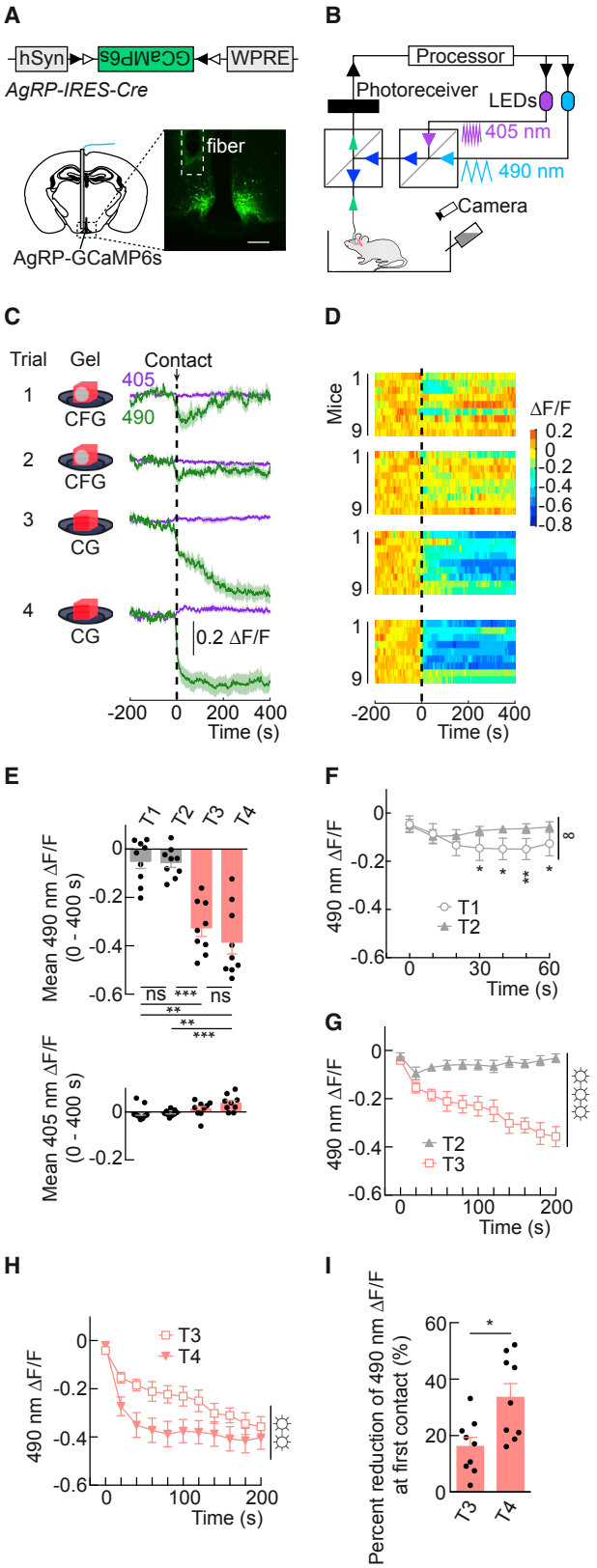

Figure 1. Nutrients Are Required for the Sustained Reduction of AgRP Neuron Activity

(A) Configuration for monitoring calcium dynamics in AgRP neurons. Scale bar, 400 μm.

(B) Dual-wavelength fiber photometry (FP) setup used to record calcium-dependent fluorescence (excited at 490 nm) and calcium-independent fluorescence (excited at 405 nm).

(C) Food-restricted mice were given two trials with calorie-free gel (CFG) followed by two trials with caloric gel (CG) during FP recordings on 4 consecutive days. Average ΔF/F of GCaMP6s signals from each trial (n = 9 mice/trial) are displayed. Signals are aligned to the first contact with gel at time 0. Green indicates the 490-nm signal; purple indicates the 405-nm control signal. Darker lines represent means, and lighter shaded areas represent SEMs.

(D) Heatmaps reporting ΔF/F of the 490-nm signal of the recordings in individual mice in (C).

(E) Top: mean ΔF/F of the 490-nm signal from 0 to 400 s among all trials in (C). Bottom: mean ΔF/F of the 405-nm signal from 0 to 400 s among all trials in (C).

(F) Mean ΔF/F of the 490-nm signal from trials 1 and 2 displayed in 10-s bins.

(G) Mean ΔF/F of the 490-nm signal from trials 2 and 3 displayed in 20-s bins.

(H) Mean ΔF/F of the 490-nm signal from trials 3 and 4 displayed in 20-s bins.

(I) Percent ΔF/F of the 490-nm signal at first contact with gel relative to maximum ΔF/F in trials 3 and 4 in (C).

Values are means ± SEMs. ns, $p > 0.05$; t tests and post hoc comparisons: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ANOVA interaction: ∞ $p < 0.05$; ANOVA main effect of group: ☼☼ $p < 0.01$; ☼☼☼ $p < 0.001$.

to high levels of AgRP neuron activity (Betley et al., 2015; Chen et al., 2015).

In this study, we used *in vivo* calcium imaging to disentangle the roles of sensory and post-ingestive signals on the regulation AgRP neuron activity. We demonstrate across multiple complementary experiments that calories, but not external sensory cues, are necessary and sufficient for sustained reductions in AgRP neuron activity. The rapid suppression of AgRP neurons by sensory cues is a transient and dynamic response that has been learned from prior consumption of caloric food. Taken together, our data show that GI nutrients provide the primary signal that teaches anticipatory responses to food cues and sustains AgRP neuron activity reductions.

RESULTS

Calories Are Required for Sustained Reductions in AgRP Neuron Activity

To gain insight into the physiological regulation of AgRP neurons, we monitored AgRP neuron calcium dynamics in freely moving animals. Fiber photometry was performed in animals engineered to express the genetically encoded calcium indicator, GCaMP6s, in AgRP neurons (Figure 1A). We measured calcium-dependent fluorescence (excited at 490 nm) as a measure of AgRP neuron activity (Cui et al., 2013; Gunaydin et al., 2014) and calcium-independent fluorescence (excited at 405 nm) as a control for movement and bleaching artifacts (Figure 1B) (Lerner et al., 2015). As previously reported (Betley et al., 2015; Chen et al., 2015; Mandelblat-Cerf et al., 2015), AgRP neuron activity was rapidly decreased by the presentation of chow and was sustained following consumption (Figures S1A and S1B).

We first sought to dissociate the role of nonnutritive sensory cues from the nutritive content of food in the regulation of AgRP neuron activity. We monitored AgRP neuron activity in

food-restricted mice while providing access to an artificially sweetened, calorie-free gel (CFG), with which they had no previous experience. Exposure to this novel, nonnutritive tastant allows the animal to experience all sensory and ingestive processes without obtaining calories, testing the sufficiency of nonnutritive sensory cues in regulating AgRP neurons. The first trial of CFG consumption resulted in a small, transient reduction in AgRP neuron activity (Figures 1C–1F) that was significantly different from encountering a non-food object (Figures S1C and S1D). This transient decrease was associated with some aspect of ingestion, as the sight and smell of an inaccessible novel food was not sufficient to suppress AgRP neuron activity (Figures S1E–S1L). Subsequent consumption of the CFG led to smaller and more transient AgRP neuron inhibition (Figures 1C–1F). This observation suggests that the sensory qualities of CFG have been devalued due to lack of calories and that sensory systems learn the contingency between cues and caloric content in a single trial.

Following two exposures to CFG, the same mice were next allowed to consume a sugar-sweetened, caloric gel (CG) with similar visual, olfactory, and gustatory profiles as those of CFG. The robust and rapid anticipatory reduction of AgRP neuron activity was not observed when the mice encountered CG for the first time (Figures 1C, 1D, and 1G–1I), likely due to previous exposure to the CFG with similar sensory profiles. However, the first trial of CG consumption led to a slower but robust and sustained AgRP neuron activity reduction (T3 in Figures 1C–1E and 1G). On the ensuing exposure to CG, the rapid and robust anticipatory reduction of AgRP neuron activity was observed (Figures 1C, 1D, 1H, and 1I), again suggesting that the contingency between sensory cues and nutritive value of food is learned in a single trial. Analogously, a single trial with caloric food is sufficient to condition visual and olfactory cues to drive the anticipatory reduction of AgRP neuron activity (Figures S1E–S1M). In all cases, the suppression of AgRP neuron activity by nonnutritive sensory cues is transient (~200 s) compared to the suppression by nutritive food, which persists during and following feeding (Figures 1C and S1E). Together, these data suggest that calories are required for sustained suppression of AgRP neuron activity. Further, these findings demonstrate that AgRP neurons dynamically calculate the contingency between sensory cues and caloric content of food so that significant changes in the neural response to food cues occur in a single trial.

We next explored the ability of AgRP circuits to "unlearn" the contingency between sensory cues and the nutritive value of calorie-containing food. We gave naive mice access to CG for two trials and, as expected, observed a sustained reduction in AgRP neuron activity (Figures 2A and 2B). The inhibition of AgRP neuron activity by CG was faster on the second trial, reflecting the learned sensory anticipation of calories (Figures 2A–2D). The mean and maximum AgRP neuron inhibition was similar in both trials (Figures 2A and 2G–2I). After two exposures to CG, we broke the contingency between sensory cues and the expected caloric content of food by giving the same mice trials with CFG. On the first trial, consumption of the CFG led to a rapid inhibition of AgRP neuron activity, which is not surprising, since similar sensory cues had previously been paired with calories (Figures 2A, 2B, and 2E). Notably, this inhibition of AgRP neurons was transient (~200 s) and was not observed with naive CFG consumption (Figure 1C). This suggests that nutrients are required for sustained AgRP neuron inhibition, despite the presence of visual, olfactory, gustatory, and non-nutritive interoceptive sensory cues. Further, a single exposure to CFG teaches a new contingency between these sensory cues and calorie content, as a subsequent exposure to CFG led to a greatly diminished anticipatory inhibition of AgRP neuron activity (Figures 2A, 2B, and 2F). The ability to reduce AgRP neuron activity is independent of the amount of the gel consumed, as animals consumed similar amounts of both the CG and CFG (Figure 2J). By isolating the sensory and nutritive properties of food, we uncovered two distinct phases of AgRP neuron inhibition: (1) a transient, sensory-mediated anticipatory inhibition and (2) a sustained, calorie-mediated inhibition.

GI Calorie Detection Is Sufficient for Sustained AgRP Neuron Activity Reduction

We next sought to understand whether calories, without sensory cues associated with ingestion, are sufficient to reduce AgRP neuron activity. To isolate the caloric content of food, we delivered calories directly to the stomach while monitoring AgRP neuron activity in food-restricted mice (Figure 3A). As previously demonstrated (Canbeyli and Koopmans, 1984; McHugh and Moran, 1978), gastric infusion of calories (Ensure) significantly reduced subsequent chow intake (Figure 3B). Gastric delivery of Ensure resulted in robust and sustained reductions in AgRP neuron activity (Figures 3C–3E). This effect was independent of osmotic, stretch, and taste signaling in the gut, as neither water, hypertonic saline, methylcellulose nor sucralose significantly altered AgRP neuron activity (Figures 3C–3E).

The magnitude and time course of AgRP neuron activity reduction is dependent on caloric content, as gastric infusion of 1/3 kcal of Ensure leads to a slower, more transient, and less substantial reduction in AgRP neuron activity compared to infusion of 1 kcal (Figures 3F and 3G). This phenomenon is also observed upon oral consumption of 1/3 and 1 kcal of chow, peanut butter, or Ensure. In each case, more calories consistently led to a more substantial and sustained reduction in AgRP neuron activity (Figures 4A–4I). In contrast to suggestions that AgRP neuron activity is reduced in proportion to palatability (Chen et al., 2015), we find similar mean and maximum AgRP neuron activity reductions following consumption of palatable foods (peanut butter or Ensure) and less palatable foods (chow) (Figures 4J–4M). Taken together, these data suggest that caloric content detected by the GI tract, rather than palatability, drives activity changes in AgRP neurons.

Are all calories capable of reducing AgRP neuron activity? Infusions of glucose, lipids, and amino acids all reduced the activity of AgRP neurons (Figures 5A–5C), demonstrating that AgRP neurons respond to the detection of various macronutrients. Similar to the activity reduction observed upon infusion of Ensure, we observed a calorie-dependent reduction of AgRP neuron activity when infusing glucose, lipids, or amino acids (Figures 5D–5F and S2). These results demonstrate that calories are both necessary and sufficient for sustained reductions in AgRP neuron activity, consistent with our observations during the consumption of CG and CFG.

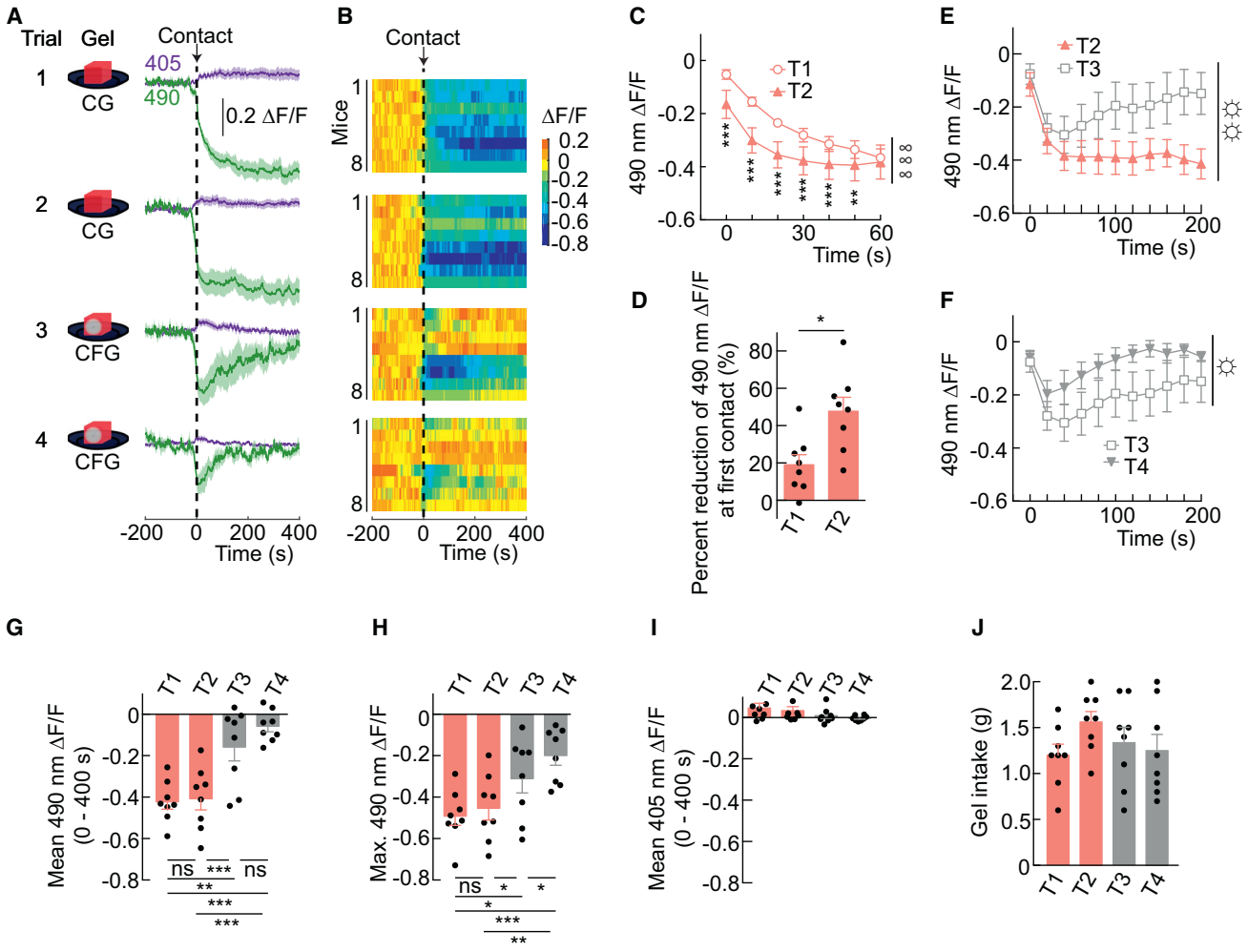

Figure 2. Nutrients Train the Sensory Regulation of AgRP Neurons in a Single Trial

(A) Food-restricted mice were given two trials with CG followed by two trials with CFG during FP recordings. Average ΔF/F of GCaMP6s signals from each trial (n = 8 mice per trial) are displayed. Individual signals were aligned to the first contact with gel at time 0. Green indicates the 490-nm signal; purple indicates the 405-nm control signal. Darker lines represent means, and lighter shaded areas represent SEMs.

(B) Heatmaps reporting ΔF/F of the 490-nm signals in individual mice in (A).

(C) Mean ΔF/F of the 490-nm signal from trials 1 and 2 displayed in 10-s bins.

(D) Percent ΔF/F of the 490-nm signal at first contact with gel relative to maximum ΔF/F in trials 1 and 2 in (A).

(E) Mean ΔF/F of the 490-nm signal from trials 2 and 3 displayed in 20-s bins.

(F) Mean ΔF/F of the 490-nm signal from trials 3 and 4 displayed in 20-s bins.

(G) Mean ΔF/F of the 490-nm signal from 0 to 400 s among all trials in (A).

(H) Maximum ΔF/F of the 490-nm signal among all trials in (A).

(I) Mean ΔF/F of the 405-nm signal from 0 to 400 s among all trials in (A).

(J) Gel intake among trials in (A).

Values are means ± SEMs. ns, $p > 0.05$; t tests and post hoc comparisons: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ANOVA interaction: ∞ ∞ ∞ $p < 0.001$; ANOVA main effect of group: ☼$p < 0.05$; ☼☼$p < 0.01$.

Satiation Signals Reduce AgRP Neuron Activity

We next sought to explore potential molecular mechanisms by which gastric nutrients inhibit AgRP neuron activity. Several GI satiation signals are released following food intake (Figure 6A) and contribute to food intake suppression (Bhavsar et al., 1998; Chaudhri et al., 2006; Erlanson-Albertsson and Larsson, 1988; Kopin et al., 1999; Neary et al., 2005; Pittner et al., 2004; Wright et al., 2012; Zhang et al., 2005). The effects of these satiation signals on *in vivo* activity in hypothalamic circuits are unknown. We combined 8 satiation peptides, each known to acutely reduce food intake when administered exogenously (Bhavsar et al., 1998; Chaudhri et al., 2006; Erlanson-Albertsson and Larsson, 1988; Kopin et al., 1999; Neary et al., 2005; Pittner et al., 2004; Wright et al., 2012; Zhang et al., 2005) and injected

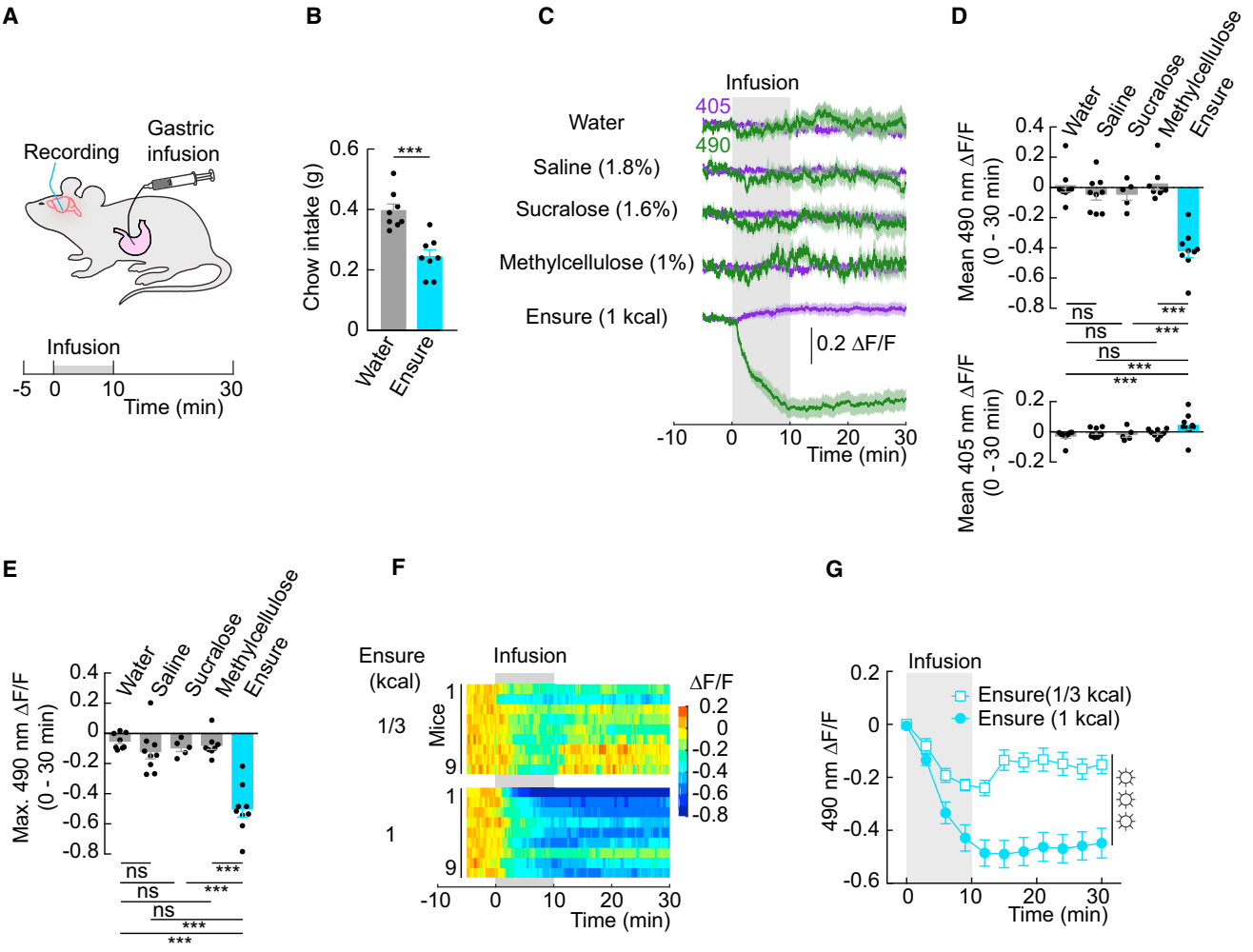

Figure 3. GI Nutrients Reduce AgRP Neuron Activity in a Calorie-Dependent Manner

(A) Experimental procedure for gastric infusion through implanted catheters during FP recordings.

(B) Food-restricted mice were infused with water or 1 kcal of Ensure (n = 8 mice) before chow intake was measured.

(C) Average ΔF/F of GCaMP6s signals in mice infused with water (n = 8 mice), hypertonic saline (1.8%, n = 9 mice), sucralose (1.6%, n = 5 mice), methylcellulose (1%, n = 8 mice), or Ensure (1 kcal, n = 9 mice). Individual signals were aligned to the start of infusion at time 0, and infusion period is indicated by gray shading. Green indicated the 490-nm signal; purple indicates the 405-nm control signal. Darker lines represent means, and lighter shaded areas represent SEMs.

(D) Top: mean ΔF/F of the 490-nm signal from 0 to 30 min for each infusate. Bottom: mean ΔF/F of the 405-nm signal from 0 to 30 min for each infusate.

(E) Maximum ΔF/F of the 490-nm signal for each infusate.

(F) Heatmaps reporting ΔF/F of the 490-nm signal for individual mice infused with either 1/3 or 1 kcal of Ensure (n = 9 mice).

(G) Mean ΔF/F of the 490-nm signal in 3-min bins from mice infused with 1/3 or 1 kcal of Ensure in (F).

Values are means ± SEMs. ns, $p > 0.05$; t tests and post hoc comparisons: ***$p < 0.001$; ANOVA main effect of group: ◦◦◦$p < 0.001$.

this cocktail intraperitoneally (i.p.) while monitoring AgRP neuron activity in food-restricted mice. The satiation peptide cocktail dramatically reduced activity in AgRP neurons (Figures 6B–6D and S3). The reduction in AgRP neuron activity was dose dependent, as a lower (1/3) dose of the peptides led to a reduced magnitude and duration of AgRP neuron activity suppression (Figures 6B–6D and S3). This reduction in AgRP neuron activity is likely independent of malaise that is often associated with satiation signaling (Deutsch and Hardy, 1977; Kanoski et al., 2012; le Roux et al., 2008), as this peptide cocktail neither induced a conditioned taste avoidance (Figure 6E) nor reduced locomotor activity (Figure 6F). Additionally, feeding-suppressive doses of noxious substances (lithium chloride [LiCl] or lipopolysaccharide [LPS]) (Mormède et al., 2004; West et al., 1987) have no impact on AgRP neuron activity (Figures 6B–6D and S3).

We next explored whether AgRP neuron activity inhibition by satiation signaling is functionally relevant for food intake control. We reasoned that if satiation signals inhibited food intake via a reduction in AgRP neuron activity, restoring AgRP neuron signaling would attenuate the anorexia resulting from peptide cocktail injection. As expected, administration of the peptide cocktail to food-deprived animals reduced food intake by 42.6 ± 10.3% in comparison to saline-treated mice (Figures 6G–6I). This effect was abrogated by optogenetically restoring

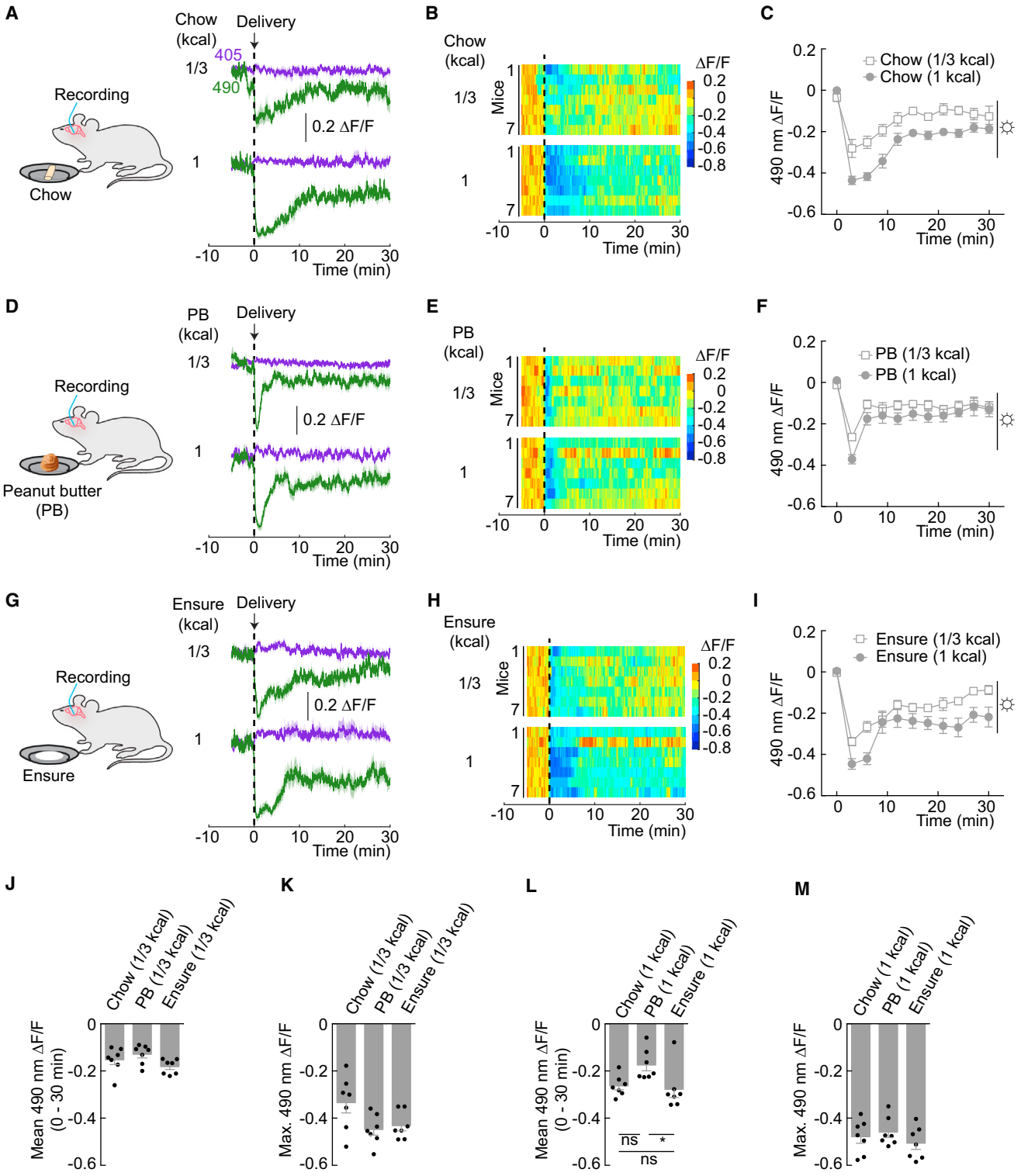

Figure 4. AgRP Neuron Response Is Proportional to Caloric Content of Food

(A) Average ΔF/F of GCaMP6s signals in food-restricted mice given 1/3 or 1 kcal of chow (n = 7 mice). Individual signals are aligned to the delivery of chow at time 0. Green indicates the 490-nm signal; purple indicates the 405-nm control signal. Darker lines represent means, and lighter shaded areas represent SEMs.

(B) Heatmaps reporting ΔF/F of the 490-nm signal during chow intake in individual mice.

(C) Mean ΔF/F of the 490-nm signal in 3-min bins in mice given 1/3 or 1 kcal of chow in (A).

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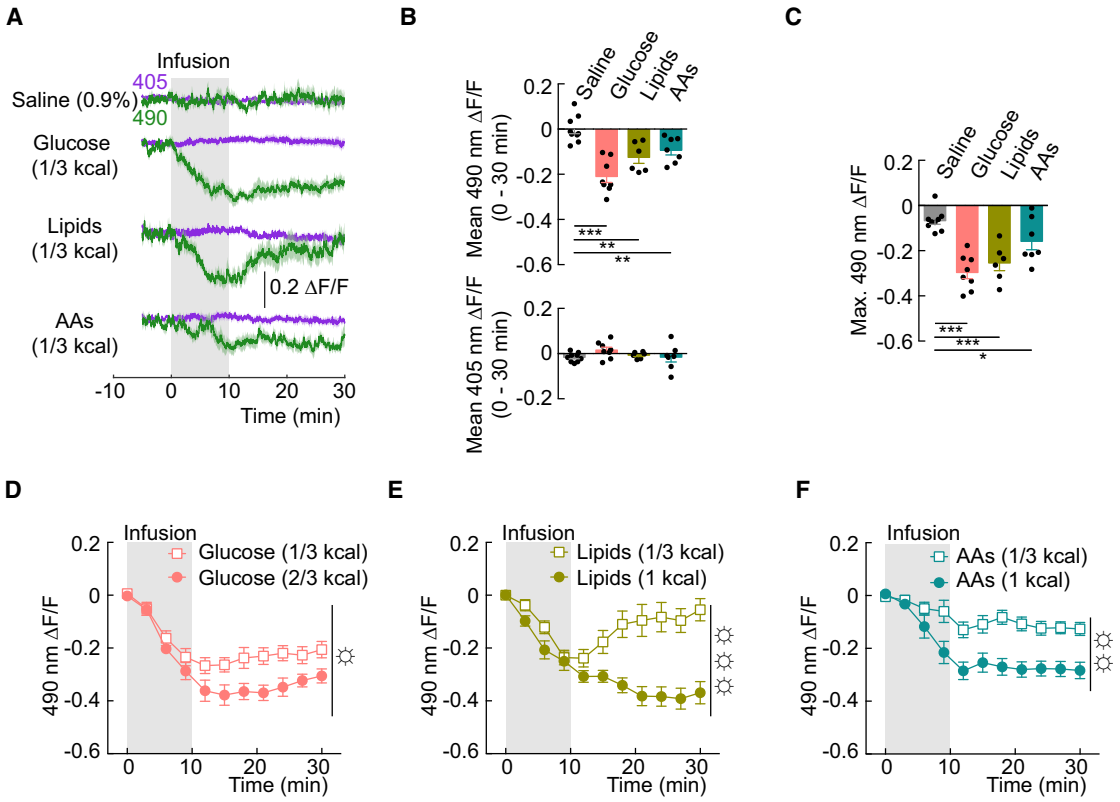

Figure 5. Individual Macronutrients Reduce AgRP Neuron Activity

(A) Average ΔF/F of GCaMP6s signals in mice infused with saline (0.9%, n = 9 mice), 1/3 kcal of glucose (n = 8 mice), 1/3 kcal of lipids (n = 6 mice), or 1/3 kcal of amino acids (AAs, n = 7 mice). Individual signals are aligned to the start of infusion at time 0. Green indicates the 490-nm signal; purple indicates the 405-nm control signal. Darker lines represent means, and lighter shaded areas represent SEMs.

(B) Top: mean ΔF/F of the 490-nm signal from 0 to 30 min for each infusate. Bottom: mean ΔF/F of the 405-nm signal from 0 to 30 min for each infusate.

(C) Maximum ΔF/F of the 490-nm signal for each infusate.

(D) Mean ΔF/F of the 490-nm signal in mice infused with 1/3 or 2/3 kcal of glucose in 3-min bins.

(E) Mean ΔF/F of the 490-nm signal in mice infused with 1/3 or 1 kcal of lipids in 3-min bins.

(F) Mean ΔF/F of the 490-nm signal in mice infused with 1/3 or 1 kcal of AAs in 3-min bins.

Values are means ± SEMs. Post hoc comparisons: *p < 0.05; **p < 0.01; ***p < 0.001; ANOVA main effect of group: ☼p < 0.05; ☼☼p < 0.01; ☼☼☼p < 0.001.

AgRP neuron activity (Figures 6G–6I), demonstrating the ability of AgRP neuron activity to overcome the intake-suppressive effect of the peptide cocktail.

To determine the specific satiation peptide or peptides that contribute to AgRP neuron inhibition, we individually evaluated the role of each satiation signal on AgRP neuron activity. Cholecystokinin (CCK), peptide tyrosine tyrosine (PYY), and amylin each suppressed AgRP neuron activity (Figures 7A and 7B; Figures S4A and S4C), albeit at higher concentrations than those used in the cocktail (Figure 6A). Conversely, the other 5 satiation signals used in the cocktail did not substantially reduce AgRP neuron activity (Figure 7B; Figures S4B and S4C), even at

(D) Average ΔF/F of GCaMP6s signals in food-restricted mice given 1/3 or 1 kcal of peanut butter (PB) (n = 7 mice). Individual signals are aligned to the delivery of PB at time 0.

(E) Heatmaps reporting ΔF/F of the 490-nm signal during PB consumption in individual mice.

(F) Mean ΔF/F of the 490-nm signal in 3-min bins in mice given 1/3 or 1 kcal of PB in (D).

(G) Average ΔF/F of GCaMP6s signals in food-restricted mice given 1/3 or 1 kcal of Ensure (n = 7 mice). Individual signals are aligned to the delivery of Ensure at time 0.

(H) Heatmaps reporting ΔF/F of the 490-nm signal during Ensure consumption in individual mice.

(I) ΔF/F of the 490-nm signal in 3-min bins in mice given 1/3 or 1 kcal of Ensure in (G).

(J) Mean ΔF/F of the 490-nm signal from 0 to 30 min in mice given 1/3 kcal of chow, PB, or Ensure.

(K) Maximum ΔF/F of the 490-nm signal in mice given 1/3 kcal of chow, PB, or Ensure.

(L) Mean ΔF/F of the 490-nm signal from 0 to 30 min in mice given 1 kcal of chow, PB, or Ensure.

(M) Maximum ΔF/F of the 490-nm signal in mice given 1 kcal of chow, PB, or Ensure.

Values are means ± SEMs. ns, p > 0.05; post hoc comparisons: *p < 0.05; ANOVA main effect of group: ☼p < 0.05.

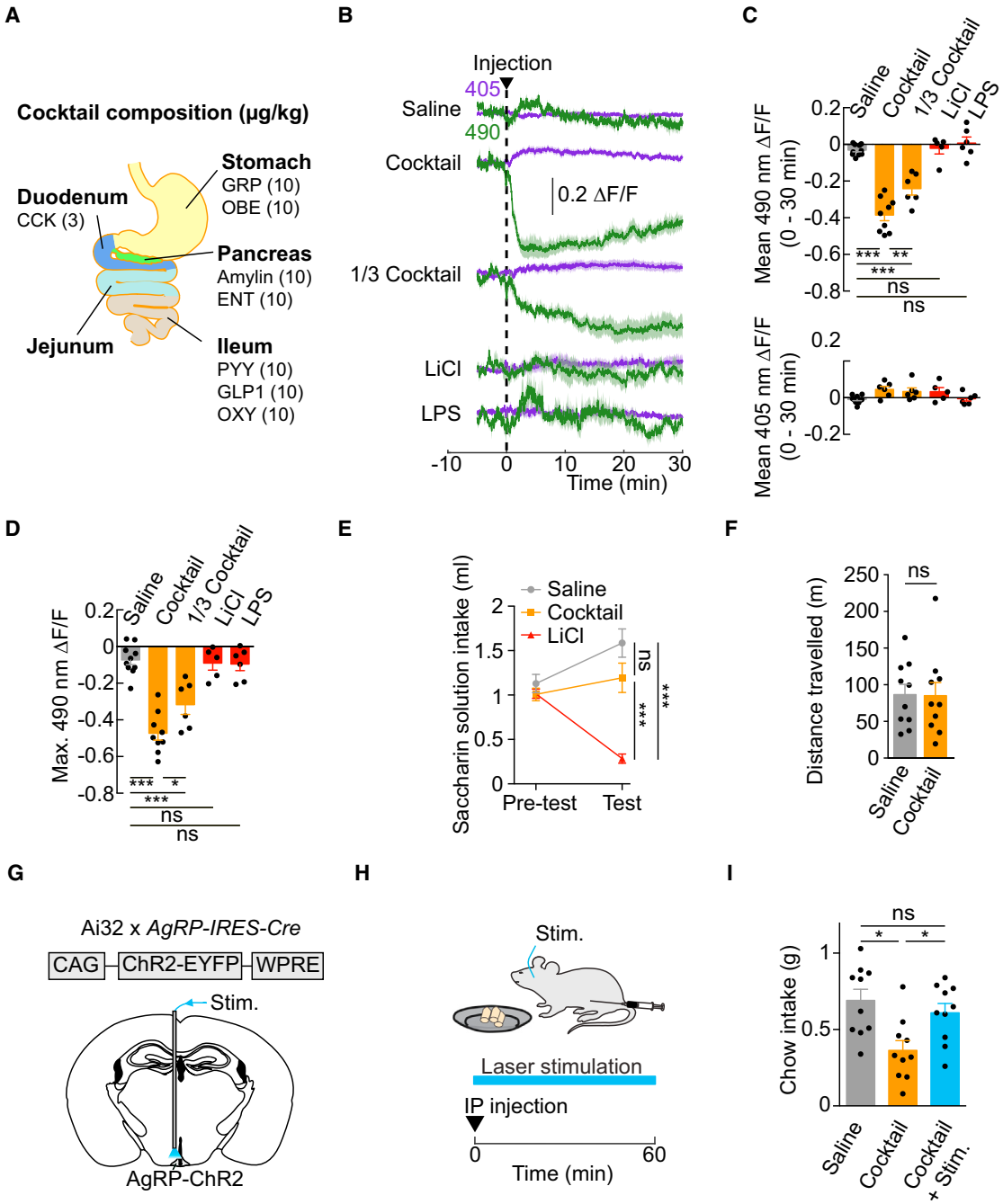

Figure 6. Satiation Signals Reduce AgRP Neuron Activity

(A) Diagram showing release sites along the GI tract for eight satiation signals.

(B) Average ΔF/F of GCaMP6s signals in mice injected with saline (0.9%, n = 10 mice), cocktail (n = 9 mice), 1/3 dose cocktail (n = 6 mice), LiCl (n = 5 mice), or lipopolysaccharide (LPS, n = 6 mice). Individual signals were aligned to the finish of injection at time 0. Green indicates the 490-nm signal; purple indicates the 405-nm control signal. Darker lines represent means, and lighter shaded areas represent SEMs.

(C) Top: mean ΔF/F of the 490-nm signal from 0 to 30 min for each substance injected. Bottom: mean ΔF/F of the 405-nm signal from 0 to 30 min for each substance injected.

(D) Maximum ΔF/F of the 490-nm signal for each substance injected.

(E) Saccharin solution intake before and 24 hr after conditioned taste avoidance pairing in mice injected with saline (n = 6 mice), cocktail (n = 7 mice), or LiCl (n = 7 mice).

(F) Total distance traveled after injection of saline or cocktail (n = 10 mice).

(G) Schematic for channelrhodopsin-2 (ChR2)-mediated *in vivo* photostimulation of AgRP neurons.

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doses known to reduce food intake (Chaudhri et al., 2006; Erlanson-Albertsson and Larsson, 1988; Neary et al., 2005; Wright et al., 2012; Zhang et al., 2005). Relative to other satiation signals, CCK reduced the activity of AgRP neurons on a fast time course so that maximal suppression was reached in 1.9 ± 0.3 min. However, CCK-induced suppression was more transient in comparison to the cocktail, PYY, or amylin (Figures 7A and 7C). In contrast, PYY-mediated suppression of AgRP neuron activity plateaued in 26.8 ± 2.0 min but continued for significantly longer than CCK, suggesting that PYY has a slower latency but a longer lasting effect on AgRP neuron activity (Figures 7A and 7C; Figure S4A). Amylin had intermediate kinetics and suppressive effects on AgRP neuron activity (Figures 7A and 7C).

Doses of CCK, PYY, and amylin used in our cocktail that are known to reduce food intake (Bhavsar et al., 1998; Kopin et al., 1999; Pittner et al., 2004) did not substantially influence AgRP neuron activity when administered individually (Figures 7D and 7E). However, combining lower doses of these 3 peptides (3 μg/kg CCK, 10 μg/kg PYY, and 10 μg/kg amylin), robustly reduced AgRP neuron activity (Figures 7D–7F; Figures S4D and S4E), suggesting a synergistic effect of these peptides. Conversely, no effect on AgRP neuron activity was observed when we applied a cocktail of the 5 satiation peptides that are individually insufficient to suppress activity (Figures 7D and 7F; Figures S4D and S4E). Together, these data suggest that CCK, PYY, and amylin synergistically interact to reduce the activity of AgRP neurons.

DISCUSSION

Detection of nutrients by the CNS is critical to food intake control. Despite compelling demonstrations that AgRP neuron activity dramatically responds to the sensory detection of food (Betley et al., 2015; Chen et al., 2015; Mandelblat-Cerf et al., 2015), our activity analyses reveal that the response of AgRP neurons is primarily driven by the homeostatic, nutritive value of food. We find that (1) AgRP neuron activity reductions only persist when coupled with the consumption of calorie-containing foods, direct infusion of nutrients, or GI satiation signals; and (2) calories are required to entrain sensory circuits that signal AgRP neurons upon the detection of food (Figure 7G).

AgRP Neuron Activity Levels Are Regulated by the Ingestion of Caloric Food

In this study, we unmask the relative contributions of the sensory and nutritive properties of food on AgRP neuron activity. We provide several lines of evidence demonstrating that calorie-containing nutrients reduce AgRP neuron activity independent of non-nutritive sensory cues. First, the sight, smell, taste, and ingestion of non-caloric but palatable (i.e., artificially sweetened) substances—such as calorie-free gel—do not lead to a sustained suppression of AgRP neuron activity. Second, the silencing of AgRP neurons is not dependent on the presence of any external sensory cues, as direct gastric infusion of nutritive substances causes a persistent reduction in AgRP neuron activity, similar to the consumption of a meal. Additionally, the sensation of substances in the GI tract is not sufficient to reduce activity of AgRP neurons, as nonnutritive and artificially sweetened solutions infused into the stomach do not change AgRP neuron activity. Third, AgRP neuron silencing scales with the number of calories ingested or infused. In addition to our direct gastric infusions, we show that consuming equicaloric quantities of palatable foods (i.e., peanut butter or Ensure) reduces AgRP neuron activity to magnitudes similar to that for chow. While previous studies suggest that palatability may drive greater reductions in AgRP neuron activity, these studies do not account for the energy density of foods consumed (Chen et al., 2015). Fourth, post-prandially released satiation signals are sufficient to inhibit AgRP neuron activity, suggesting that peptides released following food intake mediate the suppression of AgRP neuron activity. Taken together, these data show that calories are both necessary and sufficient—and, importantly, that external sensory cues are neither necessary nor sufficient—for sustained reduction in AgRP neuron activity.

Rapid Activity Reductions in Homeostatic Systems Are Learned

Although sensory cues do not sustain reductions in AgRP neuron activity, AgRP neurons are clearly wired to detect cues that predict known foods. Our findings begin to unpack how AgRP circuits learn to respond to these food cues. We find significant differences in how quickly AgRP neurons respond to novel nutritive food between the first and subsequent exposures. The single-trial learning of caloric content is also dynamic, as breaking the contingency between sensory cues and the nutritive value of food rapidly reverses previously learned associations that impact anticipatory AgRP neuron activity. These observations demonstrate that AgRP neurons are wired primarily to respond to calories and that the preemptive modulation of these circuits by sensory detection is simply a learned consequence. These data suggest that (1) calories train AgRP circuits to respond to the sight, smell, and taste of food and that (2) learning can occur in a single trial. These findings are consistent with our observations that the first exposure to a novel food or the direct infusion of calories into the stomach produces a maximal AgRP neuron activity suppression on a much slower time course (~200 s) than the consumption of a known food (<1 s). The longer time course may reflect the innate latency of calorie detection by the GI tract. The nature and site of this calorie detection and how this detection trains sensory systems remain interesting questions.

What is the purpose of the preemptive inhibition of AgRP neuron activity in response to food? In theory, a homeostatic system would not require such speed. While our data demonstrate

(H) Saline or peptide was injected i.p. before 1 hr chow re-feeding in food-restricted mice with or without photostimulation.
(I) Chow intake in mice with or without cocktail and AgRP neuron stimulation (n = 10 mice).
Values are means ± SEMs. ns $p > 0.05$, t tests and post hoc comparisons: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
Values are means ± SEMs. ns, $p > 0.05$; post hoc comparisons: **$p < 0.01$; ***$p < 0.001$; ANOVA main effect of group: ☼☼$p < 0.01$. ENT, enterostatin; GLP1, glucagon-like peptide-1; GRP, gastrin releasing peptide; OBE, obestatin; OXY, oxyntomodulin.

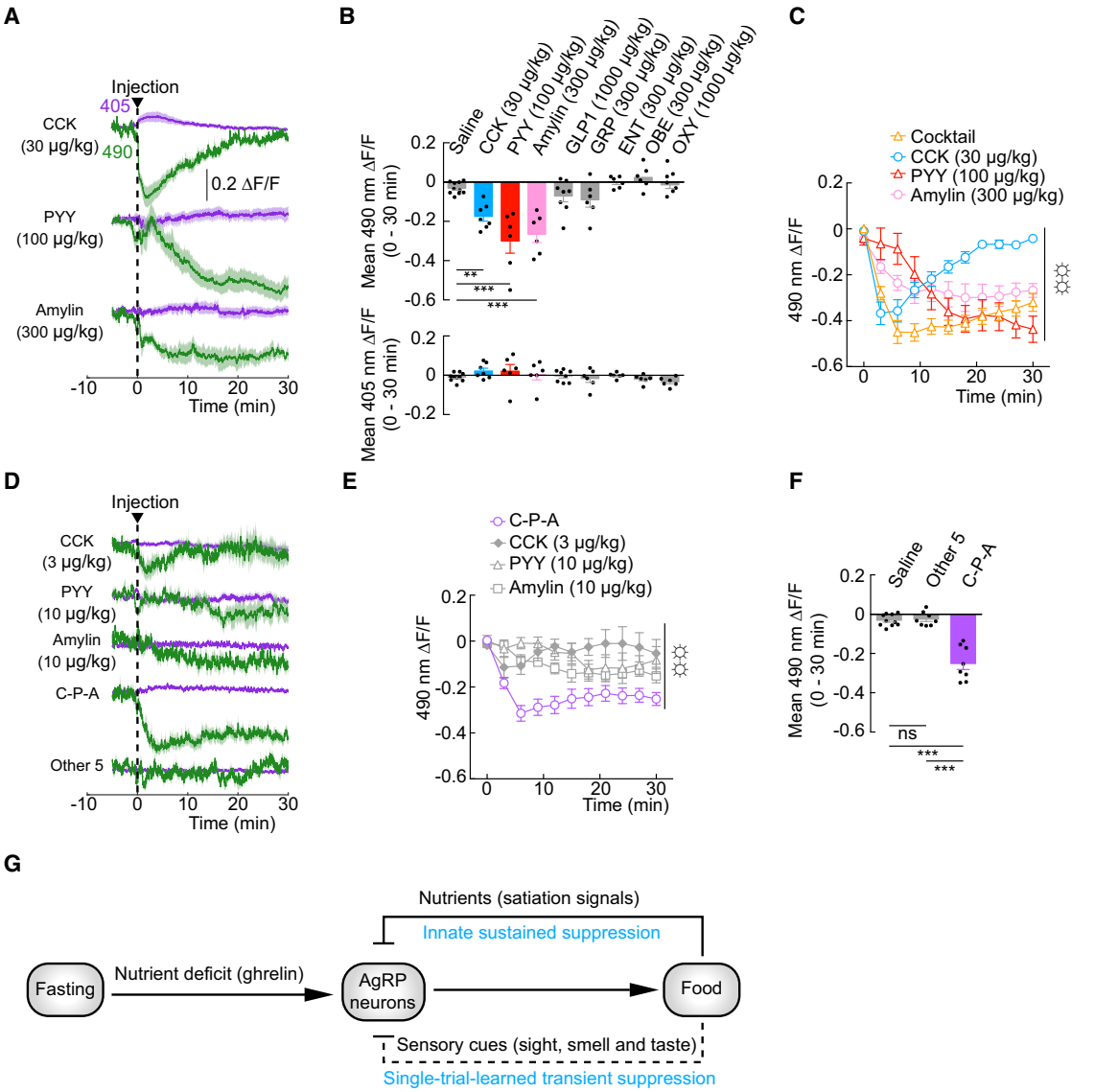

Figure 7. CCK, PYY, and Amylin Synergistically Reduce AgRP Neuron Activity

(A) Average ΔF/F of GCaMP6s signals in food-restricted mice injected with 30 μg/kg CCK (n = 7 mice), 100 μg/kg PYY (n = 6 mice), or 300 μg/kg amylin (n = 6 mice). Individual signals are aligned to the finish of injection at time 0. Green indicates the 490-nm signal; purple indicates the 405-nm control signal. Darker lines represent means, and lighter shaded areas represent SEMs.

(B) Top: mean ΔF/F of the 490-nm signal from 0 to 30 min for each substance injected. Bottom: mean ΔF/F of the 405-nm signal from 0 to 30 min for each substance injected.

(C) Mean ΔF/F of the 490-nm signal in 3-min bins from 0 to 30 min among groups injected with cocktail, CCK, PYY, or amylin.

(D) Average ΔF/F of GCaMP6s signals in food-restricted mice injected with 3 μg/kg CCK (n = 7 mice); 10 μg/kg PYY (n = 4 mice); 10 μg/kg amylin (n = 4 mice); a cocktail with CCK, PYY, and amylin (C-P-A: 3 μg/kg CCK, 10 μg/kg PYY, and 10 μg/kg amylin; n = 8 mice); or a cocktail with the other 5 peptides (10 μg/kg each of GLP1, GRP, ENT, OBE, and OXY; n = 8 mice).

(E) Mean ΔF/F of the 490-nm signal among mice injected with C-P-A, CCK, PYY, or amylin in 3-min bins.

(F) Mean ΔF/F of the 490-nm signal from 0 to 30 min among mice injected with saline (n = 10 mice), the other 5 peptides (n = 8 mice), or C-P-A (n = 8 mice).

(G) Model for regulation of AgRP neurons.

that the caloric content of food rapidly entrains the inhibition of AgRP neurons as a response to food cues, the function of this rapid sensory inhibition remains unknown. Since these neurons transmit a negative valence signal to drive food intake (Betley et al., 2015), it is possible that the rebound in AgRP neuron activity that occurs when a non-nutritive substance is identified serves as a reward prediction error (Schultz et al., 1997; Watabe-Uchida et al., 2017) that devalues non-food substances. This would be conceptually similar to the function of dopamine neurons in the ventral tegmental area, which first fire upon presentation of a

reward but, over time, fire instead at the presentation of a cue predicting a reward. Conversely, when known food is identified, AgRP neurons are inhibited, and the negative affect of being hungry is alleviated. This ability to identify food in the environment is evolutionarily advantageous and, thus, may be "rewarded" by the reduction in AgRP neuron activity and, correspondingly, a reduction in negative affect. Why does feeding persist after AgRP neurons are inhibited by sensory cues? It is possible that the animal has learned to consume a sufficient amount of food to avoid a resurgence of AgRP neuron activity levels that we observe upon inadequate consumption of calories (i.e., <1/3 kcal). This learning system may be ethologically relevant for animals seeking food in a natural environment, as detection and consumption of sufficient caloric food is important for survival.

Fast Signaling along the Gut-Brain Axis

Even though maximal AgRP neuron activity reductions induced by calories in the GI tract require ~200 s, we find that the activity begins to decline within seconds of gastric infusion of a nutritive substance (Figure 3C). This suggests that, devoid of all external sensory cues, nutrients have the ability to signal AgRP neurons along the gut-brain axis within seconds. Fast gut-brain signaling also occurs in central reward systems, which detect signals from sweet substances in the GI tract within minutes (Tellez et al., 2016). However, these rapid effects on neural activity may require more time to influence behavior, as the emergence of taste preferences for caloric substances requires ~30 min (Lucas and Sclafani, 1999). One hypothesis that explains the rapid gut-AgRP neuron signaling is that the gut has "learned" to sense incoming substances that predict calories. Given that osmotic sensing (infusion of hypertonic saline) and taste sensing (infusion of sucralose) do not reduce AgRP neuron activity, non-nutritive interoceptive sensing does not appear to drive AgRP neuron activity reductions. Since non-nutritive substances empty from the stomach rapidly (McHugh and Moran, 1979), we further explored the role of gastric distension by infusing a load of methylcellulose, a calorie-free and non-digestible constituent of fiber. Similar to the infusion of water, saline, or sucralose, mechanical distension of the stomach did not substantially reduce AgRP neuron activity. Rather, calorie sensing drives AgRP neuron activity reductions, as gastric infusions of Ensure, glucose, lipids, and amino acids are each capable of reducing AgRP neuron activity levels. Taken together, these findings demonstrate that calorie detection in the gut rapidly signals the brain to influence homeostatic systems.

A Molecular Mechanism for the Inhibition of AgRP Neurons

AgRP neurons are inhibited by gastric infusion of nutrients faster than it takes for these nutrients to reach the brain in circulation (Page et al., 2013). It is possible that cells with primary receptors in the gut detect these nutrients (Efeyan et al., 2015) and release hormones that signal to the brain (Cummings and Overduin, 2007; Sobrino Crespo et al., 2014). Here, we identify molecular mediators released by the gut upon food intake that reduce AgRP neuron activity. We demonstrate a synergistic effect of three satiation signals: CCK, PYY, and amylin. When administered together at doses that are individually subthreshold to affect AgRP neuron activity (Figures 7D and 7E), these 3 peptides strikingly recapitulate the effect of intragastric infusion of nutrients. The effect of these hormones on AgRP neurons is the first neural reflection of the behavioral observation that satiation signals can act synergistically to reduce food intake (Bhavsar et al., 1998; Hinton et al., 1986; Roth et al., 2007; Talsania et al., 2005).

Individually, CCK, PYY, and amylin are all known to reduce food intake (Bhavsar et al., 1998; Kopin et al., 1999; Pittner et al., 2004), and we demonstrate that each of these peptides reduce AgRP neuron activity. While amylin only moderately reduces AgRP neuron activity, the time course and magnitude of the neural inhibition driven by individual administration of CCK or PYY may explain both the rapid and sustained suppression observed following administration of the peptide cocktail. CCK leads to a rapid and robust inhibition of AgRP neuron activity. Since CCK is primarily released from cells in the duodenum in the proximal intestine (Buffa et al., 1976), the fast time course of action is sensible, as nutrients begin to enter the duodenum shortly after entering the stomach. Additionally, CCK is thought to act via vagal afferent CCK1 receptors (Cummings and Overduin, 2007), providing a plausible explanation for the fast rate of signaling to AgRP neurons. Conversely, PYY is released primarily by the ileum of the distal small intestine (Adrian et al., 1985) and is thought to act both through vagal afferent signaling (Koda et al., 2005) and through direct action in the hypothalamic arcuate nucleus (Batterham et al., 2002). When the 3 peptides are administered together, we observe synergistic effects on AgRP neuron activity whereby the early phase of inhibition is predominantly explained by the actions of one molecular actor (CCK) and the late phase of inhibition is explained by another (PYY).

Satiation peptides have become attractive therapeutic agents for weight loss but have side effects of nausea and vomiting (Kanoski et al., 2012; le Roux et al., 2008). Thus, we examined the possibility that the peptide cocktail reduces AgRP neuron activity by inducing visceral malaise. Our peptide cocktail did not cause a conditioned taste avoidance, which is a classic test for nausea and malaise (Ferreira et al., 2006), nor did it cause alterations in overall locomotor activity. Furthermore, noxious stimuli that are well known to cause visceral malaise and sickness (i.e., LiCl and LPS) did not affect AgRP neuron activity. Thus, the reductions we observed in AgRP neuron activity and feeding behavior induced by this cocktail are not likely due to malaise or sickness. Together with our data showing that AgRP neuron stimulation attenuates the intake inhibition by the peptide cocktail, these observations suggest that satiation signals, but not signals of visceral malaise, activate a neural pathway that converges on AgRP neurons to inhibit food intake.

Interestingly, we identified several satiation peptides known to reduce food intake that do not affect the activity of AgRP neurons. Given the anatomically distributed control of food intake (Andermann and Lowell, 2017; Berthoud et al., 2006; Denis et al., 2015; Grill and Hayes, 2012), this finding is not surprising. These peptides likely target systems that communicate with other important neural targets for the regulation of food intake, such as MC4R neurons in the paraventricular hypothalamus (PVH), the GABAergic neurons of the lateral hypothalamus (LH), and hedonic/reward pathways in the midbrain and ventral forebrain.

Deconstructing the Regulation of AgRP Neurons

Gaining insight into the coordinated regulation of food intake involves unraveling the complex biological processes that occur during hunger and satiety. In addition to recent evidence provided by Beutler et al., 2017, we demonstrate that calories in consumed nutrients are essential for sustained reductions in AgRP neuron activity and demonstrate the ability of post-prandial satiation signals to recapitulate this inhibition. Furthermore, we have demonstrated that nutrients can train sensory cues to inhibit AgRP neuron activity in a single trial. Thus, there are two distinct phases of AgRP neuron inhibition by food: an early, sensory-driven component that is learned and a slower, nutrient-driven process. These findings clarify the respective roles of sensory cues and nutritive, post-ingestive signals on AgRP neuron activity, laying the foundation for the design of weight loss strategies that are grounded in minimizing AgRP neuron activity.

EXPERIMENTAL PROCEDURES

Further details and an outline of resources used in this work can be found in the Supplemental Experimental Procedures.

Animal Strains

All protocols were conducted according to NIH guidelines for animal research and approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. Adult male and female mice (2 to 6 months old) were used. *AgRP-IRES-Cre* mice were used for monitoring AgRP neuron activity and crossed with Ai32 mice to express channelrhodopsin-2 (ChR2) for photostimulation experiments. C57BL/6J mice were used for all other experiments.

Dual-Wavelength Fiber Photometry

Two excitation wavelengths (490 nm and 405 nm) were modulated at different frequencies to detect calcium-dependent and calcium-independent GCaMP6s fluorescence signals, respectively. The emission lights were converted to electrical signals by a photoreceiver and demodulated by a real-time processor.

Photostimulation

10-ms pulses of 450-nm light were provided at 20 Hz for 1 s and repeated every 4 s. The output power at the tip of the terminal fiber was set to ensure at least 2 mW/mm^2 irradiance on AgRP neurons.

Statistical Analysis

Data are presented as means ± SEMs. Comparisons between two groups were made with paired or unpaired two-tailed t tests using Prism. One-way, two-way, and repeated-measures ANOVAs were performed in Prism to make comparisons across multiple groups. Detailed statistical analyses are summarized in Table S1.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.11.036.

AUTHOR CONTRIBUTIONS

Z.S., A.L.A., and J.N.B. initiated the project, designed experiments, performed experiments, analyzed data, and prepared the manuscript.

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