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Interplay between glucose and leptin signaling determines the strength of GABAergic synapses at POMC neurons

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Abstract

Regulation of GABAergic inhibitory inputs and alterations in POMC neuron activity by nutrients and adiposity signals regulate energy and glucose homeostasis. Thus, understanding how POMC neurons integrate these two signal molecules at the synaptic level is important. Here we show that leptin's action on GABA release to POMC neurons is influenced by glucose levels. Leptin stimulates the JAK2-PI3K pathway in both presynaptic GABAergic terminals and postsynaptic POMC neurons. Inhibition of AMPK activity in presynaptic terminals decreases GABA release at 10 mM glucose. However, postsynaptic TRPC channel opening by the PI3K-PLC signaling pathway in POMC neurons enhances spontaneous GABA release via activation of presynaptic MC3/4 and mGlu receptors at 2.5 mM glucose. High-fat feeding blunts AMPK-dependent presynaptic inhibition, whereas PLC-mediated GABAergic feedback inhibition remains responsive to leptin. Our data indicate that the interplay between glucose and leptin signaling in glutamatergic POMC neurons is critical for determining the strength of inhibitory tone towards POMC neurons.

Introduction

Leptin is an adipocyte-derived hormone whose actions are required for normal energy homeostasis[1, 2]. Amongst leptin-responsive areas in the brain, leptin receptors (LepRs) are particularly highly expressed in the arcuate nucleus of the hypothalamus (ARC)[3, 4]. The ARC contains proopiomelanocortin (POMC) neurons that are a critical regulator for energy balance and glucose homeostasis[5, 6]. Mice with targeted deletion of the *Pomc* gene and their cognate receptors MC3/4R are obese[7, 8, 9, 10]. Moreover, abnormalities in POMC synthesis

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and processing as well as defects in the action of POMC-derived peptides cause obesity in humans[11, 12, 13, 14]. Hence dysregulation in melanocortin signaling leads to obesity and metabolic disorders in animals and humans.

There are extensive studies on the regulation of POMC neurons by nutrients and circulating adiposity signals including glucose and leptin. Leptin depolarizes POMC neurons via activation of canonical transient receptor potential (TRPC) channels[15]. Activation of TRPC channels is mediated by the janus kinase 2 (JAK2) - phosphatidylinositide 3-kinases (PI3K) - phospholipase C (PLC) pathway[15]. This JAK2-PI3K-PLC pathway in POMC neurons plays an essential role in the regulation of energy and glucose homeostasis. For instance, in mice with POMC-specific ablation of phosphatidylinositol 3,4,5-trisphosphate (PIP3) phosphatase (Pten) which promotes continuous activation of the PI3K pathway, leptin is not able to elicit action potentials, although leptin stimulates signal transducer and activator of transcription 3 (STAT3) phosphorylation[16]. Furthermore, disruption of PI3K in POMC neurons blunts leptin's action on the membrane potential as well as food intake, although mice show normal long-term body weight regulation[17]. This is further supported by the study showing that mice deficient of the p110β isoform of PI3K in POMC neurons exhibit leptin resistance, increased adiposity, and increased food intake, associated with no electrical response to leptin[18]. Although the JAK2-STAT3 pathway contributes to the regulation of long-term energy homeostasis via the transcription of POMC and the inhibitory suppressor of cytokine signaling 3 (SOCS3)[19, 20, 21], the JAK2-PI3K pathway appears to be important in the regulation of POMC neuron activity, resulting in leptin-induced hypophagia.

Kahn and colleagues demonstrated another important signaling pathway that is required for leptin's anorexigenic effects[22]. Leptin decreases the activity of the α2 subunit of adenosine monophosphate-activated protein kinase (AMPK) via phosphorylation of AMPK α subunits. This appears to be a key downstream target of the JAK2-PI3K pathway[22, 23]. High-fat-feeding suppresses basal AMPK activity in the hypothalamus and, more importantly, leptin fails to attenuate hypothalamic AMPK activity in diet-induced obese mice[24]. Interestingly, POMC neurons from the animals deficient for the α2 subunit of AMPK in POMC neurons remain responsive to leptin, but do not respond to alterations in extracellular glucose levels[25]. Hence, ARC neurons integrate nutrients and adiposity signals through alterations in AMPK activity.

Alterations in POMC neuron activity by glucose and leptin modulate the release of α-melanocyte-stimulating hormone (α-MSH)[26, 27]. Importantly there exists an auto-inhibitory loop from melanocortin peptides in POMC neurons[28]. We thus investigated whether leptin signaling in ARC POMC neurons is influenced by glucose levels. In this study, leptin's inhibitory effect on spontaneous GABA release at 10 mM glucose is completely absent at 2.5 mM glucose. Rather the effect of leptin on GABA release is stimulatory at 2.5 mM glucose. Reduced GABA release is due solely to inhibition of presynaptic AMPK activity. However, leptin receptor activation on POMC neurons opens TRPC channels via the Jak2-PI3K-PLC pathway. Calcium entry through TRPC channels induces the release of α-MSH and glutamate. Activation of presynaptic MC3/4 and metabotropic glutamate (mGlu) receptors enhances GABA release. High-fat feeding elevates the JAK2-PI3K-PLC signaling

pathway, while reducing AMPK activity in POMC neurons. Hence GABAergic synapses to POMC neurons integrate glucose and leptin signaling and this integration is subject to modulation by high-fat feeding.

RESULTS

Two distinct effects of leptin on GABAergic sIPSCs

This report is based on recordings from approximately 500 neurons in acute hypothalamus slices from both male and female animals. We examined whether alterations in extracellular glucose levels influenced leptin's action on spontaneous GABAergic inhibitory currents (sIPSCs) to POMC neurons. As described in the prior studies[28, 29], leptin (100 nM) significantly depressed GABAergic transmission in a subset of POMC neurons at 10 mM glucose, consistent with a prior study[28] (Fig. 1A, B and Table 1; mean percent change in spontaneous inhibitory postsynaptic currents (sIPSCs) frequency: 60.3 ± 4.9 % of control; n = 11 out of 26 neurons). Treatment with leptin significantly reduced the mean frequency without altering the mean amplitude of sIPSCs (Fig. 1C and Table 1).

We then lowered external glucose levels from 10 to 5 mM. At 5 mM glucose, addition of leptin decreased GABAergic sIPSC frequency by 22 % in one-fifth of POMC neurons (n= 6 out of 27 neurons; Fig. 1A, B and Table 1). Surprisingly, approximately 40 % of POMC neurons responded to leptin with increased sIPSC frequency (n = 12 out of 27 neurons; Fig 1A, B and Table 1). The mean percent change in sIPSC frequency was 147.8 ± 10.5 % of control, (Fig. 1B and Table 1). When glucose levels were further lowered to 2.5 mM, the net effect of leptin on sIPSC frequency was stimulatory, which was in contrast with that observed at 10 mM glucose. In other words, leptin's inhibitory effect on sIPSCs in POMC neurons was completely absent at 2.5 mM glucose (n=0 out of 19 neurons examined). Addition of leptin raised sIPSC frequency in about 60 % of POMC neurons (n = 12 out of 19 neurons; Fig. 1B and Table 1). The fraction of POMC neurons receiving enhanced GABA release was significantly higher at 2.5 mM than at 5 and 10 mM glucose levels (Table 1). Importantly, differences in leptin's action on sIPSC frequency were not due to changes in the baseline sIPSC activity as the baseline sIPSC frequency at different glucose levels was similar ([Glucose] 2.5 mM, 2.4 ± 0.4 Hz; 5 mM, 2.6 ± 0.3 Hz; 10 mM, 2.6 ± 0.5 Hz, n= 19, 27, and 26 neurons, respectively).

Given that leptin increases GABAergic inhibitory tone in a subset of POMC neurons, we sought to determine whether leptin-mediated enhancement of sIPSCs is able to inhibit POMC neuron activity. In current clamp configuration, addition of leptin (100 nM) induced a hyperpolarization in one-third of POMC neurons in the absence of the $GABA_A$ receptor antagonist at both 2.5 and 5 mM glucose (Supplementary Fig. 1 and Supplementary Table 1). More importantly, leptin-induced hyperpolarization was completely abolished by the $GABA_A$ receptor antagonist (Supplementary Fig. 1 and Supplementary Table 1), suggesting that enhanced GABAergic tone is sufficient to inhibit POMC neuron activity.

Two distinct effects of leptin on GABAergic mIPSCs

In the presence of tetrodotoxin (TTX; 1μM) to block the neuronal network activity, treatment with leptin increased the frequency of miniature IPSCs (mIPSCs) at 2.5 and 5 mM glucose (Fig. 2A, B and Table 1). Only a few POMC neurons responded to leptin with reduced mIPSC frequency (Fig. 2B and Table 1). Unlike the situation at 2.5 and 5 mM glucose, leptin either enhanced or reduced mIPSC frequency in POMC neurons at 10 mM glucose (Fig 2A and B). Leptin had no effect on mIPSC amplitude (Fig. 2C). There was no difference in the baseline mIPSC frequency under these conditions ([glucose] 2.5 mM, 1.1 ± 0.2 Hz; 5 mM, 1.4 ± 0.3 Hz; 10 mM, 1.4 ± 0.2 Hz; n = 20, 21, and 31 neurons, respectively).

Activation of LepRs mediates synaptic upregulation

We sought to determine the cellular mechanisms underlying leptin-mediated enhancement of IPSCs. Leptin receptors are expressed exclusively in somas and dendrites of POMC neurons[30] and activation of leptin receptors on POMC neurons induces the release of the stimulatory peptide, α-MSH[27]. Cowley and colleagues[28] suggest the existence of an auto-regulatory loop from opioid and melanocortin peptides and show the stimulatory effect of the MC3R agonist on GABA release. We thus examined whether activation of postsynaptic leptin receptors induces synaptic upregulation by using transgenic animals with selective deletion of leptin receptors in POMC neurons (i.e. *POMC-Cre:: LepR*$^{-/-}$ mice[31, 32]). Leptin reduced mIPSC frequency without altering the mean amplitude in *POMC-Cre:: LepR*$^{-/-}$ mice at 5 mM glucose (Fig. 3A and E; mean amplitude, control, −63.9 ± 6.0 pA; leptin, −62.4 ± 6.6 pA; n = 7 neurons). Importantly, no POMC neurons showed increased mIPSC frequency in response to leptin, which was in contrast with the net stimulatory effect of leptin on mIPSCs under the same experimental conditions (see Fig. 2A, B and Table 1). Hence these results suggest that leptin's stimulatory effect requires activation of postsynaptic rather than presynaptic leptin receptors on POMC neurons.

Leptin induces melanocortin and glutamate release

As postsynaptic leptin receptors on POMC neurons are implicated in synaptic upregulation, it is plausible that leptin induces release of neuropeptides and neurotransmitters from POMC neurons, which up-regulates GABA release. Hence we investigated whether treatment with the MC3/4R (MTII, 100 nM) and metabotropic glutamate receptor (mGluR) (glutamate, 100 μM) agonists increases mIPSC frequency. The two reagents significantly increased mIPSC frequency (Supplementary Fig. 2). These results suggest that activation of presynaptic MC3/4Rs and mGluRs on GABAergic axon terminals enhances GABA release.

Based on these findings, we investigated whether leptin's stimulatory effect on mIPSCs is mediated, in part, by presynaptic mGluRs. Treatment with the broad spectrum mGluR antagonist MCPG (100 μM) significantly, but not completely, attenuated leptin's stimulatory effect on mIPSC frequency at 5 mM glucose without changing mIPSC amplitude (mean amplitude, control, −54.6 ± 4.3 pA; leptin, −55.8 ± 5.4 pA; n = 19 neurons). Thus the total mean percent change in mIPSC frequency was significantly reduced compared with that observed without MCPG (Fig. 3B and E).

We then examined whether pharmacological blockade of MC3/4Rs affects leptin's stimulatory action on mIPSCs. Leptin's stimulatory action on mIPSC frequency was completely absent in the presence of the MC3/4R antagonist SHU9119 (100 nM) (Fig. 3C and E; mean amplitude, control, −68.9 ± 8.3 pA; leptin, −68.4 ± 7.6 pA; n = 9 neurons). However, leptin's inhibitory effect on mIPSC frequency was still present. The total mean percent change in mIPSC frequency was 77.3 ± 7.9 % of control (Fig. 3E)

We expanded our findings to determine whether co-application of both MC3/4R and mGluR antagonists inhibits leptin's stimulatory effect. In the presence of SHU9119 and MCPG, leptin no longer facilitated mIPSC frequency (Fig. 3D and E; mIPSC amplitude, control, −66.2 ± 9.7 pA; leptin, −65.2 ± 10.1 pA; n = 7 neurons). These findings suggest that neuropeptides and neurotransmitters from POMC neurons regulate the synaptic activity of neighboring POMC neurons. To examine this possibility, we expressed a light-activated stimulatory protein channelrhodopsin exclusively in POMC neurons by crossbreeding the *POMC-Cre* with the *channelrhodopsin (ChR2(H134R))-YFP* animals. Bursts of light pulses (10 Hz) were applied for 1 s followed by a 3 s break that repeated 5 times. Blue light illumination of POMC neurons significantly increased sIPSC frequency (Supplementary Fig. 3).

A subset of Glutamatergic POMC neurons respond to leptin

Given that activation of leptin receptors in POMC neurons releases glutamate, we examined the expression of glutamatergic POMC neurons in the ARC. Glutamatergic POMC neurons were found adjacent to and/or within the median eminence, a circumventricular organ (Supplementary Fig. 4). Thus we investigated the anatomical relationship of glutamatergic POMC neurons with the brain blood barrier (BBB) by using Evans blue, a commonly used fluorescent dye for assessing BBB permeability. This approach allows us to visualize glutamatergic POMC neurons that are in direct contact with the circulation. We found that a subset of POMC neurons were Evans blue-positive cells in the ARC. Surprisingly the majority of glutamatergic POMC neurons were positive to Evans blue (Fig. 3F and H), while only one-fourth of non-glutamatergic POMC neurons were Evans blue-positive (Fig. 3H). Our results suggest that glutamatergic POMC neurons represent the predominant cell type situated outside and/or adjacent to the BBB in the ARC. Due to this anatomical feature, glutamatergic POMC neurons appear to be directly exposed to blood-borne substances such as glucose and leptin.

We then examined whether glutamatergic POMC neurons responded to leptin. We performed immunostaining with antibodies against vesicular glutamate transporter 2 (vGlut2) and phosphorylated S6 (pS6, an index of neuronal activity[33]) following i.p. injection of leptin. In fact, S6 is a structural component of the ribosome that is a distal target of PI3K signaling and activated by phosphorylation[33]. Importantly, the JAK2-PI3K pathway plays a critical role in regulating POMC neuron activity independent of leptin receptor/STAT3 signaling[34]. Hence, pS6 staining appears to be useful for measuring leptin-mediated JAK2-PI3K pathway. Our data revealed that leptin induced the expression of pS6 in the ARC and VMH of the hypothalamus (Supplementary Fig. 4). Approximately one-fourth of POMC neurons from mice injected with leptin stained positive for pS6 (n=241 out of 1070

POMC-GFP neurons, Supplementary Fig. 4). Among POMC neurons, about 5 % of POMC neurons were glutamatergic (n = 22 out of 477 neurons) and approximately 40 % of glutamatergic POMC neurons were positive for pS6 (n = 9 out of 22 neurons, Fig. 3G).

TRPC channels mediate leptin's stimulatory effect

It has been reported that calcium entry through TRPC channels triggers dendritic neurotransmitter release in the brain[35]. We investigated whether leptin-mediated TRPC channel opening induces the release of α-MSH and glutamate from POMC neurons. In the presence of the TRPC channel blocker 2-APB (100 μM), leptin failed to alter mIPSC frequency (Fig. 4A, D and Table 2). As calcium influx through TRPC channels is important for dendritic release, we tested leptin in the absence of external calcium. Under these experimental conditions, most POMC neurons responded to leptin with a decrease in mIPSC frequency. The total mean percent change in mIPSC frequency was 83.6 ± 5.3 % of control (Fig. 4B, D and Table 2). Moreover the intracellular calcium release inhibitor dantrolene completely blocked leptin's effect (Supplementary Fig. 6).

As TRPC channels are activated by PLC stimulation in POMC neurons[15, 36], we blocked PLC with U73122 (10 μM). Leptin significantly reduced, but did not increase, mIPSC frequency with the PLC inhibitor U73122 (82.4 ± 2.7 % of control; Fig. 4C and D). Hence activation of TRPC channels by leptin contributes to GABAergic enhancement.

Inhibition of AMPK by leptin decreases synaptic activity

As leptin reduces mIPSC frequency following blockade of TRPC channels, we further sought to determine how leptin down-regulates GABAergic synaptic activity. AMPK plays an important role in regulating neurotransmitter release in the ARC circuit[37] and is essential for glucose-sensing by POMC and AgRP neurons in the ARC[25]. Hence we examined whether altered AMPK activity regulates GABAergic synaptic activity to POMC neurons as well. We found that the AMPK inhibitor dorsomorphin (10 μM) had an inhibitory effect on mIPSC frequency at 2.5 mM glucose at which leptin increased mIPSC frequency (Fig. 5A and C). In contrast, the AMPK activator A769662 (100 μM) increased mIPSC frequency by 40.4 ± 11.8 % at 10 mM glucose at which leptin reduced mIPSC frequency (Fig. 5B and C).

Based on these findings, we examined whether leptin's inhibitory effect on mIPSCs is due to reduced AMPK activity at 5 mM glucose. Leptin no longer depressed, but clearly enhanced, mIPSC frequency in the presence of the AMPK inhibitor dorsomorphin (Fig. 5D and F). The total mean percent change in mIPSC frequency was 122.1 ± 6.5 % of control (Fig. 5F). These results suggest that suppression of AMPK activity in presynaptic GABA terminals reduces GABA release. Additionally, we also investigated whether blockade of K_{ATP} channels influences leptin's action on GABA release as K_{ATP} channels appears to be a downstream target of leptin[38]. However, inhibition of K_{ATP} channels did not affect leptin's action on mIPSCs (Supplementary Fig. 5).

Interestingly, leptin-mediated JAK2-PI3K pathway regulates both AMPK and PLC signaling pathways[15, 23]. It is thus possible that blockade of PI3K abolishes leptin's stimulatory and inhibitory effects on mIPSCs. Indeed, we found that leptin had no effect on mIPSCs in the presence of the PI3K inhibitor wortmannin at 5 mM glucose (100 nM; Fig.

5E and F). Furthermore blockade of JAK2 completely abolished the effects of leptin on mIPSCs (Supplementary Fig. 7). To summarize, inhibition of presynaptic AMPK activity by leptin reduces, whereas activation of postsynaptic PLC in POMC neurons enhances GABA release. Both effects are mediated by the JAK2-PI3K signaling pathway.

High fat feeding disrupts leptin's action on sIPSCs

It has been reported that the baseline AMPK activity in the hypothalamus is down-regulated in animals fed a high-fat diet (HFD). More importantly, leptin fails to further reduce AMPK activity in DIO mice[24]. We tested the hypothesis that high-fat feeding preferentially suppresses leptin's inhibitory action on GABA release to POMC neurons. We performed experiments on POMC neurons from animals fed a HFD for three weeks. Under these conditions, treatment with leptin facilitated, but did not depress, GABAergic sIPSCs at 10 mM glucose at which leptin had an inhibitory effect on sIPSCs in the control group (Fig. 6A and E). These results were in contrast with those observed in animals fed a normal chow diet (NCD; Fig. 6E). As reduced AMPK activity can result in this alteration, we further examined whether pharmacological activation of AMPK is able to reverse enhanced GABAergic sIPSCs. Incubation of hypothalamic slices with the AMPK activator for 1–2 hrs completely reversed leptin's stimulatory effect at 10 mM glucose (Fig 6B and E). We further determined if leptin's stimulatory effect is due to activation of MC3/4Rs and mGluRs as well. In the presence of the MC3/4R and mGluR antagonists, leptin no longer increased sIPSC frequency, but leptin's inhibitory effect was still present in animals on a HFD (Fig 6C, D and E).

As POMC neurons in animals fed a HFD respond to leptin with increased GABA release, we sought to determine whether short-term activation of PI3K up-regulates inhibitory tone to POMC neurons. Microinjection of the PI3K activator (5 mg/ml) into the mediobasal hypothalamus (MBH) 12 hrs prior to recording sessions significantly increased the baseline GABAergic activity from 2.5 ± 0.4 Hz to 4.8 ± 0.8 Hz (Fig. 6F). More importantly, this effect was completely abolished by co-injection of the MC3/4R and mGluR antagonists (Fig 6F). These findings suggest that up-regulation of the PI3K pathway provokes synaptic feedback inhibition of POMC neurons.

High fat feeding upregulates JAK2-PI3K signaling

We expanded our findings to determine the physiological consequences of these synaptic alterations in POMC neurons at the whole body level. We measured body weight, calorie intake, plasma glucose, leptin, and insulin levels after 3 weeks on a HFD. Body weight and calorie intake in animals fed a HFD were no different from the control (body weight, NCD 19.9 ± 0.6 g, HFD 19.8 ± 0.5 g; calorie intake, NCD, 13.5 ± 0.5 kcal/g, HFD, 12.6 ± 0.3 kcal/g; Fig. 7A and B). However, the ratio of fat tissue to lean mass was significantly higher in animals fed a HFD than in animals on NCD (0.3 ± 0.02 vs. 0.4 ± 0.02; Fig. 7C). This was associated with elevated blood glucose and leptin levels (Fig. 7D and E).

In order to determine if high-fat feeding affects leptin receptor signaling in POMC neurons, we measured both pS6 and pSTAT3 expression in POMC neurons from animals on a HFD following i.p. injection of leptin. The percent of pS6 expression in POMC neurons was

significantly higher in the HFD group than in the control NCD group (Fig. 7G and H), although there was no change in pSTAT3-positive POMC cells following high-fat feeding (Supplementary Fig. 4D). We further measured PI3K (both p110α and p110β isoforms) mRNA levels in individual POMC neurons with single-cell real time qRT-PCR. High-fat feeding robustly elevated the expression of p110β, but not p110α, mRNAs in POMC neurons from animals fed a HFD (Fig. 7I and J). We also noted that the fraction of AMPK α2-expressing POMC neurons was decreased in animals on high-fat feeding (Supplementary Fig. 8)

We then determined if increased PI3K levels in POMC neurons in the HFD group influenced leptin's effects on calorie intake. Leptin (50 ng/0.5 μl) was directly infused into the MBH. The NCD group consumed significantly less than the HFD group did after micro-injection of leptin, although leptin still reduced food intake in animals fed a HFD (Fig. 7K). Therefore leptin-mediated up-regulation of GABAergic transmission would play a role in the regulation of ingestive behavior (Fig. 8).

Discussion

In this study, we outlined the cellular mechanisms underlying the effect of leptin on GABA release. We found that inhibition of AMPK by leptin in presynaptic terminals decreased GABA release at 10 mM glucose. However, activation of somatic leptin receptors on POMC neurons stimulated PLC activity, resulting in opening of TRPC channels. Ca^{2+} permeable TRPC channels generated changes in the intracellular calcium concentration, thereby causing the release of α-MSH and glutamate from glutamatergic POMC neurons at 2.5 mM glucose. Activation of presynaptic MC3/4 and metabotropic glutamate (mGlu) receptors elevated GABA release. Hence GABAergic synapses to POMC neurons differentially responded to leptin at different levels of glucose. AMPK appears to be responsible for the interplay between glucose and leptin signaling. Furthermore, high-fat feeding blunted AMPK-dependent presynaptic inhibition, whereas PLC-mediated GABAergic feedback inhibition remained responsive to leptin. Therefore, in mice fed a HFD, leptin enhanced rather than reduced GABA release to POMC neurons. This unexpected finding appears to be paradox as inhibition of POMC neurons would increase food intake.

Previous studies describe direct and indirect regulation of POMC neurons by activation of MC3/4Rs[39] and/or μ-opioid receptors[28, 40]. Furthermore, recent studies illustrate that hypothalamic POMC neurons have distinct neurotransmitter phenotypes[29, 41, 42]. Less than half of POMC neurons are GABAergic, while a subset of non-GABAergic POMC neurons is glutamatergic[29, 42, 43]. Moreover, POMC is cleaved enzymatically into diverse peptides, including α-MSH and β-endorphin. POMC neurons thus produce and release two distinct peptides, α-MSH and β-endorphin, which have opposing actions on ingestive behavior; α-MSH is anorexigenic (appetite-suppressing), whereas β-endorphin appears to be orexigenic (appetite-stimulating)[44, 45, 46]. Recent studies also demonstrate that selective stimulation of POMC neurons appears to induce the release of either opioid alone or melanocortin alone at the cellular and whole body levels since the effects of optogenetic stimulation of POMC neurons are blocked by either the opioid or the melanocortin receptor antagonists, respectively[37, 47]. POMC neurons are thus likely to be phenotypically and perhaps

functionally heterogeneous unlike AgRP neurons which are all GABAergic[29]. This heterogeneity of POMC neurons raises fundamental questions as to their precise identity, distribution as well as function.

Although POMC neurons sense and respond to both glucose and leptin[26, 28, 48], the role of glucose-sensing by POMC neurons on leptin's action has not been well studied. Both glucose and leptin induce α-MSH release from POMC neurons[26, 27]. Hence it is important to know how POMC neurons integrate these two signal molecules at the synaptic level and whether this integration influences POMC neuronal activity, resulting in changes in feeding behavior and ultimately body weight. The cellular mechanisms that mediate glucose-sensing by POMC neurons have been well documented. For instance, opening of K_{ATP} channels by lowering glucose levels decreases POMC neuron activity[26]. Additionally, stimulation of AMPK by reducing glucose levels is responsible for glucose-sensing as mice lacking the AMPK α2 in POMC neurons do not respond to alterations in glucose levels[25]. Of particular interest is that AMPK activity is altered by leptin as well[22, 23]. Thus it seems likely that POMC neurons integrate nutrients and adiposity signals through alteration in AMPK activity.

Activation of the leptin receptor stimulates diverse signaling pathways in POMC neurons. For instance, activation of leptin receptors in POMC neurons regulates POMC mRNAs via the JAK2-STAT3 signaling[21]. Additionally, leptin stimulates the JAK2-PI3K pathway in POMC neurons. In fact, mice lacking STAT3 in POMC neurons still show leptin-induced hypophagia[49] and GABAergic inputs to POMC neurons remain responsive to leptin in animals expressing *LepRb* defective for STAT3 signaling[34]. Recent studies further highlight the contribution of the JAK2-PI3K pathway to the regulation of POMC neuronal activity. First, indirect continuous stimulation of PI3K activity through inhibition of Pten in POMC neurons affects leptin's electric effect[16]. Of particular interest is that mice lacking Pten in POMC neurons exhibit a marked hyperpolarization that is associated with a reduction in the baseline firing rate[16]. We also observed that microinjection of the PI3K activator enhanced inhibitory tone, further supporting that increased PI3K activity reduces POMC neuron activity. Second, POMC neurons in mice with genetically disrupted PI3K signaling do not respond to leptin. Third, leptin has no effect on POMC neurons in mice lacking the p110β isoform of PI3K[18]. Importantly short-term exposure to a HFD increased plasma leptin levels as well as p100β expression in POMC neurons. Fourth, leptin stimulates TRPC channels via the JAK2-PI3K-PLC pathway[15]. Finally, our current data showed the contribution of PI3K signaling to leptin's electrophysiological action as blockade of JAK2 or PI3K completely blunted leptin's presynaptic as well as postsynaptic effects. Hence, leptin-induced JAK2-PI3K pathway plays a key role in regulating POMC neuron function.

Despite the renowned effects of leptin on GABA release to POMC neurons[28, 29], the cellular mechanisms remain elusive. It has been described that stimulation of AMPK by fasting or ghrelin increases the release of neurotransmitters to AgRP neurons via ryanodine receptors[37]. In line with these findings, we showed that alterations in AMPK activity in presynaptic terminals modulated GABA release to POMC neurons. More importantly, inhibition of AMPK completely abolished leptin's inhibitory effect on GABA release. We should emphasize that, although reduced GABA release was independent of extracellular

calcium, leptin failed to reduce GABA release when intracellular calcium release was blocked with the ryanodine receptor inhibitor dantrolene. We thus propose that inhibition of AMPK in GABAergic terminals reduces calcium release from ryanodine receptors, thereby decreasing GABA release to POMC neurons. Hence our results provide detailed insights into how leptin regulates GABA release in the ARC neural circuit.

In contrast to leptin's inhibitory effect of GABA release, leptin's excitatory effect was postsynaptic in origin. In other words, activation of leptin receptors in POMC neurons enhanced GABA release because deletion of leptin receptors in POMC neurons completely blunted the effect. Moreover, the release of α-MSH and glutamate is a causal factor in enhancing GABA release. Indeed, POMC neurons receive GABAergic inputs regulated by MC3/4Rs, suggesting that melanocortin peptides have an autoinhibitory effect on POMC neuronal activity[28]. We found that TRPC channels located in POMC neurons contributed to the up-regulation of GABAergic transmission. In fact, it has been reported that calcium entry through TRPC channels controls dendritic neurotransmitter release in the thalamus[35]. Moreover, a series of experiments (i.e. 0 mM $[calcium]_{ext}$, the TRPC blocker, and PLC inhibitor) support the contribution of TRPC channels to the release of glutamate and melanocortin peptides. We also note that the release of melanocortin and glutamate was still present without neuronal activity (i.e. under TTX treatment). A recent immunohistochemical study demonstrates the expression of leptin receptors exclusively in somas and dendrites, but not axon terminals, of POMC neurons[30]. It is thus plausible that activation of somatic and/or dendritic leptin receptors stimulates TRPC channels on POMC neurons and subsequently causes calcium entry into the cytoplasm, thereby inducing neurotransmitter release from somas/dendrites of POMC neurons.

Why and how does leptin induce the release of melanocortin and glutamate especially at low levels of glucose? Among TRPC channels, TRPC1, 4 and 5, which are highly expressed in POMC neurons[15], are positively regulated by intracellular calcium[50]. Recent work by Sternson and colleagues demonstrates that activation of AMPK stimulates ryanodine receptors that can generate increased calcium levels in axon terminals[37]. Hence it is also plausible that lowering glucose levels stimulates AMPK and ryanodine receptors in POMC neurons. Sustained rise in cytosolic calcium levels from intracellular calcium pools would facilitate channel activity. As a result, calcium influx through TRPC channels by leptin would be more robust at low than at high glucose levels. Additionally, this would be further enhanced by production of inositol 1,4,5-trisphosphate (IP3) through the PLC pathway. Therefore, we propose that alterations in AMPK activity require leptin's inhibitory and excitatory effects on GABA release. AMPK plays a key role in integrating nutrients and adiposity signals at GABAergic synapses to POMC neurons.

Central leptin resistance developed after long-term exposure to a HFD (>15 weeks)[27, 51, 52, 53]. Indeed, we showed that the JAK2-PI3K signaling pathway was intact in POMC neurons, although animals fed a HFD for 3 weeks showed elevated leptin levels. In particular, we demonstrated that high-fat feeding significantly increased the expression of p110β mRNA levels specifically in POMC neurons. Moreover this was associated with reduced AMPK α2 mRNA levels in POMC neurons. Although it has been shown that high-fat-feeding lowers the basal AMPK activity and abolishes leptin's ability to reduce AMPK

activity in the hypothalamus[24], our present study further suggests that short-term high-fat feeding alters AMPK as well as PI3K activity in POMC neurons. At GABAergic synapses to POMC neurons, high-fat feeding diminishes leptin's inhibitory effect on GABA release via down-regulation of presynaptic AMPK activity. In contrast, intracellular signals via the PLC pathway are still effectively transmitted, thereby enhancing GABAergic inhibitory tone onto POMC neurons at the postsynaptic level. Leptin-mediated up-regulation of GABAergic transmission would result in inhibition of POMC neurons. Hence this feedback inhibition of POMC neurons would play a role in the regulation of ingestive behavior.

Methods

Animals

All mouse care and experimental procedures were approved by the Institutional Animal Care Research Advisory Committee of the Albert Einstein College of Medicine. Mice used in these experiments included *POMC-CRE, Z/EG, LepR$^{flox/flox}$ (POMC-Cre:: LepR$^{-/-}$*[31]) and *POMC-GFP* (The Jackson Laboratory) mice. Male and female mice of mixed C57BL/6, FVB and 129 strain background were used at postnatal age 28–35 days for all experiments.

Slice preparation

Transverse brain slices were prepared from transgenic mice. Animals were anesthetized with isoflurane. After decapitation, the brain was transferred into a sucrose-based solution bubbled with 95% O_2/5% CO_2 and maintained at ~3°C. This solution contained the following (in mM): 248 sucrose, 2 KCl, 1 $MgCl_2$, 1.25 KH_2PO_4, 26 $NaHCO_3$, 1 sodium pyruvate, and 10 glucose. Transverse coronal brain slices (200 μm) were prepared using a vibratome. Slices were equilibrated with an oxygenated artificial cerebrospinal fluid (aCSF) for > 1 hr at 32°C before transfer to the recording chamber. The slices were continuously superfused with aCSF at a rate of 1.5 ml/min containing the following (in mM): 113 NaCl, 3 KCl, 1 NaH_2PO_4, 26 $NaHCO_3$, 2.5 $CaCl_2$, 1 $MgCl_2$, and 2.5, 5 or 10 glucose in 95% O_2/5% CO_2.

Electrophysiological recordings

Brain slices were placed on the stage of an upright, infrared-differential interference contrast microscope (Olympus BX50WI) mounted on a Gibraltar X–Y table (Burleigh) and visualized with a 40× water-immersion objective by infrared microscopy (DAGE MTI camera). The internal solution contained the following (in mM): 130 CsCl (or KCl), 5 $CaCl_2$, 10 EGTA, 10 HEPES, 2 MgATP, 0.5 Na_2GTP, and 5 phosphocreatine. All recordings were made at 30 ± 2°C. GABAergic currents were isolated by addition of 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) and D-amino-phosphovaleric acid (D-AP5, 50 μM). Membrane currents were recorded with a Multiclamp 700B in whole cell configuration. Electrophysiological signals were low-pass filtered at 2–5 kHz, stored on a PC and analyzed offline with pClamp 10 software (Molecular devices, CA) and Mini Analysis software version 6 (Synaptosoft, Inc). For each recording, the averaged IPSC event number and membrane potential from every 30 s epoch were taken as 1 data point. A total of 10 data points before and after application of leptin was compared using the unpaired t-test. If the P value was less than 0.05, cells were considered to be leptin-responsive. In some

experiments, extracellular calcium ions were removed from aCSF (i.e. $[Calcium]_{ext}$ 0 mM) and 1 mM EGTA was added.

To examine whether the responses to leptin are regulated by dietary history, two groups of animals were fed a standard diet (with 60% calories provided by carbohydrates, 20% by protein and 5% by fat) or a high fat diet (with 20% calories by carbohydrate, 20% by protein and 60% by fat) for 3 weeks starting from postnatal age 21 days. The PI3K activator (5 mg/ml) alone or with HS024 (0.13 mg/ml) and MCPG (20 mg/ml) was micro-injected into the mediobasal hypothalamus of the POMC-GFP mice in some experiments as noted.

Stereotaxic surgery and leptin infusion

Mice were maintained under isoflurane anesthesia and placed in a stereotaxic apparatus (David Kopf Instruments). Under aseptic conditions, sterile custom guide cannulas (Plastics One, Inc.) were stereotaxically implanted into the MBH (AP, −1.2 mm; ML, 0; DV, −5.5 mm). Animals were allowed at least 3 days to recover from surgery. To measure food intake, animals were separated individually in single cage for 5 days and fasted for 6 hrs (from 1pm to 7pm) before leptin infusion. Leptin (50 ng/0.5 μl) was infused directly into the MBH at 6 pm.

Body composition and energy balance measurements

Total fat and lean mass of mice were analyzed using the EchoMRI system (Echo Medical systems). Food intake per mouse was measured 12 hrs post-icv injection of leptin in animals fed a NCD and a HFD. Body weights and food intake were measured on a scale with 0.1 g accuracy.

Blood glucose and plasma leptin/insulin assay

Blood glucose levels were measured every 4 hrs for 24 hrs using a Bayer Ascensia Elite glucose meter. Blood samples were collected into tubes with lithium heparin (VWR international, LLC) and centrifuged at 3,000 rpm for 15 min to collect plasma. Plasma leptin and insulin levels were quantified using ELISA assay (Mouse leptin ELISA kit, EMD Millipore; Mouse insulin ELISA kit, ALPCO Corp.) by the Albert Einstein College of medicine hormone assay core.

Immunostaining

Animals at postnatal age 6 weeks were treated with either 1 mg/kg-body weight leptin or saline for 1 hr. Mice were anesthetized with isoflurane and perfused transcardially with 10 ml of cold 1×PBS followed by 25 ml of 4% paraformaldehyde (PFA). Brains were immediately removed, and postfixed in 4% PFA at 4°C overnight. The complete rostral to caudal extension of the ARC was cut in 40 μm coronal sections with a vibratome. Sections were blocked with 5% bovine serum albumin (Sigma-aldrich) for 2 hrs at room temperature, and then incubated with anti-pS6 (Cell signaling, 2215S), anti-pSTAT3 (Cell signaling, 9145S), anti-vGlut2 (Millipore, MAB5504), and anti-GFP (Millipore, 06-896) antibodies (1:1,000; 1:500; 1:500; 1:500) diluted in 0.5% Triton X-100 in PBS for 2 days at 4 °C. For vGlut2 staining, colchicine (1 μl of 10 mg/ml) was injected into the lateral ventricle of animals 1 day before sacrifice. Sections were washed 3 times in PBS, and then incubated in

1:500 diluted Alexa Fluor 405 anti- mouse IgG (Life technologies, A31553), Alexa Fluor 488 anti-chicken IgG (Millipore, AP194F), and Alexa Fluor 568 anti-rabbit IgG (Life technologies, A11008) in 0.5% Triton X-100 in PBS for 3 hrs at room temperature. Tissues were then washed 3 times in PBS, dried, and mounted with VECTASHIELD mounting media (Vector Labs). Images were acquired using a Leica scanning confocal microscope (Heidelberg, Germany).

Single-Cell qRT-PCR analysis

Single-cell samples were collected from brain slice preparations via aspiration into the patch pipette. The initial reverse transcription (RT) reaction was conducted after pressure ejection of the single cell samples into a microcentrifuge tube with REPLI-g WTA single cell kit (Qiagen). Samples were incubated in a total volume of 2.5 l at 24°C for 5 min and cooled to 4°C. Samples were then incubated for 10 min at 42C with 0.5 l gDNA wipeout buffer prior to addition of 1.75 l RT mix to synthesize first strand cDNA (RT mix : 0.25 l oligodT primer, 1 l RT buffer, 0.25 l random primer, 0.25 l RT enzyme mix). The tubes were incubated at 42C for 1 hr, and at 95C for 3 min. The tubes then were incubated at 24C for 30 min with 2.5 μl ligation mix (2 l ligase buffer, 0.5 l ligase Mix). The reaction was stopped by incubating at 95 C for 5 min. Samples were incubated at 30C for 2 hrs after adding the amplification mix (7.25 l buffer, 0.25 l REPLI-g SensiPhi DNA polymerase) and at 65C for 5 min. The concentration of purified single cell whole cDNA was measured using a NanoDrop 8000 spectrophotometer (Thermo Scientific).

Single-cell real time qPCR was performed in sealed 96 well plates with SYBR Green I master Mix by using a Light Cycler 480 instrument (Roche Applied Science). The 18S ribosomal RNA was used as an internal positive control for normalization of each sample. qPCR reactions were prepared in a final volume of 20 μl containing 2 μl of single cell whole cDNA, and 10 μl of SYBR Green master mix in the presence of primers at 0.5 μM. The primers used for qPCR were as follows for the *PI3K 110α, PI3K 110β, AMPK α2, and 18S* genes. *PI3K 110α-F, 5'-GTGAACTGGATGGCTCACAC-3'; PI3K 110α-R, 5'-ATGGAAAGGAAACGAACAGG-3'; PI3K 110β-F, 5'-GCATTTCTTCCAGCTCATCA-3'; PI3K 110β-R, 5'-ATCAGACCATCAGGAAAGCC-3'; AMPK α2-F, 5'-CTCCCAGGGTGAAGTCTCAT-3'; AMPK α2-R, 5'- CTGAAGGACAAGTTGCCAGA-3'*. A standard curve was generated from measurements of serial dilutions of pooled cDNAs from individual POMC neurons. Cycle number was plotted against the normalized fluorescence intensity to visualize the PCR amplification and determine the amplification efficiencies. The amplification efficiency of a qPCR reaction for *p110α, p110β* and *AMPK α2* was 91, 91 and 99 %, respectively. PCR products were analyzed by melting curve analysis and agarose gel electrophoresis. All primer pairs were required to pass these quality control checks. We used the comparative C_t method ($\Delta\Delta C_t$ method) for quantification.

Statistics

Statistical analyses were performed using a paired or unpaired *t*-test as noted (Origin 8.5). The mean values were reported from the entire population tested (GraphPAD4.03 and Origin 8.5). Data were considered significantly different when the P value was < 0.05. All statistical results are given as mean ± S.E.M.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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References

1. Flier JS. Obesity wars: molecular progress confronts an expanding epidemic. Cell. 2004; 116:337–350. [PubMed: 14744442]
2. Friedman JM. Obesity in the new millennium. Nature. 2000; 404:632–634. [PubMed: 10766249]
3. Patterson CM, Leshan RL, Jones JC, Myers MG Jr. Molecular mapping of mouse brain regions innervated by leptin receptor-expressing cells. Brain research. 2011; 1378:18–28. [PubMed: 21237139]
4. Elmquist JK, Bjorbaek C, Ahima RS, Flier JS, Saper CB. Distributions of leptin receptor mRNA isoforms in the rat brain. The Journal of comparative neurology. 1998; 395:535–547. [PubMed: 9619505]
5. Cone RD. Anatomy and regulation of the central melanocortin system. Nature neuroscience. 2005; 8:571–578. [PubMed: 15856065]
6. Belgardt BF, Okamura T, Bruning JC. Hormone and glucose signalling in POMC and AgRP neurons. The Journal of physiology. 2009; 587:5305–5314. [PubMed: 19770186]
7. Yaswen L, Diehl N, Brennan MB, Hochgeschwender U. Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. Nature medicine. 1999; 5:1066–1070.
8. Challis BG, et al. Mice lacking pro-opiomelanocortin are sensitive to high-fat feeding but respond normally to the acute anorectic effects of peptide-YY(3–36). Proceedings of the National Academy of Sciences of the United States of America. 2004; 101:4695–4700. [PubMed: 15070780]
9. Huszar D, et al. Targeted disruption of the melanocortin-4 receptor results in obesity in mice. Cell. 1997; 88:131–141. [PubMed: 9019399]
10. Butler AA, et al. A unique metabolic syndrome causes obesity in the melanocortin-3 receptor-deficient mouse. Endocrinology. 2000; 141:3518–3521. [PubMed: 10965927]
11. Krude H, Biebermann H, Luck W, Horn R, Brabant G, Gruters A. Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations in humans. Nat Genet. 1998; 19:155–157. [PubMed: 9620771]
12. Jackson RS, et al. Obesity and impaired prohormone processing associated with mutations in the human prohormone convertase 1 gene. Nat Genet. 1997; 16:303–306. [PubMed: 9207799]
13. Yeo GS, Farooqi IS, Aminian S, Halsall DJ, Stanhope RG, O'Rahilly S. A frameshift mutation in MC4R associated with dominantly inherited human obesity. Nat Genet. 1998; 20:111–112. [PubMed: 9771698]
14. Vaisse C, Clement K, Guy-Grand B, Froguel P. A frameshift mutation in human MC4R is associated with a dominant form of obesity. Nat Genet. 1998; 20:113–114. [PubMed: 9771699]
15. Qiu J, Fang Y, Ronnekleiv OK, Kelly MJ. Leptin excites proopiomelanocortin neurons via activation of TRPC channels. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2010; 30:1560–1565. [PubMed: 20107083]
16. Plum L, et al. Enhanced PIP3 signaling in POMC neurons causes KATP channel activation and leads to diet-sensitive obesity. The Journal of clinical investigation. 2006; 116:1886–1901. [PubMed: 16794735]
17. Hill JW, et al. Acute effects of leptin require PI3K signaling in hypothalamic proopiomelanocortin neurons in mice. The Journal of clinical investigation. 2008; 118:1796–1805. [PubMed: 18382766]

18. Al-Qassab H, et al. Dominant role of the p110beta isoform of PI3K over p110alpha in energy homeostasis regulation by POMC and AgRP neurons. Cell metabolism. 2009; 10:343–354. [PubMed: 19883613]
19. Ernst MB, et al. Enhanced Stat3 activation in POMC neurons provokes negative feedback inhibition of leptin and insulin signaling in obesity. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2009; 29:11582–11593. [PubMed: 19759305]
20. Reed AS, Unger EK, Olofsson LE, Piper ML, Myers MG Jr, Xu AW. Functional role of suppressor of cytokine signaling 3 upregulation in hypothalamic leptin resistance and long-term energy homeostasis. Diabetes. 2010; 59:894–906. [PubMed: 20068134]
21. Bates SH, et al. STAT3 signalling is required for leptin regulation of energy balance but not reproduction. Nature. 2003; 421:856–859. [PubMed: 12594516]
22. Minokoshi Y, et al. AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. Nature. 2004; 428:569–574. [PubMed: 15058305]
23. Dagon Y, Hur E, Zheng B, Wellenstein K, Cantley LC, Kahn BB. p70S6 kinase phosphorylates AMPK on serine 491 to mediate leptin's effect on food intake. Cell metabolism. 2012; 16:104–112. [PubMed: 22727014]
24. Martin TL, Alquier T, Asakura K, Furukawa N, Preitner F, Kahn BB. Diet-induced obesity alters AMP kinase activity in hypothalamus and skeletal muscle. The Journal of biological chemistry. 2006; 281:18933–18941. [PubMed: 16687413]
25. Claret M, et al. AMPK is essential for energy homeostasis regulation and glucose sensing by POMC and AgRP neurons. The Journal of clinical investigation. 2007; 117:2325–2336. [PubMed: 17671657]
26. Parton LE, et al. Glucose sensing by POMC neurons regulates glucose homeostasis and is impaired in obesity. Nature. 2007; 449:228–232. [PubMed: 17728716]
27. Enriori PJ, et al. Diet-induced obesity causes severe but reversible leptin resistance in arcuate melanocortin neurons. Cell metabolism. 2007; 5:181–194. [PubMed: 17339026]
28. Cowley MA, et al. Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. Nature. 2001; 411:480–484. [PubMed: 11373681]
29. Vong L, Ye C, Yang Z, Choi B, Chua S Jr, Lowell BB. Leptin Action on GABAergic Neurons Prevents Obesity and Reduces Inhibitory Tone to POMC Neurons. Neuron. 2011; 71:142–154. [PubMed: 21745644]
30. Ha S, et al. Somato-Dendritic Localization and Signaling by Leptin Receptors in Hypothalamic POMC and AgRP Neurons. PloS one. 2013; 8:e77622. [PubMed: 24204898]
31. Balthasar N, et al. Leptin receptor signaling in POMC neurons is required for normal body weight homeostasis. Neuron. 2004; 42:983–991. [PubMed: 15207242]
32. Chun SK, Jo YH. Loss of leptin receptors on hypothalamic POMC neurons alters synaptic inhibition. Journal of neurophysiology. 2010; 104:2321–2328. [PubMed: 20844117]
33. Knight ZA, et al. Molecular profiling of activated neurons by phosphorylated ribosome capture. Cell. 2012; 151:1126–1137. [PubMed: 23178128]
34. Munzberg H, et al. Appropriate inhibition of orexigenic hypothalamic arcuate nucleus neurons independently of leptin receptor/STAT3 signaling. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2007; 27:69–74. [PubMed: 17202473]
35. Munsch T, Freichel M, Flockerzi V, Pape HC. Contribution of transient receptor potential channels to the control of GABA release from dendrites. Proceedings of the National Academy of Sciences of the United States of America. 2003; 100:16065–16070. [PubMed: 14668438]
36. Qiu J, et al. Insulin excites anorexigenic proopiomelanocortin neurons via activation of canonical transient receptor potential channels. Cell metabolism. 2014; 19:682–693. [PubMed: 24703699]
37. Yang Y, Atasoy D, Su HH, Sternson SM. Hunger States Switch a Flip-Flop Memory Circuit via a Synaptic AMPK-Dependent Positive Feedback Loop. Cell. 2011; 146:992–1003. [PubMed: 21925320]
38. Mirshamsi S, et al. Leptin and insulin stimulation of signalling pathways in arcuate nucleus neurones: PI3K dependent actin reorganization and KATP channel activation. BMC Neurosci. 2004; 5:54. [PubMed: 15581426]

39. Smith MA, Hisadome K, Al-Qassab H, Heffron H, Withers DJ, Ashford ML. Melanocortins and agouti-related protein modulate the excitability of two arcuate nucleus neuron populations by alteration of resting potassium conductances. The Journal of physiology. 2007; 578:425–438. [PubMed: 17068101]
40. Pennock RL, Hentges ST. Differential expression and sensitivity of presynaptic and postsynaptic opioid receptors regulating hypothalamic proopiomelanocortin neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2011; 31:281–288. [PubMed: 21209213]
41. Yee CL, Wang Y, Anderson S, Ekker M, Rubenstein JL. Arcuate nucleus expression of NKX2.1 and DLX and lineages expressing these transcription factors in neuropeptide Y(+), proopiomelanocortin(+), and tyrosine hydroxylase(+) neurons in neonatal and adult mice. The Journal of comparative neurology. 2009; 517:37–50. [PubMed: 19711380]
42. Hentges ST, Otero-Corchon V, Pennock RL, King CM, Low MJ. Proopiomelanocortin expression in both GABA and glutamate neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2009; 29:13684–13690. [PubMed: 19864580]
43. Collin M, et al. Plasma membrane and vesicular glutamate transporter mRNAs/proteins in hypothalamic neurons that regulate body weight. Eur J Neurosci. 2003; 18:1265–1278. [PubMed: 12956725]
44. Bodnar RJ. Endogenous opioids and feeding behavior: a 30-year historical perspective. Peptides. 2004; 25:697–725. [PubMed: 15165728]
45. Glass MJ, Billington CJ, Levine AS. Opioids and food intake: distributed functional neural pathways? Neuropeptides. 1999; 33:360–368. [PubMed: 10657513]
46. Kalra SP, Horvath TL. Neuroendocrine interactions between galanin, opioids, and neuropeptide Y in the control of reproduction and appetite. Ann N Y Acad Sci. 1998; 863:236–240. [PubMed: 9928174]
47. Aponte Y, Atasoy D, Sternson SM. AGRP neurons are sufficient to orchestrate feeding behavior rapidly and without training. Nature neuroscience. 2011; 14:351–355. [PubMed: 21209617]
48. Williams KW, et al. Segregation of acute leptin and insulin effects in distinct populations of arcuate proopiomelanocortin neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2010; 30:2472–2479. [PubMed: 20164331]
49. Xu AW, Ste-Marie L, Kaelin CB, Barsh GS. Inactivation of signal transducer and activator of transcription 3 in proopiomelanocortin (Pomc) neurons causes decreased pomc expression, mild obesity, and defects in compensatory refeeding. Endocrinology. 2007; 148:72–80. [PubMed: 17023536]
50. Strubing C, Krapivinsky G, Krapivinsky L, Clapham DE. TRPC1 and TRPC5 form a novel cation channel in mammalian brain. Neuron. 2001; 29:645–655. [PubMed: 11301024]
51. Lin S, Thomas TC, Storlien LH, Huang XF. Development of high fat diet-induced obesity and leptin resistance in C57Bl/6J mice. International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity. 2000; 24:639–646.
52. Munzberg H, Flier JS, Bjorbaek C. Region-specific leptin resistance within the hypothalamus of diet-induced obese mice. Endocrinology. 2004; 145:4880–4889. [PubMed: 15271881]
53. El-Haschimi K, Pierroz DD, Hileman SM, Bjorbaek C, Flier JS. Two defects contribute to hypothalamic leptin resistance in mice with diet-induced obesity. The Journal of clinical investigation. 2000; 105:1827–1832. [PubMed: 10862798]

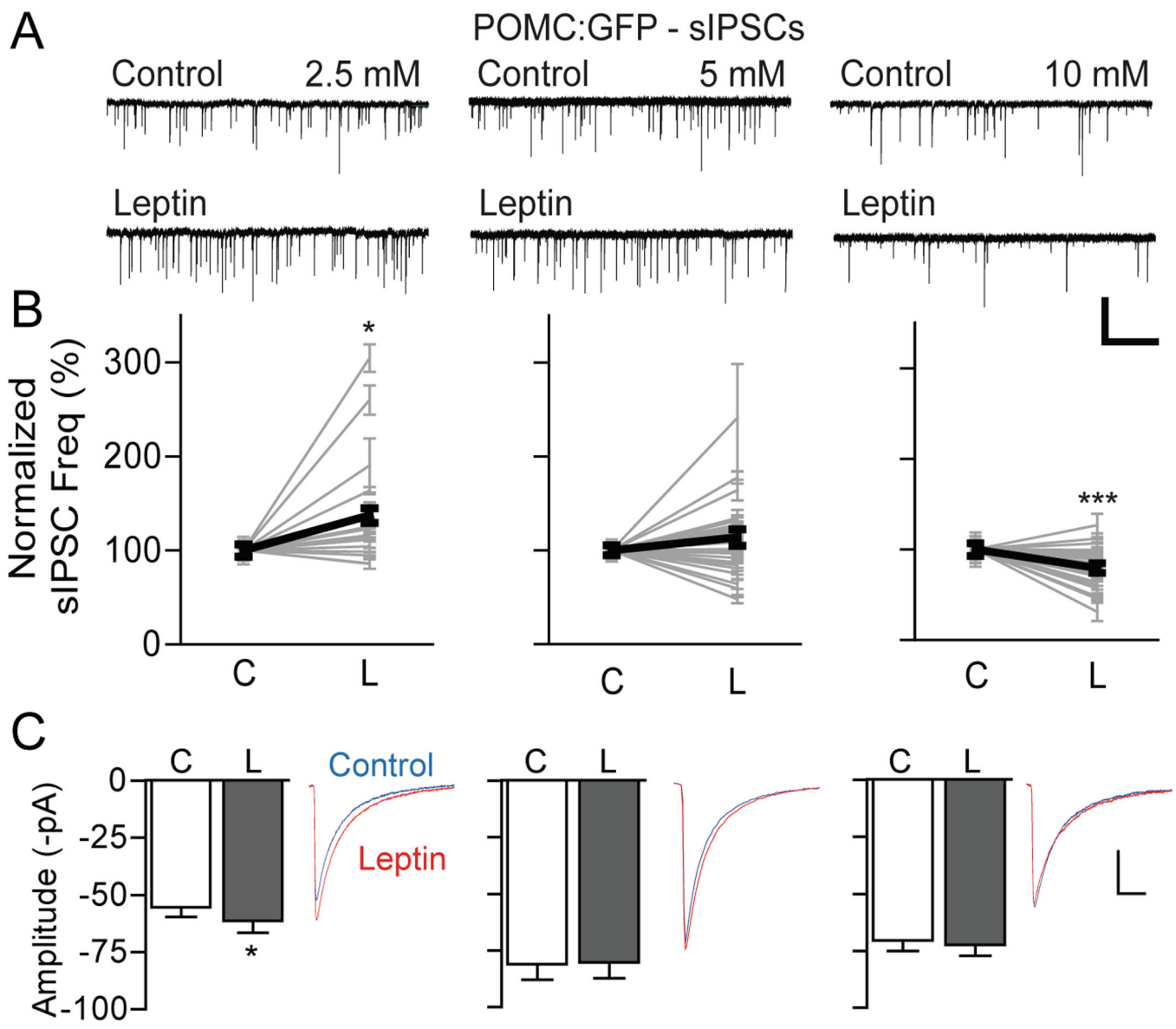

Figure 1. Two distinct effects of leptin on sIPSCs

A) Representative recording traces showing sIPSCs recorded from POMC neurons at different glucose levels. Addition of leptin (100 nM) robustly increased sIPSC frequency at 2.5 mM glucose, whereas leptin had an inhibitory effect on sIPSCs at 10 mM glucose. HP = −70 mV. Scale bar: 100 pA, 10 s.

B) Graphs showing normalized frequency of sIPSCs from individual POMC neurons before and after treatment with leptin (100 nM) at different glucose levels (Bold line: total mean change in sIPSC frequency; 2.5 mM, n = 19 neurons; 5 mM, n = 27 neurons; 10 mM, n = 26 neurons). C: control, L: leptin

C) Pooled data showing sIPSC amplitude. Superimposition of traces of sIPSCs before (blue) and after (red) application of leptin. Leptin increased the mean amplitude of sIPSCs at 2.5 mM glucose (2.5 mM: n = 19 neurons; 5 mM: n = 27 neurons; 10 mM: n = 26 neurons). Scale bar: 20 pA, 20 ms.

*p < 0.05, ***p < 0.001 vs. control (paired *t*-test). All data are shown as mean ± SEM.

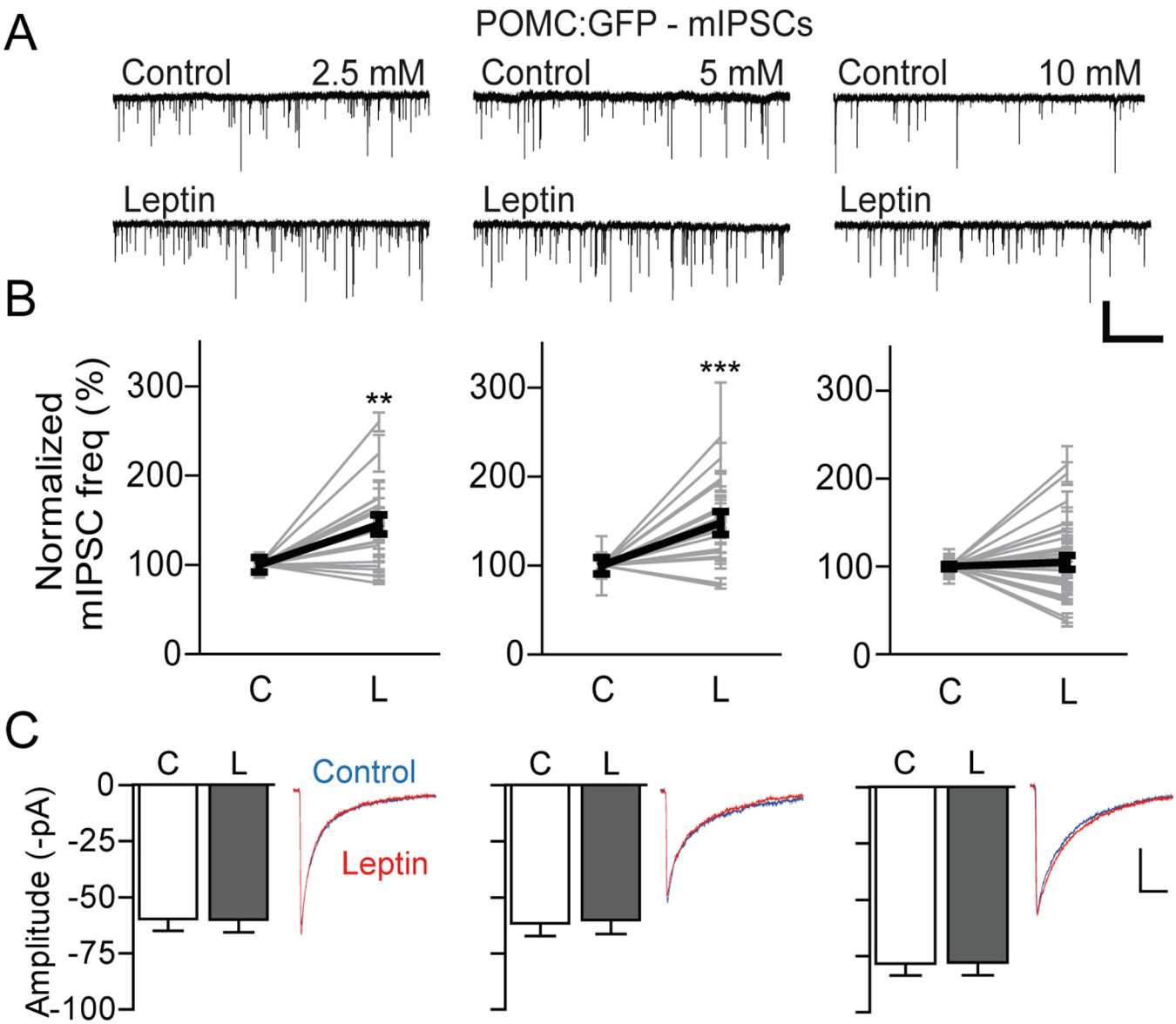

Figure 2. Two distinct effects of leptin on mIPSCs

A) Representative recording traces showing mIPSCs recorded from POMC neurons in the presence of TTX (1 μM). Treatment with leptin (100 nM) increased mIPSC frequency in a subset of POMC neurons at 2.5, 5 and 10 mM glucose. HP = −70mV. Scale bar: 100 pA, 10 s.

B) Graphs showing normalized frequency of mIPSCs from individual POMC neurons before and after treatment with leptin (Bold line: total mean change in mIPSC frequency; 2.5 mM, n = 20 neurons; 5 mM, n = 21 neurons; 10 mM, n = 31 neurons). C: control, L: leptin

C) Pooled data showing mIPSC amplitude. Both superimposition of traces of sIPSCs before (blue) and after (red) application of leptin. Leptin did not change the mean amplitude of mIPSCs (2.5 mM: n=20 neurons; 5 mM: n=21 neurons; 10 mM: n = 31 neurons). Scale bar: 20 pA, 20 ms.

**p < 0.01, ***p < 0.001 vs. control (paired *t*-test). All data are shown as mean ± SEM.

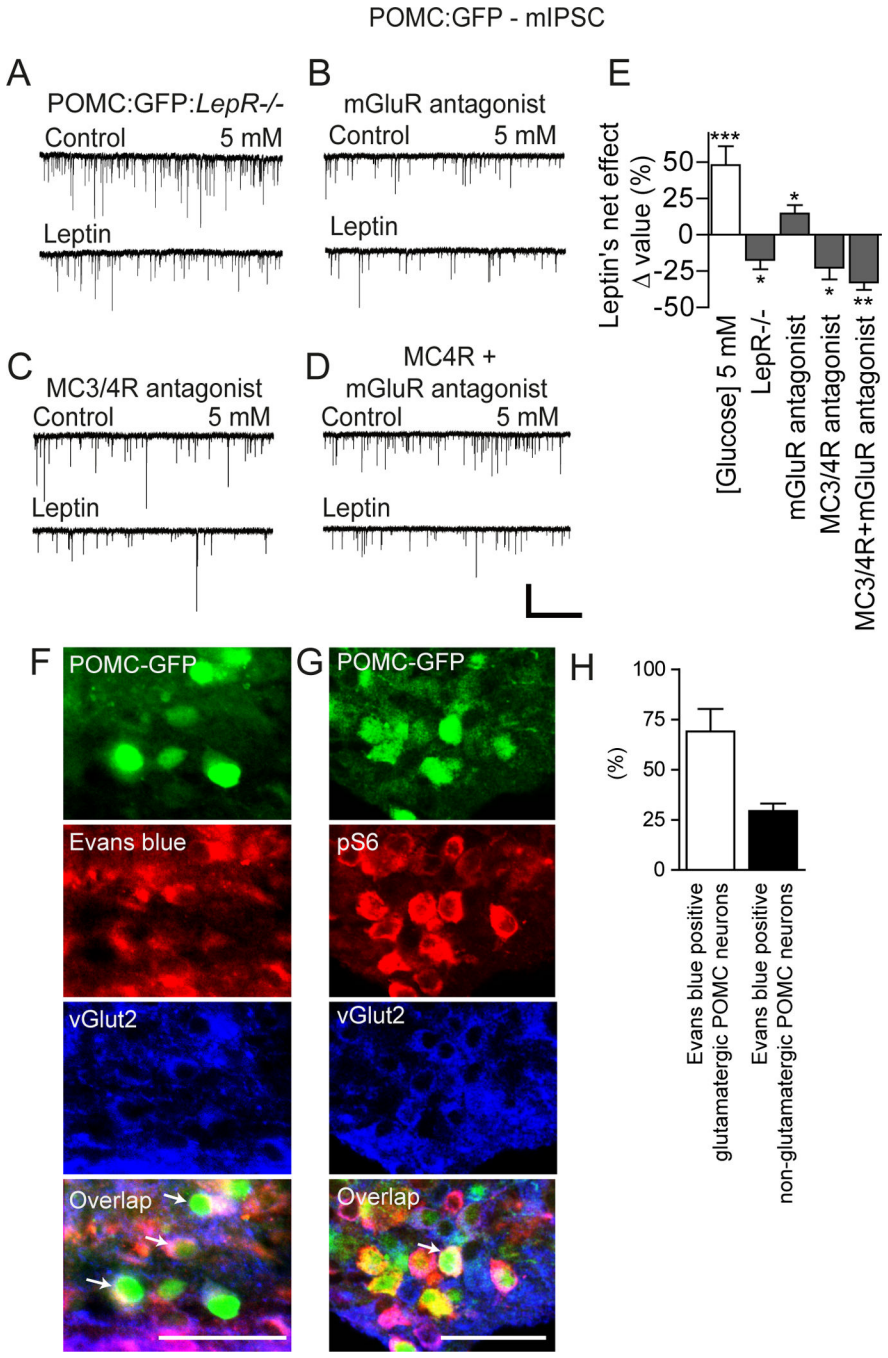

Figure 3. Blockade of both MC3/4Rs and mGluRs inhibits leptin's stimulatory effect

A) Representative traces showing leptin's effect on mIPSCs in POMC neurons from mice with selective deletion of leptin receptors in POMC neurons at 5 mM glucose levels. In contrast to the control group, leptin did not enhance the frequency of mIPSCs. HP = −70mV. B, C and D) Representative recording samples showing mIPSCs in the presence of the mGluR (MCPG; B), MC3/4R (SHU9119; C) antagonists or both (D) at 5 mM glucose. In the presence of the mGluR antagonist, leptin's effect was significantly diminished compared to the control group. In the presence of the MC3/4R blocker alone or the MC3/4R and

mGluR blockers, leptin no longer stimulated GABA release. Rather leptin had an inhibitory effect on mIPSCs. HP = −70mV. 100 pA, 10 s.

E) Summary of leptin's net effects on mIPSCs in mice lacking leptin receptors in POMC neurons and in the presence of the mGluR, MC3/4R antagonists or both. Pharmacological blockade of MC3/4Rs or mGluRs or both significantly attenuated or completely abolished leptin's stimulatory effect on mIPSCs (*LepR*$^{-/-}$, n = 7 neurons; MCPG, n = 19 neurons; SHU9119, n = 9 neurons; MCPG + SHU9119, n = 10 neurons).

F) Images of fluorescence microscopy showing the co-labeling of glutamatergic POMC neurons and Evans blue in the ARC. Glutamatergic neurons were found adjacent to and/or within the median eminence. More than two-third of glutamatergic POMC neurons were Evans blue-positive, suggesting that glutamatergic POMC neurons are directly exposed to blood glucose and leptin.

G) Images of fluorescence microscopy showing the co-expression of pS6 (red) and vGlut2 (blue) in a subset of POMC-GFP (green) neurons in the ARC of animal injected with leptin. 40% of glutamatergic POMC neurons were pS6-positive (n = 9 out of 22 neurons). White arrow represents the POMC-GFP neuron co-expressing pS6 and vGlut2. Scale bar: 50 μm

H) Percent of Evans blue-positive glutamatergic and non-glutamatergic POMC neurons. The majority of glutamatergic POMC neurons were Evans Blue-positive (n = 5 animals).

*p < 0.05, **p < 0.01 vs. control (paired *t*-test). All data are shown as mean ± SEM.

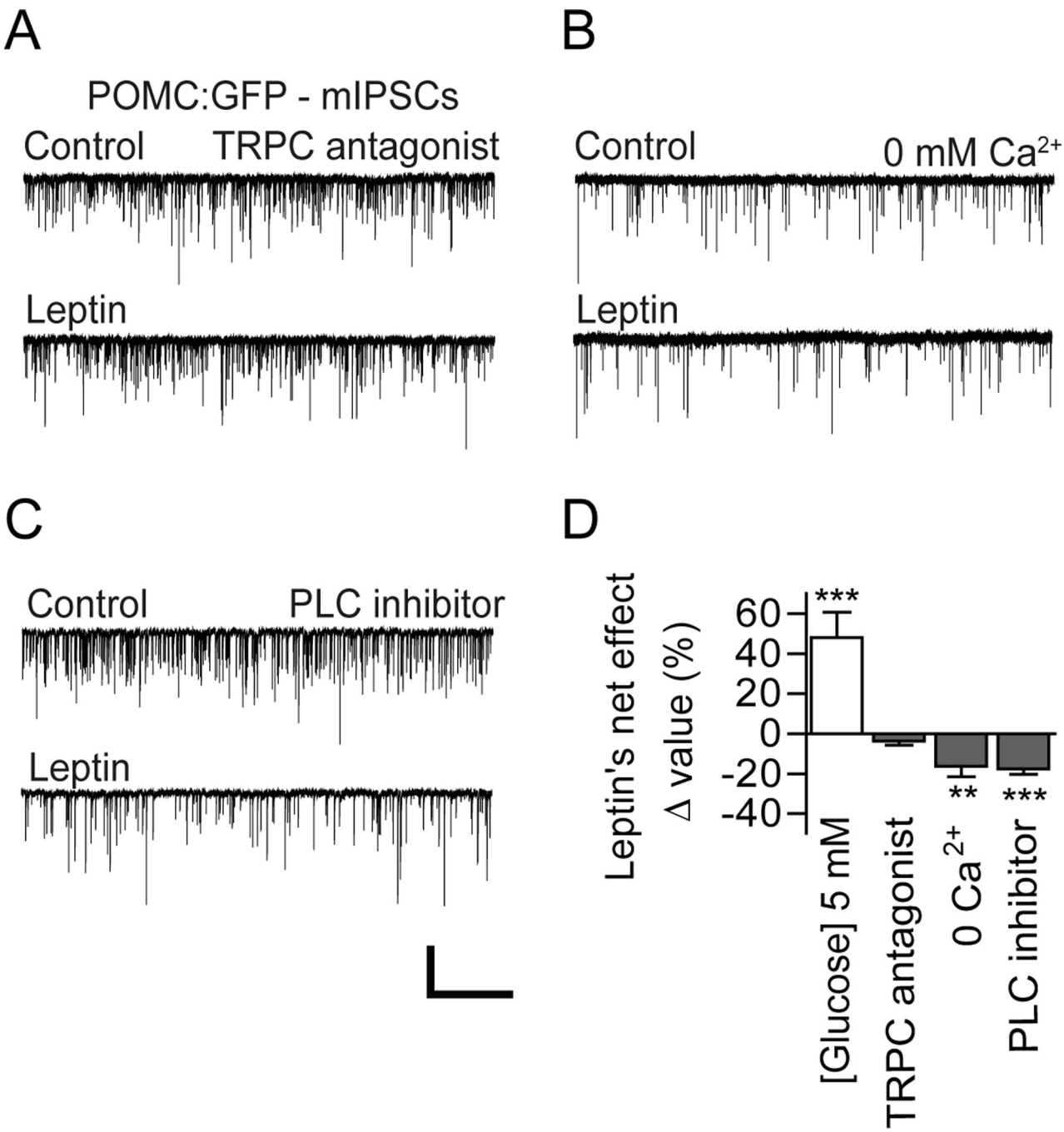

Figure 4. TRPC channels mediate leptin's stimulatory effect

A) Representative recording samples showing mIPSCs recorded from POMC neurons in the presence of the TRPC channel blocker 2-APB. Leptin no longer modulated mIPSCs under these conditions. HP = −70mV.

B) Sample traces showing mIPSCs recorded from POMC neurons following treatment with leptin in the absence of extracellular calcium. Leptin still reduced mIPSC frequency without extracellular calcium.

C) Representative recording samples showing mIPSCs recorded from POMC neurons in the presence of the PLC inhibitor U73122. Pharmacological blockade of PLC signaling abolished leptin's stimulatory effect. Under these conditions, leptin remained effective in reducing GABA release. Scale bar: 100 pA, 10 s.

D) Summary plot showing leptin's effect on mIPSC frequency following blockade of TRPC channel signaling (2-APB: n = 9 neurons; 0 calcium: n = 13 neurons; U73122: n = 10 neurons).

**p < 0.01, ***p < 0.001 vs. control (paired *t*-test). All data are shown as mean ± SEM.

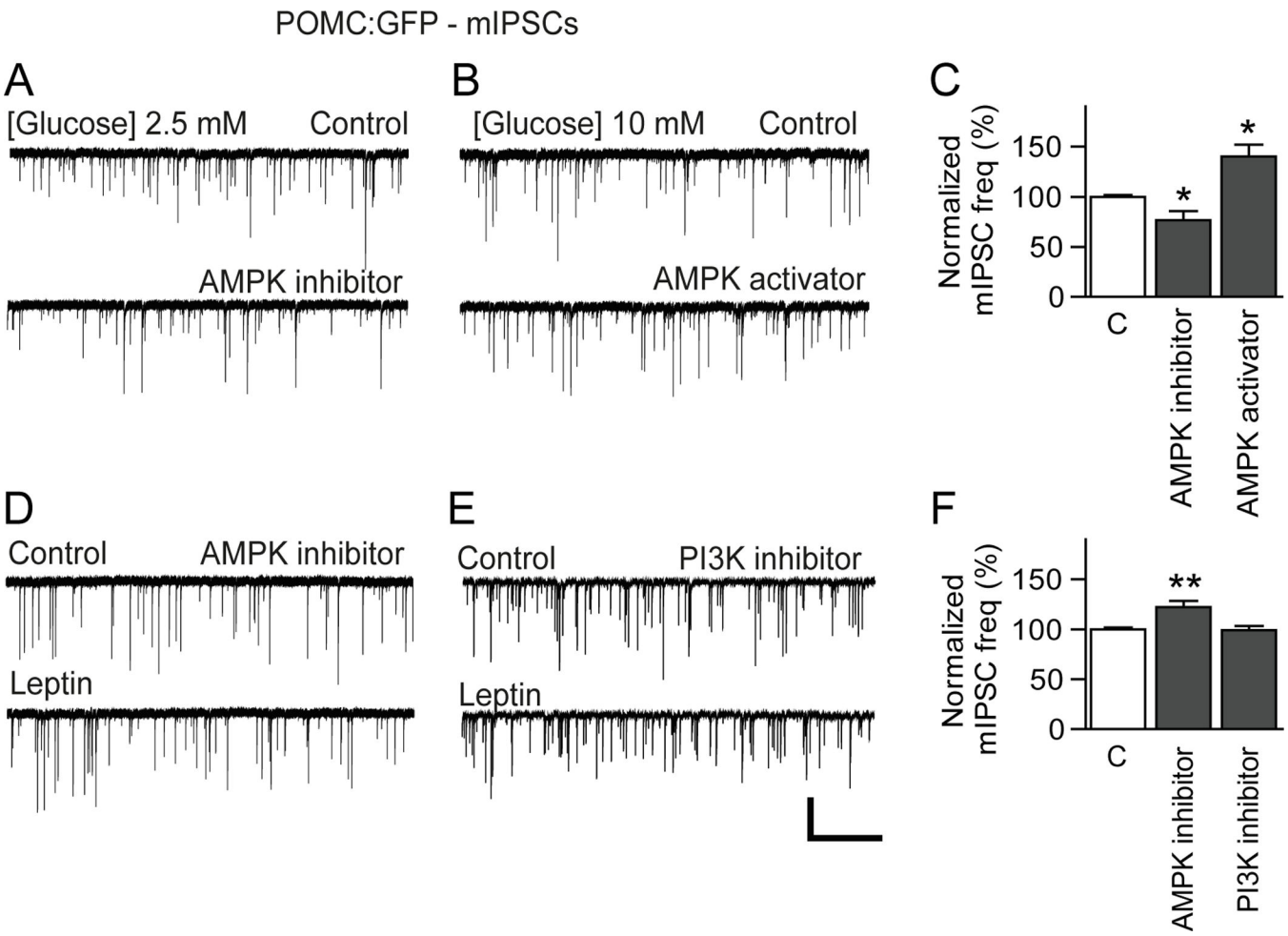

Figure 5. Inhibition of presynaptic AMPK activity reduces mIPSC frequency

A and B) Sample recording traces showing mIPSCs recorded from POMC neurons with the AMPK inhibitor dorsomorphin (A) and the AMPK activator A769662 (B). The AMPK inhibitor significantly decreased GABA release at 2.5 mM glucose (A), whereas the AMPK activator robustly increased mIPSC frequency at 10 mM glucose (B). The results suggest that presynaptic AMPK plays a major role in regulating GABA release. HP = −70 mV, Scale bar: 100 pA, 10 s

C) Summary plot showing AMPK modulators' effect on mIPSC frequency (dorsomorphin: n = 6 neurons; A769662: n = 7 neurons). C: control

D and E) Representative recording samples showing leptin's effect on mIPSCs recorded from POMC neurons in the presence of the AMPK inhibitor dorsomorphin and the PI3K inhibitor wortmannin at 5 mM glucose. Leptin increased mIPSC frequency in the presence of the AMPK inhibitor. The PI3K inhibitor completely abolished leptin's action on mIPSCs. HP = −70 mV, Scale bar: 100 pA, 10 s

F) Summary plot showing leptin's effect on mIPSC frequency in the presence of the AMPK inhibitor and the PI3K inhibitor. As leptin no longer modulates mIPSCs in the presence of the PI3K inhibitor, both pre- and postsynaptic PI3K signaling is a key factor for leptin's

effect. However, presynaptic AMPK is involved in leptin-mediated reduction of GABA release (dorsomorphin: n = 9 neurons; wortmannin: n = 10 neurons).
*p < 0.05, **p < 0.01 vs. control (paired *t*-test). All data are shown as mean ± SEM.

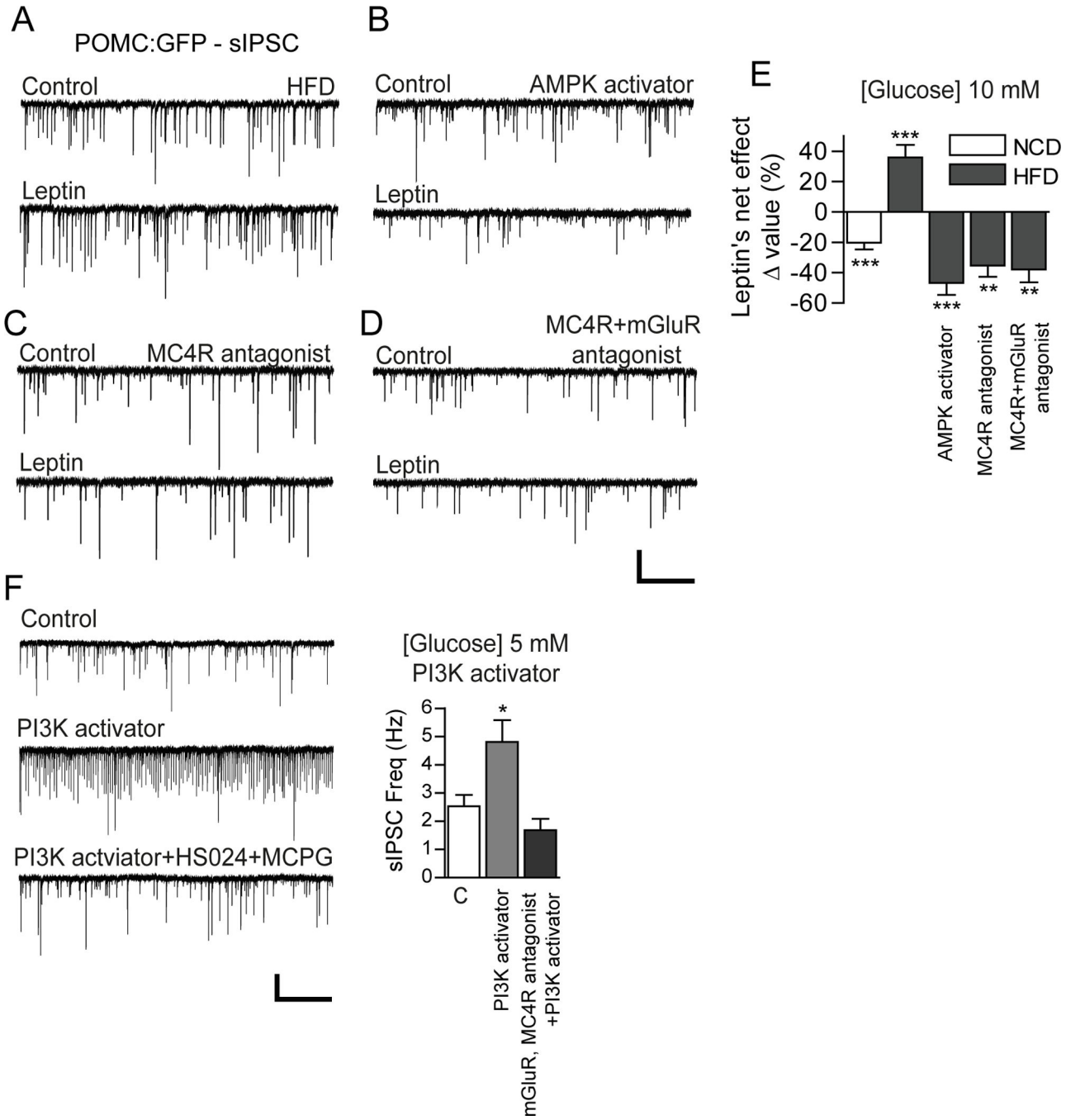

Figure 6. High-fat feeding alters leptin's action on sIPSCs

A and B) Representative sample traces showing sIPSCs recorded from POMC neurons in animals fed HFD for 3 weeks. In contrast to the control group, leptin significantly increased sIPSC frequency at 10 mM glucose (A). Leptin reduced sIPSC frequency, suggesting that the AMPK activator completely reverses leptin's action on sIPSCs. HP = −70 mV, Scale bar: C and D) Sample recording traces showing sIPSCs recorded from POMC neurons in animals on HFD in the presence of the MC3/4R antagonist alone or the MC3/4R and mGluR

antagonists. Leptin had an inhibitory effect on sIPSCs under these conditions. HP = −70 mV, Scale bar: 100 pA, 10 s.

E) Summary plot showing leptin's net effect on sIPSCs recorded from POMC neurons at 10 mM glucose in animals fed NCD and HFD. Leptin had opposing effects on sIPSCs at 10 mM glucose in animals fed NCD and HFD (n = 14 neurons). Leptin's stimulatory effect was completely inhibited by the AMPK activator and MC3/4R antagonist (A769662, n = 7 neurons; SHU9119, n = 8 neurons; MCPG + SHU9119, n = 8 neurons). NCD: normal chow diet, HFD: high-fat diet

F) Sample recording traces (left) and summary plot (right) of sIPSCs recorded from POMC neurons in animals micro-injected with the PI3K activator or the activator plus the MC3/4R and mGluR antagonists. Injection of the PI3K activator strongly increased sIPSC frequency, which was completely blocked by the MC3/4R and mGluR antagonists (n = 24 and 16 neurons, respectively). Scale bar: 100 pA, 10 s.

*p < 0.05, **p < 0.01, ***p < 0.001 vs. control (paired *t*-test). All data are shown as mean ± SEM.

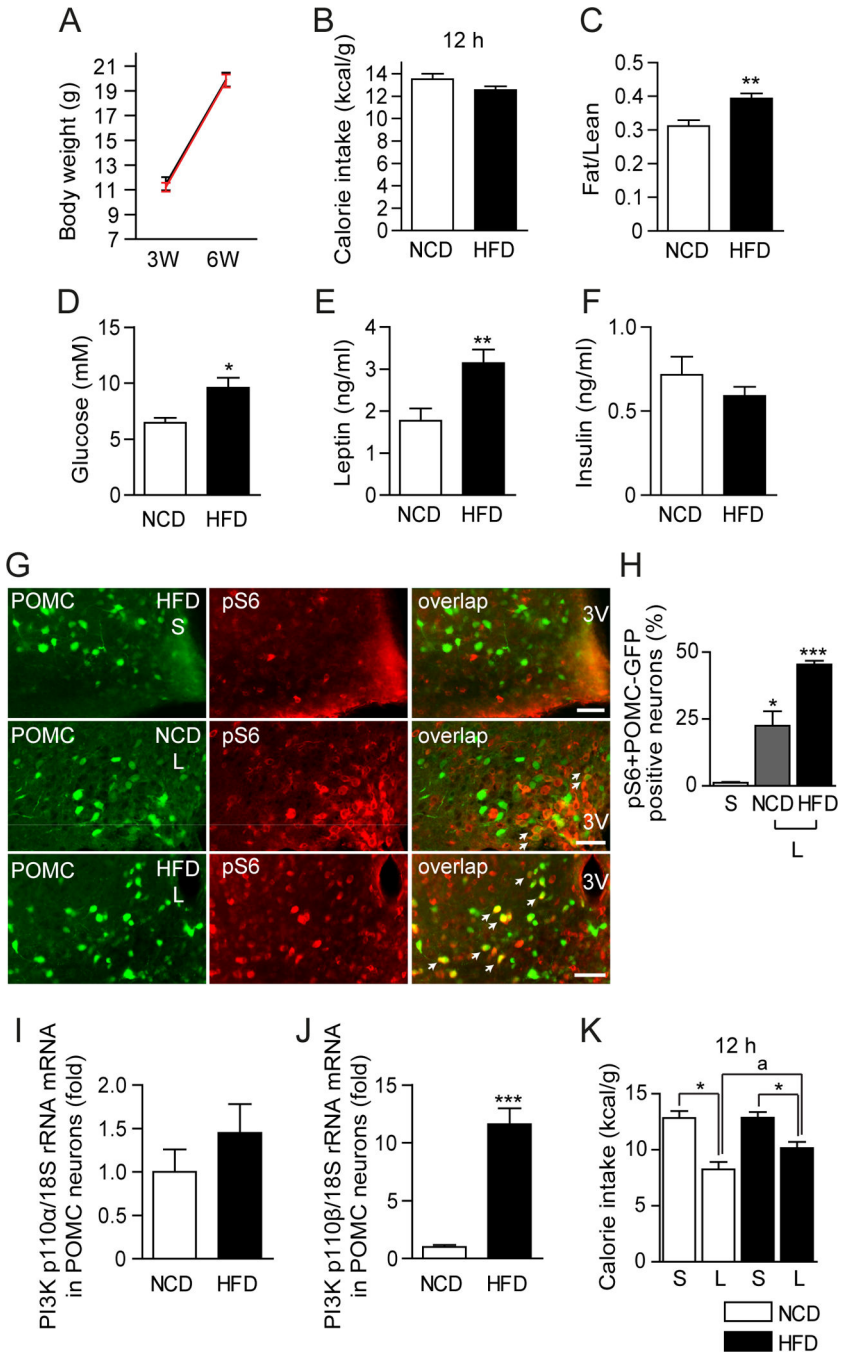

Figure 7. High-fat feeding upregulates JAK2-PI3K signaling

A and B) Body weight and calorie intake of animals fed NCD (control, n = 15 animals) and HFD (n = 18 animals) after 3 weeks. There was no difference between control (NCD) and HFD animals. NCD: normal chow diet, HFD: high-fat diet

C) Ratio of fat tissue to lean mass for control (n = 10 animals) and HFD (n = 14 animals) groups. High-fat feeding significantly increased the ratio of fat tissue to lean mass.

D, E and F) Plasma glucose, leptin and insulin levels after 3 weeks on HFD. Plasma glucose and leptin levels were significantly elevated in animals fed HFD (glucose, n = 8 animals;

leptin, n = 14 animals) compared with control (glucose, n = 7 animals; n = 16 animals). However, insulin levels were similar in both groups.

G and H) Images of fluorescence microscopy showing the co-expression of pS6 (red) and POMC neurons (green) in the ARC of animal injected with leptin (G). Summary plot (H) showing the percent of pS6-positive POMC neurons in animals fed a NCD (n = 9 animals) and a HFD (n = 8 animals). There was a significant increase in the number of pS6-positive POMC neurons in animals fed HFD. Scale bar: 50 μm. S: saline, L: leptin

I and J) PI3K p110α (I) and p110β (J) mRNA expression in POMC neurons. The dietary intervention significantly increased the expression of p110β, but not p110α, mRNAs in POMC neurons (p110α, NCD, n = 6 neurons, HFD, n = 5 neurons; p110β, NCD, n = 6 neurons, HFD, n = 9 neurons).

K) Calorie intake for control and HFD groups after micro-injection of leptin into the MBH. High-fat feeding significantly diminished leptin's anorexigenic effects (NCD, n = 14 animals, HFD, n = 18 animals).

*p < 0.05, **p < 0.01, ***p < 0.001 vs. control (unpaired *t*-test); [a]p < 0.05 vs. NCD + leptin (unpaired *t* test). All data are shown as mean ± SEM.

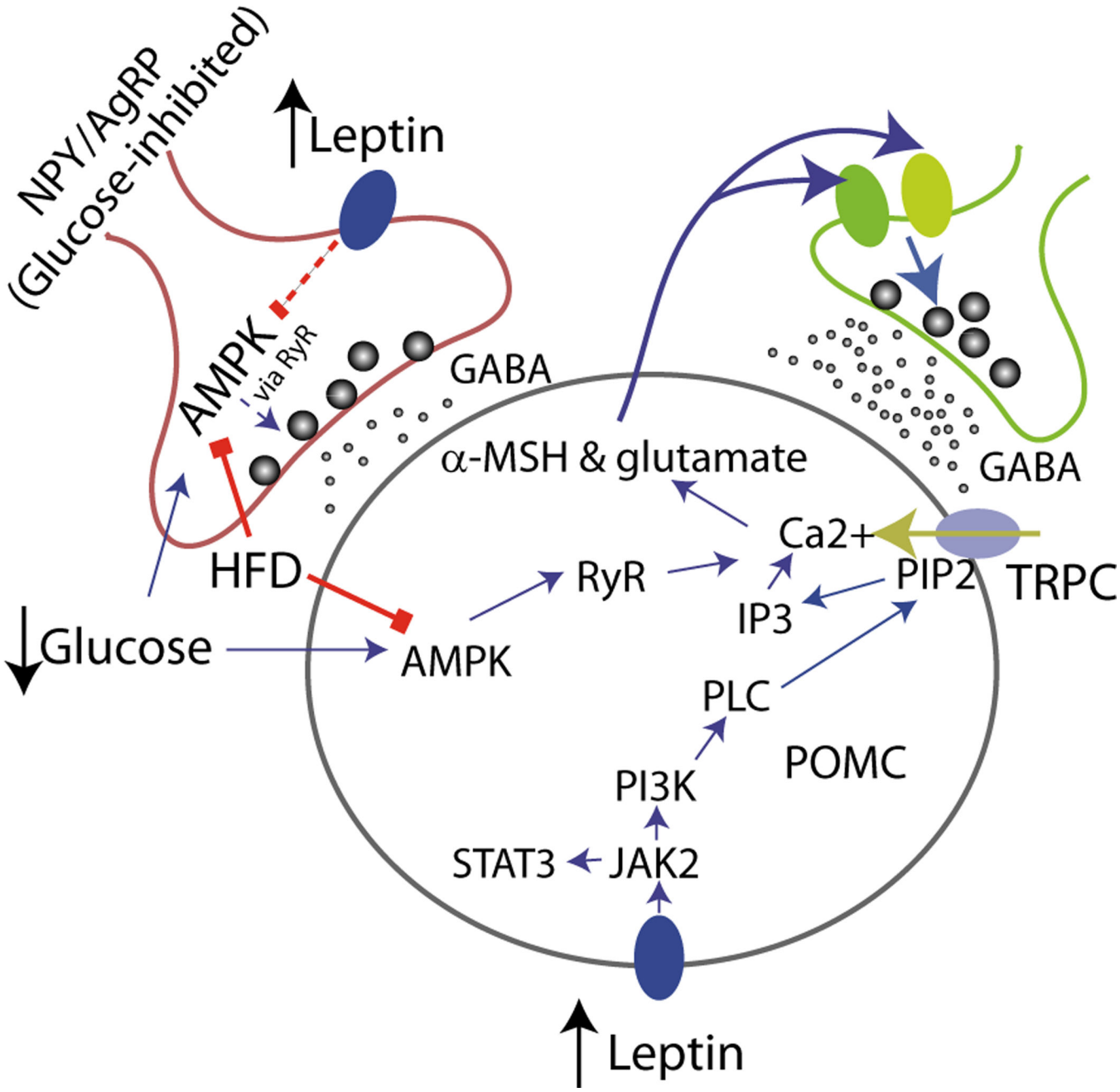

Figure 8. Schematic illustration of the proposed model

Alterations in glucose levels regulate AMPK activity in the ARC circuit. Inhibition by leptin of AMPK in presynaptic GABAergic axon terminals reduces GABA release onto POMC cells. In contrast, leptin receptor activation on POMC neurons stimulates the JAK2-PI3K-PLC pathway, resulting in opening of TRPC channels and probably IP3 receptors as well. Increased Ca^{2+} concentrations trigger the release of melanocortin and glutamate from glutamatergic POMC neurons. Short-term exposure to a high-fat diet up-regulates PI3K activity specifically in POMC neurons. Under these conditions, leptin no longer reduces GABA release from presynaptic GABAergic neurons. However, the JAK2-PI3K-PLC

pathway by leptin in POMC neurons remains effectively transmitted, thereby enhancing GABAergic inhibitory tone to POMC neurons. This feedback inhibition of POMC neurons in animals on high-fat feeding may play a role in the regulation of overall energy balance.

Table 1

Mean data of sIPSC and mIPSC frequency before and after addition of leptin

	[Glucose] (mM)	Total (%, n)	Increase (%, n)	Decrease (%, n)
Normalized sIPSC freq (%)	2.5	137.3 ± 7.8*, 19	158.9 ± 18.1**, 12/19	0/19
	5	111.6 ± 7.7, 27	147.8 ± 10.5***, 12/27	66.5 ± 5.5**, 6/27
	10	79.8 ± 4.2***, 26	0/26	60.3 ± 4.9***, 11/26
Normalized mIPSC freq (%)	2.5	145.3 ± 10.6**, 20	171.3 ± 12.2**, 12/20	79.7 ± 1.1, 1/20
	5	148.0 ± 12.8***, 21	174.3 ± 9.3***, 13/21	76.9 ± 2.3, 1/21
	10	104.8 ± 7.8, 31	158.9 ± 13.0**, 8/31	56.6 ± 3.9***, 8/31

	[Glucose] (mM)	Increase (control vs. leptin, n)	Decrease (control vs. leptin, n)
sIPSCfreq (Hz)	2.5	2.6 ± 0.6 vs. 3.3 ± 0.7***, 12	0
	5	2.4 ± 0.5 vs. 3.3 ± 0.6***, 12	2.2 ± 0.8 vs. 1.6 ± 0.7**, 6
	10	0	2.2 ± 0.6 vs. 1.3 ± 0.3*, 11
mIPSC freq (Hz)	2.5	1.1 ± 0.3 vs. 1.8 ± 0.3***, 12	3.2 ± 0.1 vs. 2.5 ± 0.04, 1
	5	1.0 ± 0.2 vs. 1.8 ± 0.3**, 13	7.4 ± 0.3 vs. 5.7 ± 0.2, 1
	10	1.4 ± 0.4 vs. 2.0 ± 0.5**, 8	2.0 ± 0.6 vs. 1.2 ± 0.4**, 8

		Fisher's exact test	
sIPSC freq	2.5 vs. 5	2.5 vs. 10***	5 vs. 10***
mIPSC freq	2.5 vs. 5	2.5 vs. 10*	5 vs. 10*

* $p < 0.05$,

** $p < 0.01$,

*** $p < 0.001$ (paired *t*-test).

All data are shown as mean ± SEM of sIPSC and mIPSC frequency.

Table 2

Summary of leptin's effect on mIPSC and sIPSC frequency with drugs

	[Glucose] (mM)	Drugs or deficiency	Control (Hz)	Leptin (Hz)	N
mIPSC freq (Hz) (NCD)	5	$LepR^{-/-}$	1.3±0.3	0.9±0.2*	7
		MCPG	1.2±0.1	1.3±0.2**	19
		SHU9119	1.0±0.2	0.8±0.2*	9
		MCPG+SHU9119	1.7±0.3	1.1±0.2**	10
		2-APB	6.9±1.1	6.6±1.0	9
		0 Ca^{2+}	1.1±0.3	0.9±0.2*	13
		U73122	1.2±0.5	1.0±0.4*	10
		Dorsomorphin	1.0±0.3	1.2±0.4*	9
		Wortmannin	1.4±0.2	1.3±0.2	10
sIPSC freq (Hz) (HFD)	10	HFD	2.9±0.8	3.8±1.1*	14
		A769662	1.2±0.3	0.6±0.2**	7
		SHU9119	2.4±1.0	1.6±0.7*	8
		MCPG+SHU9119	2.0±0.6	1.3±0.5**	8

* $p<0.05$,

** $p<0.01$ vs. control (paired *t*-test).

All data are shown as mean ± SEM of mIPSC and sIPSC frequency.