Cell

# Hunger States Switch a Flip-Flop Memory Circuit via a Synaptic AMPK-Dependent Positive Feedback Loop

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DOI 10.1016/j.cell.2011.07.039

## SUMMARY

Synaptic plasticity in response to changes in physiologic state is coordinated by hormonal signals across multiple neuronal cell types. Here, we combine cell-type-specific electrophysiological, pharmacological, and optogenetic techniques to dissect neural circuits and molecular pathways controlling synaptic plasticity onto AGRP neurons, a population that regulates feeding. We find that food deprivation elevates excitatory synaptic input, which is mediated by a presynaptic positive feedback loop involving AMP-activated protein kinase. Potentiation of glutamate release was triggered by the orexigenic hormone ghrelin and exhibited hysteresis, persisting for hours after ghrelin removal. Persistent activity was reversed by the anorexigenic hormone leptin, and optogenetic photostimulation demonstrated involvement of opioid release from POMC neurons. Based on these experiments, we propose a memory storage device for physiological state constructed from bistable synapses that are flipped between two sustained activity states by transient exposure to hormones signaling energy levels.

## INTRODUCTION

Neurons that express *Agouti-related protein* (*Agrp*) are a molecularly defined population localized in the hypothalamic arcuate nucleus, and increasing their electrical activity is sufficient to rapidly induce voracious feeding behavior (Aponte et al., 2011). They are intermingled with a separate and functionally opposed population that is delineated by expression of *Pro-opiomelanocortin* (*Pomc*), which inhibits feeding (Aponte et al., 2011). These neurons form a core circuit regulating food intake and energy expenditure in which AGRP neurons inhibit POMC neurons (Cowley et al., 2001). Both populations behave as interoceptive sensory neurons, modulating their electrical activity in response to hormonal signals of metabolic state. In addition, AGRP and POMC neurons receive synaptic inputs that are also sensitive to the adipocyte-derived anorexigenic hormone leptin (Pinto et al., 2004). Thus, there is a close interaction between synaptic and hormonal regulation of these neurons.

Nevertheless, the influence of synaptic input on the activity of AGRP neurons is not known. In light of the capacity of AGRP neurons to stimulate feeding (Aponte et al., 2011), the relationship between excitatory synaptic input and neuronal activity is expected to be behaviorally important. Synaptic regulation of neuron activity is also of general interest in physiological circuits because synapses have a potential memory capacity through synaptic plasticity (Gordon and Bains, 2006). Here, we investigate these issues by first mapping a signaling pathway for synaptic plasticity at AGRP neurons and then selectively manipulating this pathway to test its influence on AGRP neuron electrical activity. The properties of this pathway endow this circuit with a memory mechanism.

We find that food deprivation results in persistent upregulation of excitatory synaptic input to AGRP neurons, which is also sufficient to increase their firing rate. We further examined this persistent synaptic activity and identified presynaptic signaling pathways and neural circuits that are responsible for its induction, maintenance, and offset. Plasticity was induced by the gut-derived orexigenic hormone ghrelin, which triggered a positive feedback loop dependent on AMP-activated kinase (AMPK) that maintained elevated synaptic activity even after ghrelin was removed. Conversely, persistent activity was switched off by leptin, acting through a previously undescribed opioid receptor-dependent pathway, which optogenetic experiments showed could be mediated by opioid release from POMC neurons. Based on these experiments, we propose a memory device for physiological state constructed from bistable synapses that are flipped between sustained high- and low-activity states by transient exposure to hormones associated with energy deficit and surfeit, respectively.

## RESULTS

### Deprivation-Induced Synaptic Plasticity

To investigate a potential role for synaptic plasticity in hunger, synaptic inputs onto AGRP neurons were compared between food-deprived and ad libitum fed mice during the early light period, when mice consume little. AGRP neurons were identified for whole-cell patch clamp recordings in brain slices as the subpopulation of neuropeptide Y (NPY)-expressing neurons

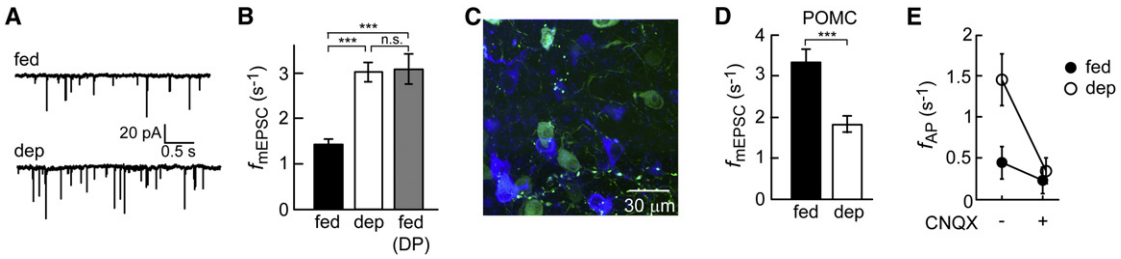


**Figure 1. Deprivation-Induced Synaptic Plasticity in AGRP and POMC Neurons**

(A) mEPSCs from fed and food-deprived (dep) mice.

(B) $f_{mEPSC}$ in AGRP neurons from deprived mice in the light period or from fed mice at the transition to the dark period (DP) were both significantly increased over $f_{mEPSC}$ from fed mice in the light period.

(C) Fluorescence micrograph of the arcuate nucleus from a *Pomc-topazFP, Npy-sapphireFP* double-transgenic mouse. POMC neurons (green) and AGRP neurons (blue) are intermingled.

(D) In POMC neurons, $f_{mEPSC}$ is decreased by food deprivation.

(E) Elevated spontaneous firing rate in AGRP neurons from deprived mice (n = 11) is reduced to the level of fed mice (n = 12) by CNQX.

***p < 0.001. Data are represented as mean ± SEM. See also Figure S1.

(Hahn et al., 1998) located in the arcuate nucleus of *Npy-sapphireFP* transgenic mice (Pinto et al., 2004). Because two measures of postsynaptic plasticity, AMPA-R/NMDA-R synaptic current ratio (Figure S1A available online) and the rectification of glutamatergic synaptic currents (Figure S1B), did not show significant differences between fed and food-deprived mice, we focused on presynaptic plasticity.

We examined presynaptic function by measuring the frequency of miniature excitatory postsynaptic currents ($f_{mEPSC}$). mEPSCs reflect spontaneous neurotransmitter release from the presynaptic terminal (Fatt and Katz, 1952) under pharmacologic conditions that block action potentials and, by extension, network activity (tetrodotoxin, TTX, 1 μM). For AGRP neurons, we found that $f_{mEPSC}$ was approximately doubled after deprivation or when measured at the beginning of the dark period (DP) in fed mice (fed: 1.4 ± 0.1 s$^{-1}$, n = 62; deprived: 3.0 ± 0.2 s$^{-1}$, n = 62; fed(DP): 3.1 ± 0.3 s$^{-1}$, n = 26; $F_{2,147}$ = 30.5, p < 0.001; Figures 1A and 1B), whereas mEPSC amplitudes were not significantly different ($F_{2,145}$ = 2.2, p = 0.11; Figure S1C). Thus, in AGRP neurons, $f_{mEPSC}$ increased in association with the animal's tendency to consume food, either after food deprivation or at the start of the dark period. Furthermore, deprivation-induced synaptic upregulation was cell type selective as POMC neurons, which are intermingled with AGRP neurons (Figure 1C) but are functionally opposed, experienced marked reduction in $f_{mEPSC}$ with deprivation (fed: 3.3 ± 0.3 s$^{-1}$, n = 30; deprived: 1.8 ± 0.2 s$^{-1}$, n = 22; unpaired t test, p < 0.001; Figure 1D), consistent with previous experiments measuring synaptic inputs to these neurons (Sternson et al., 2005).

Activation of AGRP neurons increases feeding behavior (Aponte et al., 2011), so we considered the impact of elevated spontaneous synaptic input on AGRP neuron firing rate. Because neuron firing is measured in the absence of TTX, we first recorded the frequency of spontaneous excitatory synaptic currents under this condition, which showed a significant deprivation-induced increase (fed: 2.4 ± 0.3 s$^{-1}$, n = 9; deprived: 5.4 ± 0.7 s$^{-1}$, n = 10; unpaired t test, p = 0.009). In line with elevated excitatory synaptic input, AGRP neurons from deprived mice also have a higher spontaneous firing rate ($f_{AP}$) than neurons from fed mice. This difference was eliminated by blocking excitatory synaptic activity with the glutamate receptor antagonist CNQX (2 μM) (±CNQX: $F_{1,21}$ = 15.8, p < 0.001; fed/dep: $F_{1,21}$ = 3.2, p = 0.09; interaction: $F_{1,21}$ = 6.2, p = 0.02; Figure 1E). A previous report showed that AGRP neuron firing rate was elevated after food deprivation in the absence of all synaptic input (Takahashi and Cone, 2005). For pharmacological conditions similar to those used in this prior study, we find comparable $f_{AP}$ in slices from deprived mice ($f_{AP}$, CNQX/picrotoxin: 1.2 ± 0.4 s$^{-1}$, n = 10); however, our results indicate that in the presence of synaptic inhibition, a more physiologically relevant condition, excitatory synaptic upregulation is necessary for increased electrical activity. Together with AGRP neuron photostimulation-evoked feeding (Aponte et al., 2011), these experiments suggest a relationship between synaptic activity, AGRP neuron firing, and feeding behavior. Because of the potential behavioral importance of this synaptic control point, we further investigated synaptic regulation of these neurons.

## Synaptic Upregulation Requires Ryanodine-Sensitive Calcium Stores

To probe the mechanism of synaptic upregulation at AGRP neurons, we first manipulated intracellular $Ca^{2+}$ buffering in brain slices with the membrane-permeable $Ca^{2+}$ buffer, BAPTA-AM (25 μM). This treatment reduced $f_{mEPSC}$ in slices from deprived mice to the level observed in BAPTA-AM-treated slices from fed mice (fed: 1.1 ± 0.1 s$^{-1}$, n = 24; deprived: 1.4 ± 0.1 s$^{-1}$, n = 27; Figure 2A, left). These data implicate $Ca^{2+}$-dependent processes in synaptic plasticity observed after food deprivation.

One potential pathway for $Ca^{2+}$ is through voltage-gated $Ca^{2+}$ channels (VGCCs). Blockade of VGCCs with $CdCl_2$ (200 μM) reduced $f_{mEPSC}$ in both fed and deprived mice (fed: 0.7 ± 0.1 s$^{-1}$, n = 26; deprived: 1.8 ± 0.3 s$^{-1}$, n = 21; Figure 2A, right); however, $f_{mEPSC}$ in AGRP neurons from deprived mice was still significantly greater than that observed in fed mice. Thus, VGCCs contribute to $f_{mEPSC}$ in AGRP neurons, but they are not required for deprivation-induced synaptic upregulation. Moreover, in the presence of $CdCl_2$, $f_{mEPSC}$ in AGRP neurons from deprived mice was still reduced by BAPTA-AM to the level

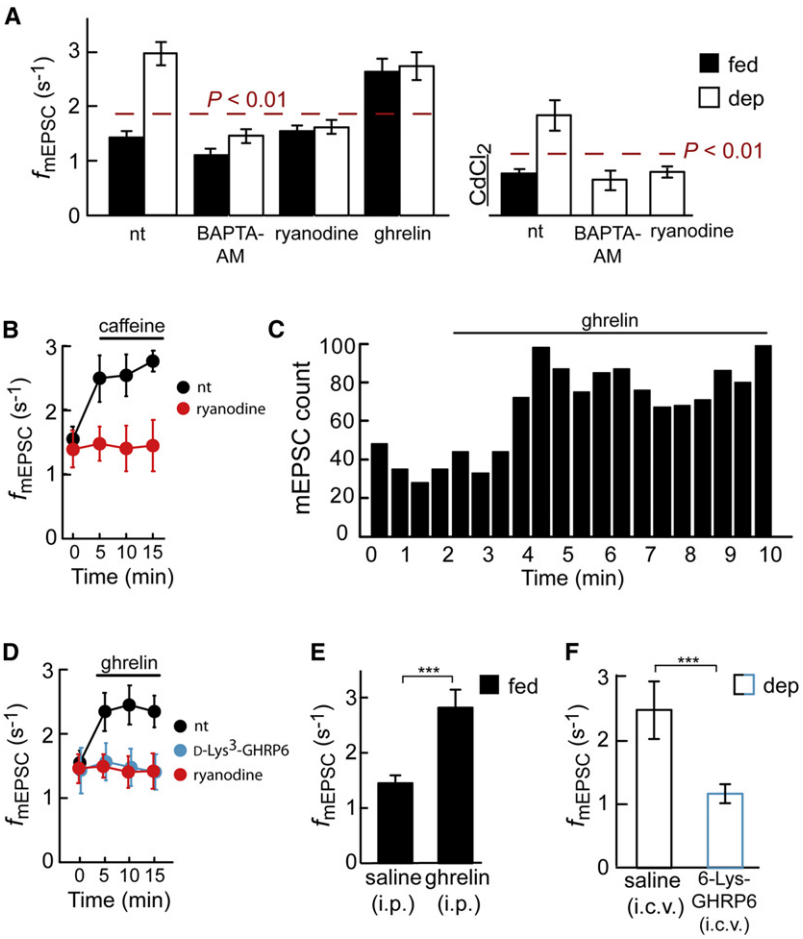


**Figure 2. Role of Calcium and Ghrelin in Synaptic Plasticity at AGRP Neurons**

(A) Comparison of $f_{mEPSC}$ in AGRP neurons from fed and food-deprived (dep) mice with no treatment (nt, data from Figure 1B) or treated with BAPTA-AM, ryanodine, or ghrelin (left panel). The right panel shows treatments in the presence of $CdCl_2$, which blocks VGCCs. All pairwise interactions were tested and p values were corrected with Holm's method. Significant differences are denoted by any interaction across the red dashed line; interactions on the same side of the line are not significant ($p > 0.05$). Left and right panels were analyzed separately.

(B) Caffeine increases $f_{mEPSC}$ (n = 8), which is blocked by ryanodine pretreatment (red, n = 3).

(C) Time course of ghrelin-mediated $f_{mEPSC}$ increase in an AGRP neuron.

(D) Ghrelin increases $f_{mEPSC}$ in AGRP neurons (n = 6), which is blocked by D-Lys3-GHRP6 (blue, n = 5) or ryanodine (red, n = 7).

(E) For fed mice, ghrelin injection increased $f_{mEPSC}$ relative to saline treatment.

(F) i.c.v. D-Lys3-GHRP6 (blue) during deprivation blocked the $f_{mEPSC}$ increase observed with i.c.v. saline (black).

***$p < 0.001$. Data are represented as mean ± SEM.

observed in slices from fed mice (0.6 ± 0.2 s$^{-1}$, n = 11; Figure 2A, right), which indicated that a different source of $Ca^{2+}$ was responsible for elevated $f_{mEPSC}$ in deprived mice.

We next perturbed $Ca^{2+}$ release from internal stores by blocking ryanodine receptors (RyR) with ryanodine (10 μM). Under these conditions, $f_{mEPSC}$ in AGRP neurons from deprived mice was reduced to the level observed for fed mice (fed: 1.5 ± 0.1 s$^{-1}$, n = 26; deprived: 1.6 ± 0.1 s$^{-1}$, n = 21; Figure 2A, left), and this was also the case in the presence of $CdCl_2$ (deprived: 0.8 ± 0.1 s$^{-1}$, n = 10; Figure 2A, right). This indicates that internal stores serve as a source of $Ca^{2+}$ required for elevated $f_{mEPSC}$ in deprived mice. In addition, caffeine (10 mM), which activates RyR-mediated $Ca^{2+}$ release, increased $f_{mEPSC}$ in AGRP neurons from fed mice to the level observed after deprivation, and this was blocked by ryanodine pretreatment (Figure 2B). It has been shown previously that increased spontaneous neurotransmitter release is associated with $Ca^{2+}$ release from internal stores in the presynaptic terminal (Emptage et al., 2001). Our experiments are consistent with a role for $Ca^{2+}$ release from internal stores in deprivation-induced plasticity.

## Ghrelin Mediates Deprivation-Induced Synaptic Plasticity

Synaptic plasticity was associated with conditions that increase food intake, such as deprivation or dark-period onset. Therefore, we tested the role of the gut-derived orexigenic hormone ghrelin, which is elevated in circulation after food deprivation (Tschöp et al., 2000). We found that ghrelin treatment (30 nM) of brain slices from fed mice (n = 23) increased $f_{mEPSC}$ to levels similar to those observed in food-deprived mice (n = 17, Figure 2A). This synaptic upregulation was rapid (Figures 2C and 2D) and was blocked by pretreatment with a ghrelin receptor (Ghsr1) antagonist, D-Lys3-GHRP6 (100 μM) (Howard et al., 1996) (Figure 2D). Moreover, pretreatment with ryanodine also blocked the ghrelin-mediated increase in $f_{mEPSC}$ (Figure 2D), consistent with our proposal that RyR-mediated $Ca^{2+}$ release is needed for this pathway.

Next, we tested the necessity and sufficiency of ghrelin for deprivation-induced synaptic plasticity. We found that the upregulation of $f_{mEPSC}$ could also be induced by intraperitoneal (i.p.) administration of ghrelin (1 μg/g) to ad libitum fed mice in the early light period, only 30 min before preparing brain slices (fed/saline: 1.4 ± 0.1 s$^{-1}$, n = 20; fed/ghrelin: 2.8 ± 0.3 s$^{-1}$, n = 24; unpaired t test, $p < 0.001$; Figure 2E). In addition, the deprivation-induced increase of $f_{mEPSC}$ onto AGRP neurons was blocked by intracerebroventricular (i.c.v.) administration of D-Lys3-GHRP6 (0.35 nmol, 3 times during 24 hr deprivation period; saline: 2.5 ± 0.5 s$^{-1}$, n = 8; D-Lys3-GHRP6: 1.2 ± 0.1 s$^{-1}$, n = 14; unpaired t test, p = 0.003; Figure 2F). Together, these results are consistent with a key role for ghrelin and Ghsr1 signaling for upregulation of synaptic activity in AGRP neurons after food deprivation.

## Presynaptic AMPK Signaling Mediates Synaptic Upregulation and AGRP Neuron Activation

Ghrelin-evoked feeding requires both AMPK signaling (Andrews et al., 2008; López et al., 2008) and AGRP neurons (Luquet et al.,

2007). Furthermore, we showed that, in brain slices, excitatory synaptic input is necessary for elevated firing in AGRP neurons after food deprivation. Thus, the influence of ghrelin on presynaptic activity suggested the possibilities that (1) AMPK regulates presynaptic activity and (2) presynaptic upregulation of glutamate release activates AGRP neuron firing. To test this, we used pharmacological activation and inhibition of AMPK signaling to examine the role of AMPK for ghrelin- and deprivation-induced synaptic activation and its impact on electrical activity in AGRP neurons.

A small-molecule activator of AMPK, AICAR, is transported into cells and phosphorylated to generate ZMP (Corton et al., 1995), a mimetic of adenosine monophosphate, which allosterically activates AMPK. AICAR (500 μM) increased $f_{mEPSC}$ in AGRP neurons in brain slices from fed but not food-deprived mice (±AICAR: $F_{1,14}$ = 8.5, p = 0.011; fed/dep: $F_{1,14}$ = 1.7, p = 0.21; interaction: $F_{1,14}$ = 14.8, p = 0.002; Figure 3A, dose response in Figure S2A). Conversely, the AMPK antagonist, Compound C (Cpd C, 150 μM), reduced $f_{mEPSC}$ in brain slices from deprived but not fed mice and also blocked subsequent ghrelin-mediated activation of $f_{mEPSC}$ (treatment: $F_{2,12}$ = 5.4, p = 0.012; fed/dep: $F_{1,12}$ = 3.5, p = 0.09; interaction: $F_{2,12}$ = 5.2, p = 0.013; Figure 3B, dose response in Figure S2B). Pharmacological control experiments testing alternative modes of action for AICAR and Cpd C on $f_{mEPSC}$ did not reveal off-target effects (Figures S2C–S2H). These results show that AMPK is a necessary and sufficient signaling component mediating synaptic upregulation after food deprivation or application of ghrelin in brain slices.

Modulation of $f_{mEPSC}$ indicated that AMPK may act presynaptically. Because presynaptic AMPK signaling has not been reported previously, we manipulated AMPK in the postsynaptic patch-clamped AGRP neuron in additional experiments to rule out a postsynaptic mechanism. Dialysis of AGRP neurons with the membrane-impermeant AMPK activator ZMP (3 mM) (Corton et al., 1995) in the patch pipette did not increase $f_{mEPSC}$ in brain slices from fed mice (1.2 ± 0.1 $s^{-1}$, n = 11; Figure 3C and Figure S2I). In brain slices from deprived mice, dialysis with a high concentration of Cpd C (500 μM) did not significantly decrease $f_{mEPSC}$ (break-in: 3.1 ± 0.4 $s^{-1}$, n = 7; 15 min cell dialysis: 3.0 ± 0.4 $s^{-1}$, n = 7; paired t test, p = 0.25; Figure 3D). These results indicate that AMPK signaling in the postsynaptic AGRP neuron does not influence $f_{mEPSC}$, which is consistent with a presynaptic AMPK signaling pathway.

Because deprivation-induced upregulation of excitatory synaptic input increases the action potential firing rate ($f_{AP}$) of AGRP neurons (Figure 1E), we next determined the contribution of AMPK-mediated presynaptic activation to electrical activity in AGRP neurons. For this, we blocked AMPK signaling in the postsynaptic AGRP neuron with dialysis of Cpd C from the patch pipette while perturbing the presynaptic pathway with subsequent bath application of AMPK activators and inhibitors. In brain slices from fed mice, AICAR increased $f_{AP}$ in AGRP neurons under conditions of postsynaptic AMPK inhibition (control: 0.11 ± 0.05 $s^{-1}$; AICAR: 0.9 ± 0.3 $s^{-1}$, n = 5; paired t test, p = 0.033; Figure 3E). Conversely, AMPK blockade by extracellular but not intracellular Cpd C reduced $f_{AP}$ in slices from deprived mice (control: 1.3 ± 0.4 $s^{-1}$; Cpd C: 0.2 ± 0.1 $s^{-1}$, n = 5; paired t test, p = 0.037; Figure 3F). Together, these experiments show that AMPK signaling is sufficient to increase AGRP neuron firing rate to levels observed in deprived mice, and that this is not due to AMPK in the postsynaptic AGRP neuron.

Ghrelin activation of synaptic activity, which is sensitive to Cpd C-mediated AMPK inhibition (Figure 3B), was also unaffected by Cpd C targeted to the postsynaptic AGRP neuron (control: 1.4 ± 0.1 $s^{-1}$, ghrelin: 2.5 ± 0.2 $s^{-1}$, n = 6; paired t test, p < 0.001; Figure 3G), consistent with a presynaptic effect. Further supporting this, mEPSC amplitude was not significantly changed by ghrelin treatment (control: 17.8 ± 0.9 pA; ghrelin: 19.2 ± 1.6 pA, n = 6; paired t test, p = 0.34). In addition, ghrelin elevated $f_{mEPSC}$ in AGRP neurons dialyzed with high BAPTA (10 mM, control: 1.7 ± 0.3 $s^{-1}$, ghrelin: 2.9 ± 0.1 $s^{-1}$, n = 4; paired t test, p = 0.007, Figure S2J). These experiments indicate that neither postsynaptic AMPK nor postsynaptic $Ca^{2+}$ signaling was necessary for upregulation of $f_{mEPSC}$ by ghrelin, which is in agreement with a presynaptic mechanism.

Consistent with elevated excitatory synaptic input frequency, AGRP neuron firing was increased by ghrelin in slices from fed but not food-deprived mice with AMPK signaling blocked in the postsynaptic AGRP neuron (±ghrelin: $F_{1,13}$ = 5.1, p = 0.041; fed/dep: $F_{1,13}$ = 8.1, p = 0.014; interaction: $F_{1,13}$ = 6.9, p = 0.014; Figure 3H). Also, ghrelin-mediated activation of $f_{AP}$ in AGRP neurons was prevented by excitatory synaptic blockade with CNQX (control/CNQX: 0.1 ± 0.03 $s^{-1}$; ghrelin/CNQX: 0.1 ± 0.05 $s^{-1}$, n = 9; paired t test, p = 0.57; Figure 3I). Both of these results indicate that ghrelin acts directly on presynaptic terminals, which is consistent with a report showing ghrelin-binding sites associated with axonal boutons in the arcuate nucleus (Cowley et al., 2003). Our data also indicate a presynaptic site of action for ghrelin activation of AGRP neuron firing rate and not a direct effect on AGRP neurons as has been previously suggested (Andrews et al., 2008; Cowley et al., 2003).

To further examine the presynaptic mechanism described here, we also considered the influence of this signaling pathway on activity-evoked synaptic release. The ratio of synaptic responses ($EPSC_2/EPSC_1$, paired-pulse ratio, PPR) from a pair of electrical stimuli (50 ms interval) to afferent axons is a property associated with presynaptic function, where lower PPR values (synaptic depression) are indicative of higher synaptic release probability (Dobrunz and Stevens, 1997). A positive relationship might be expected between release probability and elevated $f_{mEPSC}$. However, despite divergent $f_{mEPSC}$ levels, we found that PPR in AGRP neurons was not significantly different in brain slices from fed and food-deprived mice (fed: 0.67 ± 0.09, n = 15; dep: 0.59 ± 0.08, n = 12; unpaired t test, p = 0.53; Figure 3J). In both conditions, low PPR was not due to glutamate receptor desensitization, as similar results were obtained under partial receptor block by γ-D-glutamylglycine (2 mM), a competitive glutamate receptor antagonist (Figure S2K). Next, we considered the possibility that because synapses onto AGRP neurons from fed mice show high release probability, they may have limited capacity for further reduction of PPR after food deprivation or treatment with ghrelin. To test this, we shifted synaptic responses from depressing to facilitating by reducing external [$Ca^{2+}$] from our standard conditions (2 mM) to 0.5 mM, which resulted in PPR > 1 in AGRP neurons from fed mice (Figure 3K). We found that treatment of brain slices with ghrelin under these

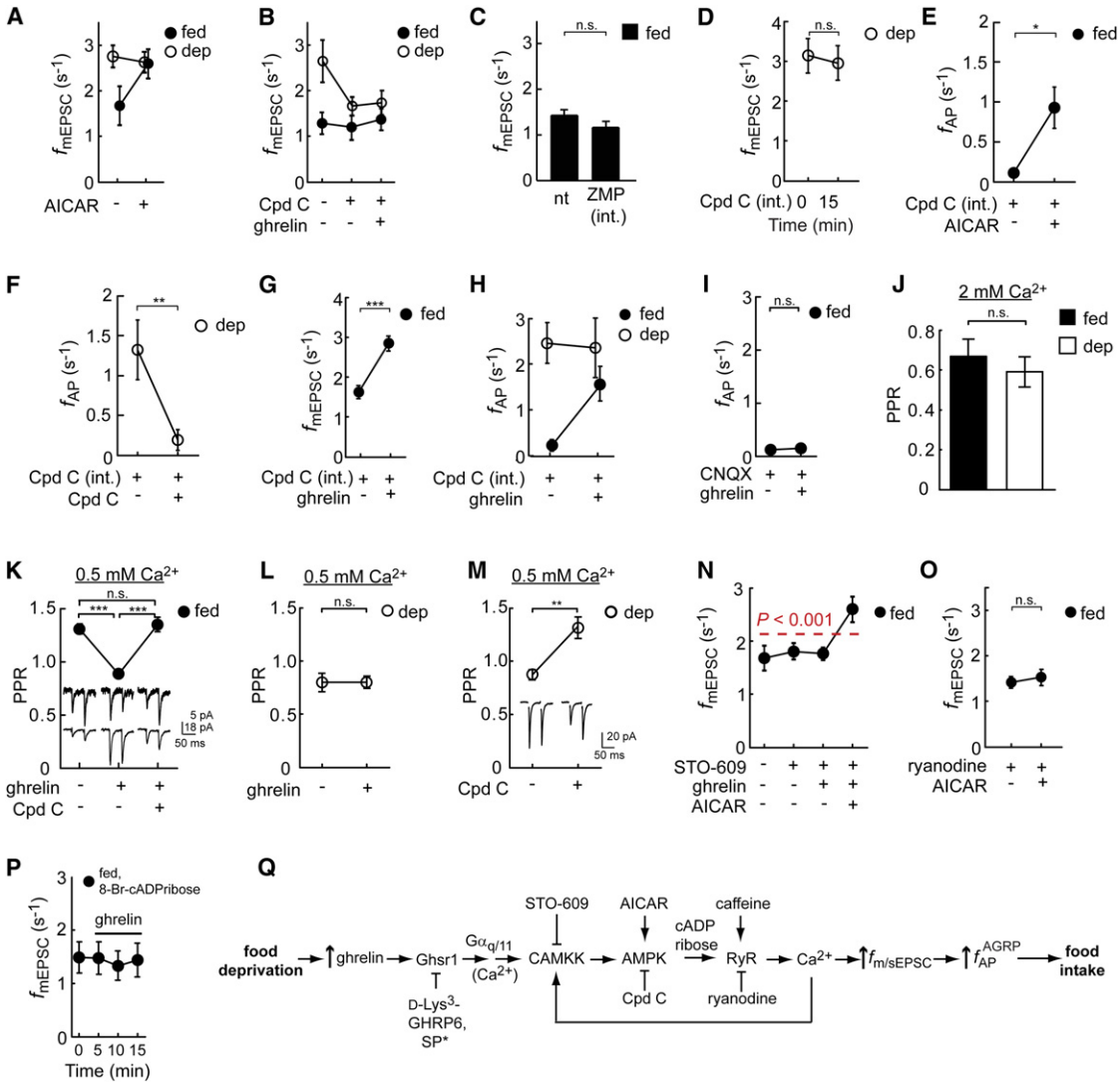


**Figure 3. AMPK Signaling Mediates Deprivation- and Ghrelin-Induced Synaptic Activity**

(A) AICAR increases $f_{mEPSC}$ in AGRP neurons from fed (n = 8) but not food-deprived (n = 8) mice.

(B) Cpd C reduces $f_{mEPSC}$ in AGRP neurons from deprived (n = 8) but not fed (n = 6) mice and blocks the ghrelin-mediated increase in $f_{mEPSC}$.

(C) AGRP neuron dialysis with the AMPK activator ZMP (3 mM) in the patch pipette internal solution (int.) does not significantly increase $f_{mEPSC}$ relative to AGRP neurons recorded with standard internal solution (nt, data from Figure 1B).

(D) Targeted AMPK inhibition with Cpd C (int.) in AGRP neurons does not significantly ($p > 0.05$) change $f_{mEPSC}$ after neuron dialysis (15 min).

(E) AGRP neuron firing rate is increased by bath-applied AICAR after intracellular blockade of AMPK with Cpd C (int.).

(F) AGRP neuron firing is decreased by bath-applied Cpd C after dialysis with Cpd C (int.).

(G) Targeted AMPK inhibition with Cpd C (int.) does not block ghrelin-mediated $f_{mEPSC}$ increase.

(H) Ghrelin activates firing in AGRP neurons from fed (n = 8) but not deprived (n = 7) mice with postsynaptic AMPK blockade by Cpd C (int.).

(I) Glutamate receptor blockade prevents ghrelin activation of AGRP neuron firing.

(J) Paired-pulse ratio (PPR) in AGRP neurons from fed and food-deprived mice in 2 mM $Ca^{2+}$.

(K) PPR in AGRP neurons from fed mice (0.5 mM external $Ca^{2+}$) is reduced by ghrelin, and this is reversed by Cpd C. Average EPSC responses from two cells are also shown (inset).

(L and M) PPR in AGRP neurons from deprived mice (0.5 mM external $Ca^{2+}$) is unaffected by ghrelin and increased by Cpd C. (M, inset) Average EPSC response from one cell is shown.

(N) Inhibition of CAMKK with STO-609 in AGRP neurons from fed mice blocks ghrelin-mediated but not AICAR-mediated increase of $f_{mEPSC}$.

(O) AICAR does not significantly increase $f_{mEPSC}$ in the presence of ryanodine.

(P) 8-Br-cADP ribose blocks the ghrelin-mediated increase of $f_{mEPSC}$.

(Q) Diagram of the signaling pathway supported by these experiments. Pointed and "T" arrows represent activation and inhibition, respectively.

n.s., $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data are represented as mean ± SEM. See also Figure S2.

conditions reduced PPR and switched synaptic responses from facilitating to depressing, and that this was reversed by treatment with Cpd C (control: 1.3 ± 0.05; ghrelin: 0.88 ± 0.03; ghrelin+Cpd C: 1.4 ± 0.07, n = 7; Figure 3K). Conversely, even in 0.5 mM $Ca^{2+}$, synaptic depression was observed in AGRP neurons from deprived mice and was not significantly altered by ghrelin (control: 0.80 ± 0.09; ghrelin: 0.80 ± 0.06, n = 3; paired t test, p = 0.98; Figure 3L). Notably, AMPK inhibition with Cpd C significantly increased PPR, which produced facilitating synaptic responses in AGRP neurons from food-deprived mice (control: 0.85 ± 0.05; Cpd C: 1.3 ± 0.1, n = 4; paired t test, p = 0.007; Figure 3M). These experiments indicate that a ghrelin- and AMPK-sensitive signaling pathway in presynaptic terminals influences both spontaneous release and evoked release; however, the observable effect on evoked release might be limited at physiologic [$Ca^{2+}$] due to high baseline release probability.

## A Signaling Pathway for AMPK-Mediated Synaptic Activity

Elevated AMPK activity is typically associated with energy deficit and a high cellular AMP/ATP ratio. In our experiments, brain slices were maintained in an energy-rich, high-glucose solution for several hours. Thus, it is unlikely that cellular depletion of ATP after food deprivation is the mechanism for AMPK activation, and we considered other pathways. $Ca^{2+}$/calmodulin-dependent protein kinase kinase (CAMKK) is a direct activator of AMPK (Hawley et al., 2005; Hurley et al., 2005; Woods et al., 2005) and has been shown to be important for ghrelin-mediated feeding (Anderson et al., 2008). Inhibition of CAMKK with the inhibitor STO-609 (3–15 μM, dose response in Figure S2L) blocked the ghrelin-mediated increase in $f_{mEPSC}$ onto AGRP neurons from fed mice; however, AICAR was still capable of increasing $f_{mEPSC}$ after treatment with STO-609, confirming that CAMKK signaling is upstream of AMPK (control: 1.7 ± 0.2 $s^{-1}$; STO-609: 1.8 ± 0.2 $s^{-1}$; ghrelin: 1.8 ± 0.1 $s^{-1}$; AICAR: 2.6 ± 0.2 $s^{-1}$, n = 10; Figure 3N). We note that higher concentrations of STO-609 blocked AICAR-mediated synaptic upregulation (Figure S2L), in line with known "off-target" AMPK inhibition by high STO-609 doses (Hawley et al., 2005). However, dose-response curves for $f_{mEPSC}$ and western blots of brain slice lysates show a dosage window in brain slice preparations, consistent with STO-609 inhibition of CAMKK without AMPK blockade (Figures S2L–S2N).

We also investigated the downstream signaling pathway by which AMPK increases $f_{mEPSC}$. Ryanodine blockade of AICAR upregulation of $f_{mEPSC}$ showed that RyRs are downstream of AMPK (Figure 3O). Furthermore, AMPK has been shown previously to elicit $Ca^{2+}$ release in smooth muscle tissue via the endogenous RyR potentiator, cADP ribose (Evans et al., 2005). The cADP ribose antagonist, 8-Br-cADP ribose (100 μM), blocked the ghrelin-mediated increase in $f_{mEPSC}$ (Figure 3P), indicating that cADP ribose production is likely the link from AMPK to RyR activation.

Collectively, these experiments are consistent with a deprivation-induced, ghrelin-sensitive pathway wherein signaling through Ghsr1, a $G\alpha_{q/11}$-coupled pathway that mobilizes $Ca^{2+}$ from $IP_3$-sensitive internal stores (Smith, 2005), leads to activation of CAMKK, which signals through AMPK to release $Ca^{2+}$ from ryanodine-sensitive internal stores, thereby increasing spontaneous excitatory synaptic activity and release probability (Figure 3Q). Elevated synaptic glutamate release results in increased AGRP neuron firing, a response that has been shown to elicit elevated food intake in mice (Aponte et al., 2011).

## Persistently Elevated Synaptic Activity Results from an AMPK-Dependent Positive Feedback Loop

In light of this ghrelin-dependent pathway, it was puzzling that elevated synaptic activity in brain slices from food-deprived mice persisted for hours after preparation and incubation of brain slices in ghrelin-free artificial cerebrospinal fluid (aCSF). We found that persistent activity could be sustained even after Ghsr1 blockade by D-Lys3-GHRP6 or by the Ghsr1 inverse agonist [D-Arg1,D-Phe5,D-Trp7,9,Leu11]-Substance P (SP*, 100 μM) (Holst et al., 2003) (Figure S3), indicating that ghrelin-mediated or ghrelin-independent constitutive Ghsr1 signaling were not responsible for the sustained activity.

To explore this further, we used transient exposure to ghrelin (5 min) followed by washout and treatment with SP* to block subsequent Ghsr1 signaling in order to examine the potential for ghrelin to elicit persistent activity in brain slices from fed mice. This sequence led to elevation of $f_{mEPSC}$ and demonstrated marked hysteresis, remaining high after ghrelin washout and treatment with SP* (n = 6; Figure 4A).

Hysteresis, a memory capacity observed in diverse biological processes, frequently involves positive feedback (Ferrell, 2002). We considered the possibility that, in this system, hysteresis resulted from positive feedback in the AMPK-dependent signaling pathway (Figure 3Q). This ghrelin-responsive pathway results in $Ca^{2+}$ release from ryanodine-sensitive internal stores but also involves the upstream $Ca^{2+}$-sensitive kinase CAMKK, raising the possibility for positive feedback to sustain the pathway in the absence of ghrelin. Consistent with the high cooperativity associated with positive feedback (Ferrell, 2002), dose-response curves for AICAR, Cpd C, and STO-609 showed switch-like ultrasensitivity (Koshland et al., 1982) (Figures S2A, S2B, and S2L).

To further explore the possibility of positive feedback, we reasoned that persistent synaptic activity should also be sensitive to activation and blockade at any node in the loop. To test this, we released $Ca^{2+}$ from ryanodine-sensitive internal stores using a brief exposure (5 min) to caffeine, which we have shown elevates $f_{mEPSC}$ (Figure 2B). Under these conditions, synaptic activity also exhibited hysteresis; after washout of caffeine, $f_{mEPSC}$ was still elevated (Figure 4B). Subsequent blockade of the "upstream" AMPK node of the pathway with Cpd C reversed $f_{mEPSC}$ to precaffeine levels (Figure 4B; compare with Figure S2F), as expected from a positive feedback loop. Toggling from the high- to the low-activity state in the absence of ghrelin shows that these synapses are bistable.

## Duration of the Persistently Activated State

To measure the duration of the persistently activated state, brain slices from fed mice were treated briefly with ghrelin (30 nM, 5 min), washed by transfer to a ghrelin-free bathing solution, and then transferred again for incubation with the Ghsr1 antagonist, D-Lys3-GHRP6 (100 μM), to ensure the absence of ongoing ghrelin signaling for 3–5 hr, after which they were used for

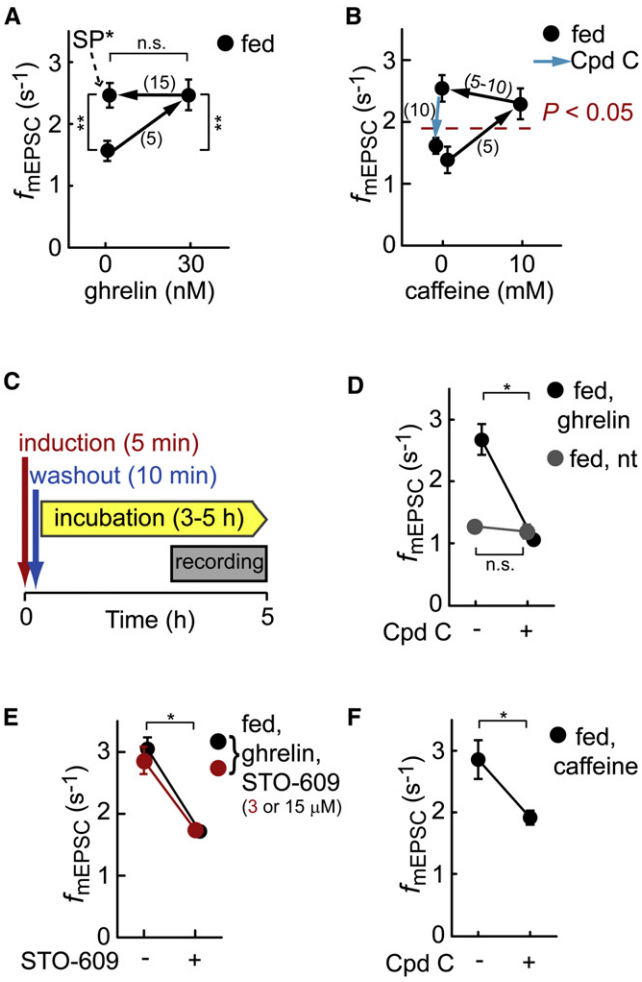


**Figure 4. Synaptic Hysteresis Resulting from an AMPK-Dependent Positive Feedback Loop**

(A) Ghrelin upregulation of $f_{mEPSC}$ shows hysteresis; $f_{mEPSC}$ is sustained after ghrelin washout and Ghsr1 blockade with SP*. Duration of each transition is in parentheses (minutes).

(B) Caffeine increases $f_{mEPSC}$, which remains elevated after washout. Synaptic activity returned to baseline after treatment with Cpd C, consistent with a positive feedback loop. Significant differences are denoted by any interaction across the red dashed line.

(C–F) Procedure for testing duration of persistent activity by transient exposure of brain slices to ghrelin or caffeine (5 min), transfer to a wash solution (10 min), and transfer again to a solution containing either aCSF alone or with D-Lys3-GHRP6 (3–5 hr). Slices were subsequently transferred to a recording chamber for electrophysiology.

(D and E) After transient ghrelin exposure and prolonged D-Lys3-GHRP6 incubation, $f_{mEPSC}$ remained elevated but was rapidly (10 min) reduced by (D) Cpd C or (E) STO-609. Control brain slices that were not treated (nt) with ghrelin but otherwise incubated as above (D) had low $f_{mEPSC}$.

(F) After transient exposure to caffeine and prolonged incubation in caffeine-free aCSF (3–5 hr), $f_{mEPSC}$ was still elevated but was rapidly (10 min) reduced by Cpd C, consistent with the operation of a positive feedback loop.

n.s., $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data are represented as mean ± SEM. See also Figure S3.

electrophysiology in D-Lys3-GHRP6-containing solution (Figure 4C). Remarkably, AGRP neurons from these slices still showed elevated $f_{mEPSC}$ levels that were not significantly different from those observed in ghrelin treatment experiments that involved continuous exposure to the hormone (Figure 2D and Figure 3G). Furthermore, $f_{mEPSC}$ was still rapidly reversed by AMPK blockade with Cpd C (ghrelin/wash/D-Lys3-GHRP6: 2.7 ± 0.2 $s^{-1}$; +Cpd C: 1.1 ± 0.1 $s^{-1}$, n = 3; paired t test, p = 0.023; Figure 4D) or CAMKK inhibition by STO-609 (3 or 15 μM; Figure 4E). As a control, we incubated slices with D-Lys3-GHRP6 without pre-exposure to ghrelin; these showed low $f_{mEPSC}$ and no effect of Cpd C (D-Lys3-GHRP6, 3–5 hr: 1.3 ± 0.02 $s^{-1}$; +Cpd C: 1.2 ± 0.1 $s^{-1}$, n = 3; paired t test, p = 0.51; Figure 4D).

In addition, we tested brief caffeine exposure (10 mM, 5 min) in brain slices from fed mice followed by incubation in caffeine-free aCSF for 3–5 hr (Figure 4C). This treatment also elevated $f_{mEPSC}$, which was rapidly reversed by Cpd C (caffeine/wash: 2.9 ± 0.3 $s^{-1}$; +Cpd C: 1.9 ± 0.1 $s^{-1}$, n = 6; paired t test, p = 0.026; Figure 4F). Thus, synaptic activation persists for at least several hours after induction, is AMPK sensitive throughout this timeframe, and can be induced upstream and downstream of AMPK signaling.

## Counter-regulation of Persistent Synaptic Activity

Next, we investigated the pathway by which persistent synaptic upregulation is switched off. We observed that $f_{mEPSC}$ from mice that were food deprived and then refed was still elevated 24 hr after refeeding but was reduced to baseline by 48 hr (Figure 5A); notably, mEPSC amplitude did not change significantly throughout this time course (data not shown, $F_{4,187}$ = 1.22, p = 0.31). Consistent with the elevated excitatory synaptic input, the action potential firing rate of AGRP neurons was also elevated 24 hr after refeeding, which required glutamatergic synaptic input (24 hr refed: 1.5 ± 0.2 $s^{-1}$; 24 hr refed/CNQX: 0.2 ± 0.1 $s^{-1}$, n = 6; paired t test, p = 0.002; Figure 5B).

This timescale led us to examine the role of the hormone leptin, which is associated with long-term regulation of energy homeostasis. Leptin reduces ghrelin-induced feeding (Nakazato et al., 2001) and deprivation-induced AMPK phosphorylation in the hypothalamus (Minokoshi et al., 2004). Leptin administration (1 μg/g) to deprived mice 3 hr before brain slice preparation decreased $f_{mEPSC}$ to levels found in fed mice (i.p. saline: 2.8 ± 0.4 $s^{-1}$, n = 9; i.p. leptin: 1.7 ± 0.2 $s^{-1}$, n = 10; unpaired t test, p = 0.024; Figure 5C). Leptin (100 nM) treatment of brain slices from deprived mice did not affect $f_{mEPSC}$ onto AGRP neurons (2.7 ± 0.3 $s^{-1}$, n = 11), so we considered an indirect interaction.

One possibility is that leptin activation of POMC neurons in the arcuate nucleus (Cowley et al., 2001) could release a neuropeptide modulator of AMPK activity. Because α-MSH is a POMC neuron-derived neuropeptide that reduces feeding through melanocortin receptor signaling, we tested the influence of MTII (500 nM), a synthetic agonist of melanocortin receptors, on $f_{mEPSC}$. However, this treatment did not decrease $f_{mEPSC}$ onto AGRP neurons in brain slices from deprived mice (control: 3.0 ± 0.7 $s^{-1}$; MTII: 3.1 ± 0.6 $s^{-1}$, n = 6; paired t test, p = 0.93).

We next considered the possibility that an opioid such as β-endorphin could reverse persistent synaptic upregulation in AGRP neurons. β-endorphin is also produced in POMC neurons and is a μ-opioid receptor (MOR) agonist (Silva et al., 2001). Brain slices from food-deprived mice were treated with a synthetic

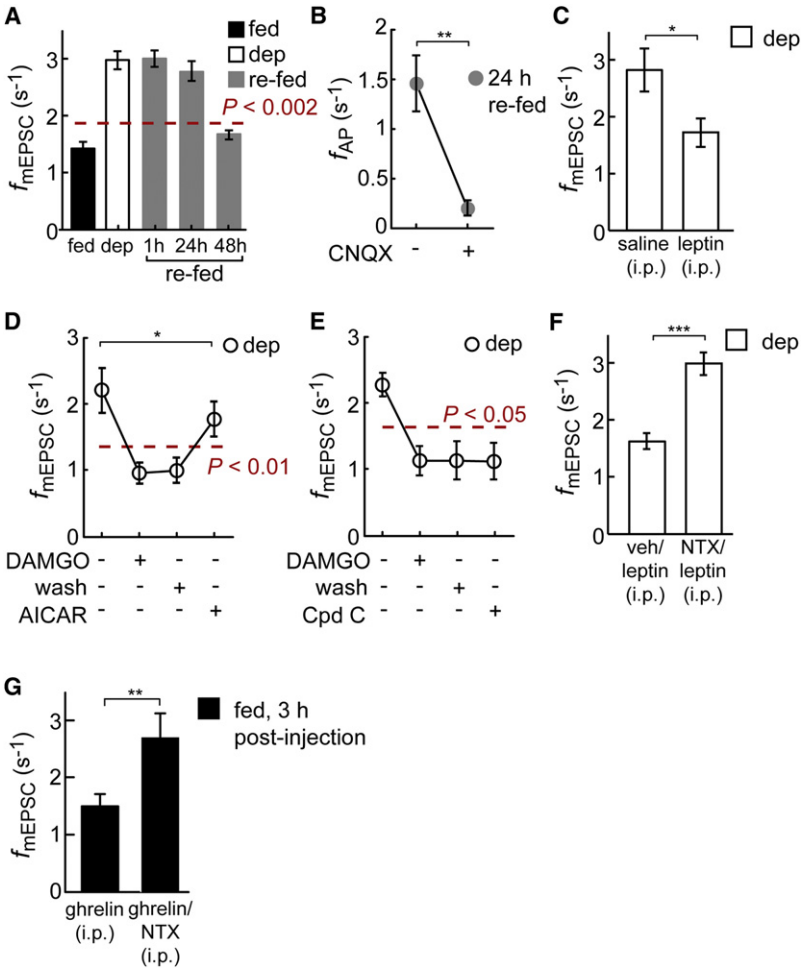


**Figure 5. Persistent Synaptic Upregulation Is Reversed by Leptin-Mediated Opioid Release**

(A) Synaptic activity before, during, and after food deprivation and refeeding (fed and deprived data from Figure 1C, 1 hr: n = 21, 24 hr: n = 27, 48 hr: n = 23). p values for multiple pairwise comparisons were adjusted with Holm's correction. The significant differences are denoted by any interaction across the red dashed line.

(B) AGRP neuron firing rate 24 hr after refeeding was still elevated, which was dependent on glutamatergic synaptic input.

(C) Injection of leptin in deprived mice reduced $f_{mEPSC}$ in AGRP neurons relative to saline injection.

(D) DAMGO reduced $f_{mEPSC}$ in AGRP neurons under VGCC block ($CdCl_2$). The $f_{mEPSC}$ remained at this level during a 15 min wash but was increased by treatment with AICAR.

(E) $f_{mEPSC}$ in AGRP neurons from deprived mice treated with DAMGO is insensitive to Cpd C.

(F) NTX pretreatment of deprived mice blocks leptin-mediated reduction in $f_{mEPSC}$ observed with saline pretreatment.

(G) Coinjection of ghrelin and NTX, but not ghrelin alone, leads to elevated $f_{mEPSC}$ in brain slices prepared after 3 hr.

*p < 0.05, **p < 0.01, ***p < 0.001. Data are represented as mean ± SEM.

selective MOR agonist, DAMGO (1 μM). This was done in the presence of $CdCl_2$ because MORs can also influence synaptic release by inhibition of VGCCs (Endo and Yawo, 2000), which we have shown to be unrelated to deprivation-induced plasticity (Figure 2A). Under these conditions, we found that, after treatment and washout of DAMGO (see Experimental Procedures), $f_{mEPSC}$ in AGRP neurons was similar to levels from fed mice. Furthermore, these synapses were now sensitive to AICAR, which increased $f_{mEPSC}$ (control: 2.2 ± 0.3 $s^{-1}$; DAMGO: 0.9 ± 0.2 $s^{-1}$; wash: 1.0 ± 0.2 $s^{-1}$; AICAR: 1.8 ± 0.3 $s^{-1}$, n = 9; Figure 5D). Conversely, treatment with Cpd C after washout of DAMGO did not change $f_{mEPSC}$, indicating that AMPK had been fully inactivated by MOR signaling (control: 2.3 ± 0.2 $s^{-1}$; DAMGO: 1.1 ± 0.2 $s^{-1}$; wash: 1.1 ± 0.3 $s^{-1}$; Cpd C: 1.1 ± 0.3 $s^{-1}$, n = 5; Figure 5E).

The negative regulation of AMPK by opioid receptor signaling raised the question of whether tonic opioid activity was required to prevent ghrelin-independent activation of the AMPK-mediated positive feedback loop. Incubation of brain slices from ad libitum fed mice with the opioid receptor antagonist naltrexone (NTX, 500 nM) for 3–5 hr did not increase $f_{mEPSC}$ or induce sensitivity to Cpd C (NTX: 1.3 ± 0.2 $s^{-1}$; NTX/Cpd C: 1.4 ± 0.1 $s^{-1}$, n = 3; paired t test, p = 0.84), indicating that AMPK is not disinhibited by NTX blockade of opioid receptors. This suggests that additional control pathways restrain this presynaptic positive feedback loop in the absence of ghrelin.

We also tested the leptin-opioid relationship in vivo by treating food-deprived mice with NTX (10 μg/g) followed by leptin. This combination blocked leptin reduction of $f_{mEPSC}$ in brain slices from deprived mice (vehicle/leptin: 1.6 ± 0.1 $s^{-1}$, n = 26; NTX/leptin: 3.0 ± 0.2 $s^{-1}$, n = 34; unpaired t test, p < 0.001; Figure 5F), which confirms that leptin downregulates AMPK-mediated synaptic activity through an opioid receptor-dependent mechanism.

Next, we examined the persistence of the ghrelin-induced upregulation of $f_{mEPSC}$ after ghrelin injection to ad libitum fed mice. Because injected ghrelin levels are reported to decline to baseline over 1–2 hr (Tschöp et al., 2000), we looked at the effect of ghrelin treatment on $f_{mEPSC}$ 3 hr post-injection, during which time mice were not exposed to food. Based on our analysis of persistent activity at these synapses, we expected $f_{mEPSC}$ to be elevated as it is 30 min after ghrelin administration (Figure 2E). Instead, we found that $f_{mEPSC}$ was similar to that in uninjected fed mice (ghrelin, 3 hr: 1.5 ± 0.2 $s^{-1}$, n = 22). We tested the possibility that the opioid tone in fed mice was sufficient to switch off elevated synaptic activity once injected ghrelin was cleared. Consistent with this, cotreatment of ad libitum fed mice with ghrelin and NTX led to $f_{mEPSC}$ that was still elevated 3 hr after ghrelin treatment (ghrelin/NTX 3 hr: 2.7 ± 0.4 $s^{-1}$, n = 10; unpaired t test, p = 0.009; Figure 5G). Fed mice treated solely with NTX did not increase $f_{mEPSC}$ after 3 hr (1.5 ± 0.3 $s^{-1}$, n = 11). These experiments are consistent with the persistence of ghrelin-induced synaptic upregulation and indicate that the

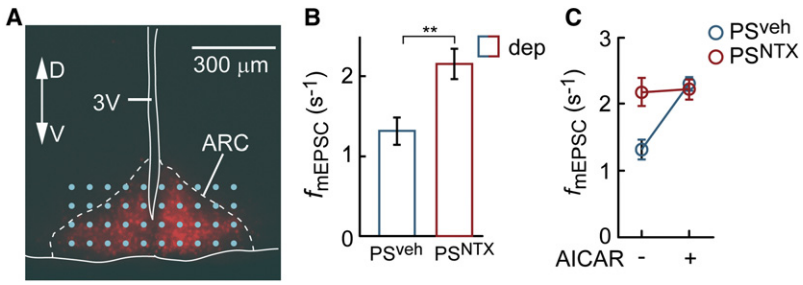


**Figure 6. POMC Neurons Release an Opioid that Resets Persistent Synaptic Activity**

(A) Epifluorescence micrograph of brain slice with POMC neurons expressing ChR2-tdtomato. Blue circles: photostimulation sites; ARC: arcuate nucleus; 3V: third ventricle; D: dorsal; V: ventral.

(B) ChR2-mediated photostimulation of POMC neurons in brain slices from deprived mice in the presence or absence of NTX. Photostimulation of POMC neurons reduces $f_{mEPSC}$ unless performed in the presence of NTX.

(C) Subset of neurons in (B) subjected to AICAR after photostimulation. POMC neuron photostimulation in the absence of NTX (n = 5) rendered $f_{mEPSC}$ in AGRP neurons sensitive to AICAR, whereas those photostimulated in the presence of NTX (n = 5) were insensitive to AICAR, indicating that a POMC neuron-derived opioid inactivates AMPK.

**p < 0.01. Data are represented as mean ± SEM.

baseline opioid tone in fed mice can reverse this after ghrelin levels fall.

### POMC Neurons Release an Opioid that Resets Persistent Synaptic Activity

Although opioid receptors are involved in the leptin-mediated inactivation of presynaptic AMPK, it was unclear whether POMC neurons could serve as the source of these opioids. We tested this using an optogenetic approach. We expressed the light-activated cation channel channelrhodopsin-2 (ChR2) (Boyden et al., 2005) in POMC neurons, rendering them selectively photoexcitable. POMC neurons were targeted using the Cre recombinase (Cre)-dependent viral vector rAAV-FLEX-*rev*-ChR2-tdtomato, which we have described previously for POMC neuron photostimulation (Aponte et al., 2011; Atasoy et al., 2008).

In brain slices from food-deprived *Pomc-Cre;Npy-sapphireFP* double-transgenic mice expressing ChR2, we photostimulated the area containing the arcuate nucleus with focused laser light in a 4 × 10 spatial pattern of stimulation sites (Figure 6A). Our expectation was that a neuromodulator would be released that could potentially downregulate AMPK signaling. Next, the slice was treated with TTX to record mEPSCs and $CdCl_2$ in order to eliminate any contribution of VGCCs. POMC neuron photostimulation led to a significant reduction in $f_{mEPSC}$ in AGRP neurons, and this effect was blocked if photostimulation was performed in the presence of NTX (500 nM) (control: 1.3 ± 0.2 $s^{-1}$, n = 15; NTX: 2.2 ± 0.2 $s^{-1}$, n = 14; unpaired t test, p = 0.003; Figure 6B). When NTX was present during photostimulation, AGRP neurons from deprived mice maintained insensitivity to AICAR (Figure 6C). However, after photostimulation in the absence of NTX, $f_{mEPSC}$ could be increased in response to subsequent AICAR administration (±AICAR: $F_{1,8}$ = 43.3, p < 0.001; ±NTX: $F_{1,8}$ = 3.6, p = 0.096; interaction: $F_{1,8}$ = 36.8, p < 0.001; Figure 6C). These results indicate that a POMC neuron-derived opioid, likely β-endorphin, reverses AMPK-mediated persistent synaptic upregulation.

## DISCUSSION

Homeostasis requires increased food intake as energy stores are depleted. In this study, we provide evidence for a dynamic neural circuit (Figure 7A) with a reversible memory storage capacity that regulates feeding behavior in response to energy deficit. Previous work showed a core circuit in which AGRP neurons inhibit POMC neurons and electrical activity is regulated by hormones that signal physiological state (Cowley et al., 2001, 2003). We report here that a key control point for this circuit is excitatory synapses onto AGRP neurons. These synapses endow this circuit with unexpected memory properties based on a presynaptic AMPK-dependent positive feedback loop and regulation by a previously undescribed POMC neuron-derived opioid pathway.

The investigation of synaptic regulation of AGRP neurons provided insight into the mechanism of ghrelin-evoked feeding. AGRP neurons are required for ghrelin's orexigenic properties (Luquet et al., 2007). Previous reports concluded that ghrelin activates AGRP neurons directly (Andrews et al., 2008; Cowley et al., 2003). A model by Andrews et al. (2008) of ghrelin-induced feeding suggests that Ghsr1 signaling in AGRP neurons elicits firing by AMPK-mediated alteration of fatty-acid oxidation and regulation of mitochondrial uncoupling proteins. However, it is unclear how these metabolic pathways influence electrical activity, except perhaps by increasing available intracellular energy stores.

Our findings support an alternative model in which ghrelin acts at presynaptic receptors to increase glutamate release and activate AGRP neurons through ionotropic glutamate receptors. This is unlikely to be related to increased excitatory synapse number based on previous ultrastructural quantification following ghrelin treatment (Pinto et al., 2004). We suggest that metabolic pathways for ghrelin action in AGRP neurons (Andrews et al., 2008) are likely responsible for altering neuropeptide gene expression or addressing metabolic requirements to maintain AGRP neuron firing but are not directly involved in the increase of neuron firing following ghrelin administration.

We also find that AMPK participates in a positive feedback loop, resulting in synaptic hysteresis. Hysteresis for AMPK activation was investigated previously in *Xenopus* oocytes, but, although nonreversing activation was observed with transient antimycin treatment, this was attributed to irreversible metabolic block, not bistability, and other activators of AMPK did not show hysteresis (Martiáñez et al., 2009). Thus, hysteresis for AMPK activity is not elicited in all cells.

Hysteresis is a hallmark of bistability (Bhalla and Iyengar, 1999), which confers memory capacity in biological systems, often in conjunction with positive feedback loops (Ferrell,

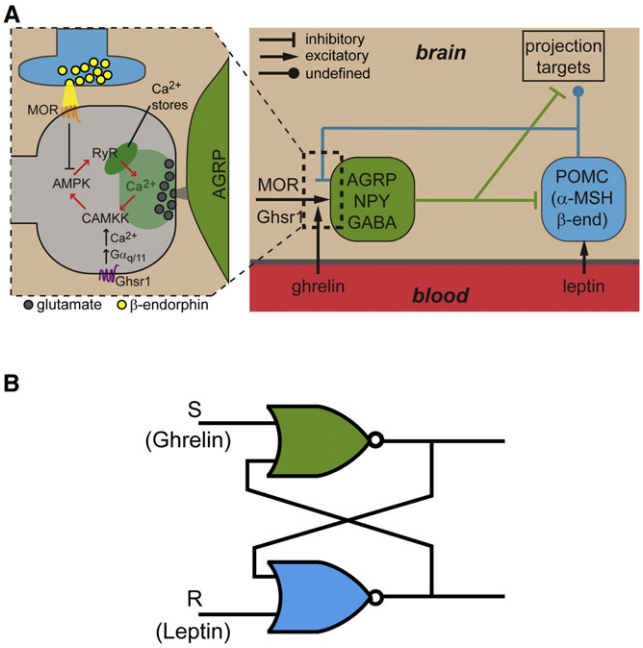


**Figure 7. SR Flip-Flop Model of a Neural Circuit with Synaptic Memory of Physiological State**

(A) A core circuit in which AGRP neurons synaptically inhibit POMC neurons and are regulated by circulating hormones is controlled by ghrelin-responsive excitatory synapses. These synapses give this circuit a memory property based on an AMPK-dependent positive feedback loop (inset), which can be reversed by POMC neuron output, likely β-endorphin.

(B) A heuristic for the logic of this circuit is the SR flip-flop memory storage circuit. In the analogy with the neural circuit reported here, the set signal is ghrelin, which activates the green NOR gate, representing the conglomeration of AGRP neurons and their ghrelin-sensitive excitatory presynaptic terminals. The reset signal is leptin, which interacts with POMC neurons represented as the blue NOR gate. Notably, when R and S are both high, the circuit does not support memory, and this condition is consistent with the case of ghrelin treatment of fed mice where opioid signaling is sufficiently high to prevent persistent synaptic upregulation (Figure 5G).

2002). Other kinase-dependent positive feedback loops have been considered previously for long-term information storage in the central nervous system (Bhalla and Iyengar, 1999; Tanaka and Augustine, 2008). The AMPK-mediated feedback described here persistently upregulates activity in presynaptic terminals for at least 5 hr, which could serve as a memory of the hormone ghrelin in the form of sustained synaptic activity onto AGRP neurons.

Synapses with positive feedback also require a separate inhibitory signal to reverse synaptic upregulation, for example once energy balance is restored. Leptin is well suited for this role as it signals energy reserves by falling rapidly during food deprivation and rises with refeeding (Boelen et al., 2006). We find that leptin is sufficient to counter-regulate elevated presynaptic activity onto AGRP neurons through an opioid receptor-dependent pathway. Leptin activates POMC neurons (Cowley et al., 2001), and it is likely that β-endorphin is the opioid released from POMC neurons to downregulate elevated synaptic activity. Interestingly, rapid synaptic plasticity at AGRP neurons was previously described in leptin-deficient *ob/ob* mice after leptin administration (Pinto et al., 2004); future work could investigate whether this involves the opioid-dependent mechanism described here.

Previous studies showed that selective ablation of only the β-endorphin-encoding portion of the *Pomc* gene yields mice that are hyperphagic and overweight (Appleyard et al., 2003). This was unexpected in the context of β-endorphin intracranial administration, which activates multiple opioid pathways and leads to overeating (Grandison and Guidotti, 1977), but it is consistent with the anorexigenic function of POMC neurons. In light of the experiments reported here, β-endorphin ablation appears to disable a key regulatory system that may include ghrelin-mediated synaptic activity onto AGRP neurons, consequently potentiating ghrelin's feeding stimulatory function.

The circuit described here could be further elaborated by considering the origin of the ghrelin-sensitive glutamatergic inputs. Currently, these neurons are not known, but they should originate from Ghsr1-expressing populations, which are clustered in several brain regions (Zigman et al., 2006). Retrograde tracing studies (Li et al., 1999; DeFalco et al., 2001) indicate that a subset of these brain regions project to the arcuate nucleus and to AGRP neurons. Future work could examine the identity of these presynaptic neurons, which would provide an entry point for genetically encoded tools to perturb presynaptic pathways with kinase mutants or optogenetic tools. In addition, upstream populations projecting to AGRP neurons might also be expected to influence feeding behavior, possibly extending control of AGRP neuron electrical activity beyond the contexts of hormonal and homeostatic regulation discussed in this study.

## Why Might Neural Circuits Responding to Hormonal Cues Require a Memory Capacity?

These experiments provide evidence of a circuit that has the capacity to store a memory of specific hormonal states (Figure 7A). Heuristically, its properties are analogous to the set/reset (SR) flip-flop memory storage circuit from digital electronics, which consists of two interconnected NOR logic gates (Horowitz and Hill, 1989) (Figure 7B). Such a memory mechanism is consistent with the logic for hormonal control of synaptic upregulation and counter-regulation described in this study.

SR flip-flop memory has useful features for physiologic regulation. In this model, two hormones ("set": ghrelin and "reset": leptin) separately signal deficit or surfeit. The memory properties of the SR flip-flop act as an "until" loop, maintaining activity in response to S (even after S is turned off) until R is true. By using two discrete signals that reflect different physiological states, the circuit detects a state change. This model does not have a set point, but instead, it has a set range that is defined by the respective thresholds for activation of S and R. The operation of this circuit differs from homeostatic set point control theory in which a physiological parameter is continuously compared to a reference value and results in commensurate adjustment of physiological or behavioral output. This two-component circuit model is, however, evocative of Walter Cannon's original formulation of the homeostasis concept, which referred to the balance of opposing factors (Cannon, 1929). In this circuit, though, we

add the provision that these signals operate as discrete Boolean logical operations (see Figure 7B) due to an AMPK-dependent positive feedback loop. Importantly, this circuit displays memory properties only under sufficient deprivation to significantly reduce R (leptin levels or opioid tone), which is what we observe experimentally.

A potential advantage of a physiological set range for energy balance is that it avoids constant readjustment of energy stores as required for defending a set point, which may facilitate pursuit of other behaviors when energy repletion is not critical. Moreover, modulation of the response threshold for ghrelin, leptin, or opioids could shift this range and, potentially, food intake and body weight. Another implication of this state-sensing property is that it could, in principle, simplify requirements for the regulatory control of ghrelin and leptin release from peripheral endocrine cells such that they need not reflect the integrated energy balance of the organism but instead simply report a state of deficit and surfeit, respectively. For example, high ghrelin levels resulting from food deprivation have been shown to fall within 1–2 hr of refeeding, due to negative regulation by nutrients such as carbohydrates (Tschöp et al., 2000), even though energy homeostasis may not be achieved in this short time. However, the ghrelin-initiated synaptic upregulation and the corresponding firing rate elevation in AGRP neurons were maintained after 24 hr of refeeding (Figures 5A and 5B), during which energy stores are restored. The operation of this memory system extends the responsiveness of the circuit beyond the lifetime of the triggering hormone until a signal of energy surfeit resets the circuit, in this case by opioid receptor activation. Such a mechanism may also be important in neural circuits governing behavioral responses to other physiological conditions. Furthermore, the relative ubiquity of these signaling pathways may extend the findings of positive feedback and set/reset bistability to other neuromodulators in the brain.

## EXPERIMENTAL PROCEDURES

Experimental protocols were conducted according to U.S. National Institutes of Health guidelines for animal research and were approved by the Institutional Animal Care and Use Committee at Janelia Farm Research Campus. Food deprivation was for 24 hr.

For several experiments, the experimenter was blinded to the identity of the pharmacological reagent or to the deprivation state of the animal (Figures 1B, 1D, and 1E; Figure S1; Figure 2A [except ghrelin treatment]; Figures 3A and 3B; Figure 5F; Figure 6).

Electrophysiology and photostimulation methods are provided in Extended Experimental Procedures.

In figures, bars represent separate groups of cells, and symbols connected by lines represent manipulations applied across a group of cells. Data are represented as mean ± standard error of the mean (SEM). p values for pairwise comparisons were calculated by two-tailed Student's t test. p values for comparisons across more than two groups were adjusted with Holm's correction (Holm, 1979). Tests involving one-way ANOVA or two-way ANOVA with one factor repeated-measures were calculated with SigmaPlot (Systat). Not significant (n.s.): $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and three figures and can be found with this article online at doi:10.1016/j.cell.2011.07.039.

## ACKNOWLEDGMENTS

This research was funded by the Howard Hughes Medical Institute. We thank L. Scheffer for discussion of digital electronic circuits; J. Cox for mouse husbandry; Y. Aponte for assistance with animal studies; Y. Li and Z. Zhang for assistance with biochemistry; and K. Moses, S. Eddy, K. Svoboda, G. Murphy, and C. Zuker for comments on the manuscript. Leptin was provided by Amylin Pharmaceuticals. Y.Y. performed the experiments. H.S. performed western blots. D.A. made the initial finding of deprivation-induced plasticity. Y.Y. and S.M.S. designed the study, analyzed the data, and wrote the paper.

Received: November 10, 2010
Revised: June 15, 2011
Accepted: July 15, 2011
Published: September 15, 2011

## REFERENCES

Anderson, K.A., Ribar, T.J., Lin, F., Noeldner, P.K., Green, M.F., Muehlbauer, M.J., Witters, L.A., Kemp, B.E., and Means, A.R. (2008). Hypothalamic CaMKK2 contributes to the regulation of energy balance. Cell Metab. *7*, 377–388.

Andrews, Z.B., Liu, Z.W., Walllingford, N., Erion, D.M., Borok, E., Friedman, J.M., Tschöp, M.H., Shanabrough, M., Cline, G., Shulman, G.I., et al. (2008). UCP2 mediates ghrelin's action on NPY/AgRP neurons by lowering free radicals. Nature *454*, 846–851.

Aponte, Y., Atasoy, D., and Sternson, S.M. (2011). AGRP neurons are sufficient to orchestrate feeding behavior rapidly and without training. Nat. Neurosci. *14*, 351–355.

Appleyard, S.M., Hayward, M., Young, J.I., Butler, A.A., Cone, R.D., Rubinstein, M., and Low, M.J. (2003). A role for the endogenous opioid beta-endorphin in energy homeostasis. Endocrinology *144*, 1753–1760.

Atasoy, D., Aponte, Y., Su, H.H., and Sternson, S.M. (2008). A FLEX switch targets Channelrhodopsin-2 to multiple cell types for imaging and long-range circuit mapping. J. Neurosci. *28*, 7025–7030.

Bhalla, U.S., and Iyengar, R. (1999). Emergent properties of networks of biological signaling pathways. Science *283*, 381–387.

Boelen, A., Kwakkel, J., Vos, X.G., Wiersinga, W.M., and Fliers, E. (2006). Differential effects of leptin and refeeding on the fasting-induced decrease of pituitary type 2 deiodinase and thyroid hormone receptor beta2 mRNA expression in mice. J. Endocrinol. *190*, 537–544.

Boyden, E.S., Zhang, F., Bamberg, E., Nagel, G., and Deisseroth, K. (2005). Millisecond-timescale, genetically targeted optical control of neural activity. Nat. Neurosci. *8*, 1263–1268.

Cannon, W.B. (1929). Organization for physiological homeostasis. Physiol. Rev. *9*, 399–431.

Corton, J.M., Gillespie, J.G., Hawley, S.A., and Hardie, D.G. (1995). 5-aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? Eur. J. Biochem. *229*, 558–565.

Cowley, M.A., Smart, J.L., Rubinstein, M., Cerdán, M.G., Diano, S., Horvath, T.L., Cone, R.D., and Low, M.J. (2001). Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. Nature *411*, 480–484.

Cowley, M.A., Smith, R.G., Diano, S., Tschöp, M., Pronchuk, N., Grove, K.L., Strasburger, C.J., Bidlingmaier, M., Esterman, M., Heiman, M.L., et al. (2003). The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. Neuron *37*, 649–661.

DeFalco, J., Tomishima, M., Liu, H., Zhao, C., Cai, X., Marth, J.D., Enquist, L., and Friedman, J.M. (2001). Virus-assisted mapping of neural inputs to a feeding center in the hypothalamus. Science *291*, 2608–2613.

Dobrunz, L.E., and Stevens, C.F. (1997). Heterogeneity of release probability, facilitation, and depletion at central synapses. Neuron *18*, 995–1008.

Emptage, N.J., Reid, C.A., and Fine, A. (2001). Calcium stores in hippocampal synaptic boutons mediate short-term plasticity, store-operated Ca2+ entry, and spontaneous transmitter release. Neuron *29*, 197–208.

Endo, K., and Yawo, H. (2000). mu-Opioid receptor inhibits N-type Ca2+ channels in the calyx presynaptic terminal of the embryonic chick ciliary ganglion. J. Physiol. *524*, 769–781.

Evans, A.M., Mustard, K.J., Wyatt, C.N., Peers, C., Dipp, M., Kumar, P., Kinnear, N.P., and Hardie, D.G. (2005). Does AMP-activated protein kinase couple inhibition of mitochondrial oxidative phosphorylation by hypoxia to calcium signaling in O2-sensing cells? J. Biol. Chem. *280*, 41504–41511.

Fatt, P., and Katz, B. (1952). Spontaneous subthreshold activity at motor nerve endings. J. Physiol. *117*, 109–128.

Ferrell, J.E., Jr. (2002). Self-perpetuating states in signal transduction: positive feedback, double-negative feedback and bistability. Curr. Opin. Cell Biol. *14*, 140–148.

Gordon, G.R., and Bains, J.S. (2006). Can homeostatic circuits learn and remember? J. Physiol. *576*, 341–347.

Grandison, L., and Guidotti, A. (1977). Stimulation of food intake by muscimol and beta endorphin. Neuropharmacology *16*, 533–536.

Hahn, T.M., Breininger, J.F., Baskin, D.G., and Schwartz, M.W. (1998). Coexpression of Agrp and NPY in fasting-activated hypothalamic neurons. Nat. Neurosci. *1*, 271–272.

Hawley, S.A., Pan, D.A., Mustard, K.J., Ross, L., Bain, J., Edelman, A.M., Frenguelli, B.G., and Hardie, D.G. (2005). Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. Cell Metab. *2*, 9–19.

Holm, S.A. (1979). A simple sequentially rejective multiple test procedure. Scand. J. Stat. *6*, 65–70.

Holst, B., Cygankiewicz, A., Jensen, T.H., Ankersen, M., and Schwartz, T.W. (2003). High constitutive signaling of the ghrelin receptor—identification of a potent inverse agonist. Mol. Endocrinol. *17*, 2201–2210.

Horowitz, P., and Hill, W. (1989). The Art of Electronics, Second Edition (Cambridge, UK: Cambridge University Press).

Howard, A.D., Feighner, S.D., Cully, D.F., Arena, J.P., Liberator, P.A., Rosenblum, C.I., Hamelin, M., Hreniuk, D.L., Palyha, O.C., Anderson, J., et al. (1996). A receptor in pituitary and hypothalamus that functions in growth hormone release. Science *273*, 974–977.

Hurley, R.L., Anderson, K.A., Franzone, J.M., Kemp, B.E., Means, A.R., and Witters, L.A. (2005). The Ca2+/calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases. J. Biol. Chem. *280*, 29060–29066.

Koshland, D.E., Jr., Goldbeter, A., and Stock, J.B. (1982). Amplification and adaptation in regulatory and sensory systems. Science *217*, 220–225.

Li, C., Chen, P., and Smith, M.S. (1999). Identification of neuronal input to the arcuate nucleus (ARH) activated during lactation: implications in the activation of neuropeptide Y neurons. Brain Res. *824*, 267–276.

López, M., Lage, R., Saha, A.K., Pérez-Tilve, D., Vázquez, M.J., Varela, L., Sangiao-Alvarellos, S., Tovar, S., Raghay, K., Rodríguez-Cuenca, S., et al. (2008). Hypothalamic fatty acid metabolism mediates the orexigenic action of ghrelin. Cell Metab. *7*, 389–399.

Luquet, S., Phillips, C.T., and Palmiter, R.D. (2007). NPY/AgRP neurons are not essential for feeding responses to glucoprivation. Peptides *28*, 214–225.

Martiáñez, T., Francès, S., and López, J.M. (2009). Generation of digital responses in stress sensors. J. Biol. Chem. *284*, 23902–23911.

Minokoshi, Y., Alquier, T., Furukawa, N., Kim, Y.B., Lee, A., Xue, B., Mu, J., Foufelle, F., Ferré, P., Birnbaum, M.J., et al. (2004). AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. Nature *428*, 569–574.

Nakazato, M., Murakami, N., Date, Y., Kojima, M., Matsuo, H., Kangawa, K., and Matsukura, S. (2001). A role for ghrelin in the central regulation of feeding. Nature *409*, 194–198.

Pinto, S., Roseberry, A.G., Liu, H., Diano, S., Shanabrough, M., Cai, X., Friedman, J.M., and Horvath, T.L. (2004). Rapid rewiring of arcuate nucleus feeding circuits by leptin. Science *304*, 110–115.

Silva, R.M., Hadjimarkou, M.M., Rossi, G.C., Pasternak, G.W., and Bodnar, R.J. (2001). Beta-endorphin-induced feeding: pharmacological characterization using selective opioid antagonists and antisense probes in rats. J. Pharmacol. Exp. Ther. *297*, 590–596.

Smith, R.G. (2005). Development of growth hormone secretagogues. Endocr. Rev. *26*, 346–360.

Sternson, S.M., Shepherd, G.M., and Friedman, J.M. (2005). Topographic mapping of VMH —> arcuate nucleus microcircuits and their reorganization by fasting. Nat. Neurosci. *8*, 1356–1363.

Takahashi, K.A., and Cone, R.D. (2005). Fasting induces a large, leptin-dependent increase in the intrinsic action potential frequency of orexigenic arcuate nucleus neuropeptide Y/Agouti-related protein neurons. Endocrinology *146*, 1043–1047.

Tanaka, K., and Augustine, G.J. (2008). A positive feedback signal transduction loop determines timing of cerebellar long-term depression. Neuron *59*, 608–620.

Tschöp, M., Smiley, D.L., and Heiman, M.L. (2000). Ghrelin induces adiposity in rodents. Nature *407*, 908–913.

Woods, A., Dickerson, K., Heath, R., Hong, S.P., Momcilovic, M., Johnstone, S.R., Carlson, M., and Carling, D. (2005). Ca2+/calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells. Cell Metab. *2*, 21–33.

Zigman, J.M., Jones, J.E., Lee, C.E., Saper, C.B., and Elmquist, J.K. (2006). Expression of ghrelin receptor mRNA in the rat and the mouse brain. J. Comp. Neurol. *494*, 528–548.