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A Postsynaptic AMPK→p21-Activated Kinase Pathway Drives Fasting-Induced Synaptic Plasticity in AgRP Neurons

Dong Kong^{1,2,3,5,*}, Yossi Dagon^{1,5}, John N. Campbell¹, Yikun Guo^{1,3}, Zongfang Yang¹, Xinchu Yi³, Pratik Aryal¹, Kerry Wellenstein¹, Barbara B. Kahn^{1,6,*}, Bernardo L. Sabatini^{2,4,6,*}, and Bradford B. Lowell^{1,4,6,*}

¹Division of Endocrinology, Diabetes and Metabolism, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, 3 Blackfan Cir, Boston, Massachusetts 02215, USA

²Howard Hughes Medical Institute, Department of Neurobiology, Harvard Medical School, 220 Longwood Ave, Boston, Massachusetts 02215, USA

³Department of Neuroscience, Tufts University School of Medicine, 136 Harrison Ave, Boston, Massachusetts 02135, USA

⁴Program in Neuroscience, Harvard Medical School, Boston, Massachusetts 02115, USA

SUMMARY

AMP-activated protein kinase (AMPK) plays an important role in regulating food intake. The downstream AMPK substrates and neurobiological mechanisms responsible for this, however, are ill-defined. Agouti-related peptide (AgRP)-expressing neurons in the arcuate nucleus regulate hunger. Their firing increases with fasting, and once engaged they cause feeding. AgRP neuron activity is regulated by state-dependent synaptic plasticity; fasting increases dendritic spines and excitatory synaptic activity, feeding does the opposite. The signaling mechanisms underlying this, however, are also unknown. Using neuron-specific approaches to measure and manipulate kinase activity specifically within AgRP neurons, we establish that fasting increases AMPK activity in AgRP neurons, that increased AMPK activity in AgRP neurons is both necessary and sufficient for fasting-induced spinogenesis and excitatory synaptic activity, and that the AMPK phosphorylation target mediating this plasticity is p21-activated kinase. This provides a signaling and

*Correspondence: blowell@bidmc.harvard.edu (B.B.L.); bernardo_sabatini@hms.harvard.edu (B.L.S.); bkahn@bidmc.harvard.edu (B.B.K.), or dong.kong@tufts.edu (D.K.).

⁵Co-first authors

⁶Co-senior authors

SUPPLEMENTAL INFORMATION

Supplemental Information includes full description of Experimental Procedures and one Figure and can be found with this report online at

AUTHOR CONTRIBUTIONS

D.K. and B.B.L. conceived the project. D.K., Y.D., B.B.K., B.L.S., and B.B.L. designed the experiments and analyzed data. D.K. constructed AAVs, performed electrophysiology and multiphoton imaging. Y.D. performed biochemistry studies. J.N.C. performed single cell gene expression. Y.G., Z.Y., P.A., X.Y., and K.W. assisted in experiments. D.K. and B.B.L. prepared the manuscript with contributions from Y.D., B.B.K., and B.L.S..

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neurobiological basis for both AMPK regulation of energy balance and AgRP neuron state-dependent plasticity.

INTRODUCTION

AMP-activated protein kinase (AMPK) is an evolutionarily conserved serine/threonine kinase stimulated by both decreased cellular energy status and increased calcium (Hardie et al., 2012). In the hypothalamus, it is inhibited by leptin (Andersson et al., 2004; Dagon et al., 2012; Minokoshi et al., 2004) and activated by fasting (Minokoshi et al., 2004), ghrelin (Andersson et al., 2004; Andrews et al., 2008; Lopez et al., 2008), and neuronal activity (Hawley et al., 2005; Kawashima et al., 2012). Notably, manipulation of AMPK activity in the hypothalamus affects energy balance (Andersson et al., 2004; Claret et al., 2007; Minokoshi et al., 2004). However, the neurobiological mechanism and downstream AMPK target responsible for these effects are not known.

In this context, hypothalamic agouti-related peptide (AgRP)-expressing neurons, and their excitatory synaptic inputs, are of interest. AgRP neurons are activated by fasting (Takahashi and Cone, 2005), and once engaged, they induce intense hunger and reduce energy expenditure (Aponte et al., 2011; Gropp et al., 2005; Krashes et al., 2011; Luquet et al., 2005). Chemogenetic activation or inhibition of the excitatory neuronal drive to AgRP neurons stimulates/inhibits hunger, respectively (Krashes et al., 2014). Indeed, synaptic plasticity of these excitatory afferents is an important control point. Fasting, ghrelin and low leptin increases excitatory synapses, dendritic spines and excitatory synaptic activity in AgRP neurons (Liu et al., 2012; Pinto et al., 2004; Yang et al., 2011), and this fasting-induced plasticity, which requires NMDA receptors on AgRP neurons, contributes importantly to activation (Liu et al., 2012).

AMPK in AgRP neurons could trigger this plasticity because a) it is activated in the hypothalamus by fasting and by ghrelin, although it is not known if this occurs specifically in AgRP neurons, b) when stimulated pharmacologically in isolated neurons, brain slices, or in vivo in mice, it increases AgRP neuronal activity (Kohno et al., 2008; Kohno et al., 2011) and excitatory input to AgRP neurons (Yang et al., 2011), although the later was reported to be mediated by AMPK in the presynaptic neurons, and c) of significant interest, p21-activated kinase (PAK), a known inducer of spinogenesis and excitatory synaptic plasticity (Hayashi et al., 2004; Kreis and Barnier, 2009; Penzes et al., 2003), was recently identified in an unbiased chemical genetic screen in cultured cells as a novel AMPK substrate (Banko et al., 2011). In the present study, we use neuron-specific approaches to test the following two hypotheses: 1) a postsynaptic AMPK \rightarrow PAK pathway drives state-dependent excitatory synaptic plasticity in AgRP neurons, and 2) the plasticity brought about by this AMPK \rightarrow PAK pathway accounts for effects of AMPK on energy balance.

RESULTS

Fasting Increases AMPK Activity in AgRP neurons

AMPK activity in the hypothalamus, including the arcuate nucleus (ARC), is higher in fasted versus re-fed mice (Minokoshi et al., 2004). However, since AgRP neurons are just one of many subpopulations of neurons in the ARC, and since the other neurons have opposite or unrelated functions, it is unknown if fasting increases AMPK activity specifically in AgRP neurons. To monitor activity selectively in AgRP neurons, we constructed a cre-dependent adeno-associated virus (AAV) expressing FLAG-tagged $\alpha 2$ AMPK (Figure 1A) and stereotaxically injected it into the arcuate nucleus of *Agrp-IRES-Cre* mice (Figure 1B). Mice were then studied in the fed or fasted state (food was removed at 9 AM and assays were performed 24 hrs later). AgRP neuron-specific $\alpha 2$ AMPK was then immunoprecipitated (Figure 1C) and assayed for kinase activity as described previously (Dagon et al., 2012; Minokoshi et al., 2004). Of note, AMPK activity was increased more than two-fold in AgRP neurons of fasted versus fed mice (Figure 1D). Thus, marked fasting-feeding regulation of AMPK occurs specifically in AgRP neurons.

Stimulation of AMPK Activity in AgRP neurons Drives Plasticity

To stimulate AMPK selectively in AgRP neurons, we constructed and stereotaxically injected cre-dependent AAV co-expressing mCherry and a constitutively active (CA) mutant (H150R) of the $\gamma 1$ subunit of AMPK (Minokoshi et al., 2004) into the arcuate nucleus of *Agrp-IRES-Cre* mice (Figure 1E, 1F). We chose this mutant over constitutively active truncated $\alpha 2$ AMPK lacking the autoinhibitory domain to preserve the normal subcellular localization of activated AMPK. Expression occurred in a pattern consistent with AgRP neurons (Figure 1G) and increased $\alpha 2$ AMPK activity in the arcuate nucleus (Figure 1H). To assess effects of AMPK activation on synaptic plasticity, we injected AAV-DIO-CA-AMPK unilaterally into the arcuate nucleus of *Agrp-IRES-Cre, Npy-hrGFP* mice (Figure 1I) and then assessed various parameters, within the same mice in the *ad libitum* fed state, in CA-AMPK-expressing (mCherry⁺, hrGFP⁺) versus “control” non-expressing (hrGFP⁺) AgRP neurons (Figure 1J). As AgRP neurons co-express neuropeptide Y (NPY), the *Npy-hrGFP* BAC transgene allows visualization of AgRP neurons (van den Pol et al., 2009). We employed 2-photon laser scanning microscopy combined with whole-cell patch clamp electrophysiology (Kozorovitskiy et al., 2012) to analyze synaptic plasticity of AgRP neurons (Figure 1K). CA-AMPK expression in fed mice increased dendritic spines (Figure 1L, 1M) and the frequency of mEPSCs (Figure 1N, 1O) but not their amplitude (Figure 1P). CA-AMPK expression also activated AgRP neurons as judged by their depolarization (Figure 1Q, 1R) and increased firing rate (Figure 1Q, 1S). Furthermore, in animals bilaterally injected with AAV-DIO-CA-AMPK, the amount of food eaten (Figure 1T), body weight (Figure 1U), and body fat (Figure 1V) also increased. Thus, activation of AMPK in AgRP neurons increases dendritic spines and excitatory synaptic transmission, AgRP neuron firing rate, and consequently hunger.

Inhibition of AMPK Activity in AgRP Neurons Blocks Fasting-Induced Plasticity

To inhibit AMPK activity selectively in AgRP neurons, we next constructed and stereotaxically injected cre-dependent AAV co-expressing mCherry and dominant negative

(DN) kinase dead (K45R) $\alpha 2$ AMPK (Minokoshi et al., 2004) into the arcuate nucleus of *Agrp-IRES-Cre* mice (Figure 2A, 2B). Expression occurred in a pattern consistent with AgRP neurons (Figure 2C) and lowered total $\alpha 2$ AMPK activity in the arcuate nucleus (Figure 2D) where AgRP neurons are located. Of note, DN-AMPK expression did not cause death of AgRP neurons (Figure S1). To assess effects of AMPK inhibition on synaptic plasticity, we injected AAV-DIO-DN-AMPK unilaterally into the arcuate nucleus of *Agrp-IRES-Cre, Npy-hrGFP* mice (Figure 2E) and then assessed various parameters, within the same mice, in DN-AMPK-expressing (mCherry⁺, hrGFP⁺) versus “control” non-expressing (hrGFP⁺) AgRP neurons (Figure 2F). In control AgRP neurons, as previously observed (Liu et al., 2012), fasting increased dendritic spines (Figure 2G, 2H) and the frequency of mEPSCs (Figure 2I, 2J), but not their amplitude (Figure 2K). Fasting also activated control AgRP neurons as judged by their depolarization (Figure 2L, 2M) and increased firing rate (Figure 2L, 2N). Notably, these effects of fasting on both synaptic plasticity and activation of AgRP neurons were absent in DN-AMPK-expressing AgRP neurons (Figure 2G–2N). Also, in animals bilaterally injected with AAV-DIO-DN-AMPK, the amount of food eaten following 24-hr fasting was reduced (Figure 2O). Thus, activation of AMPK in AgRP neurons is both sufficient (CA-AMPK studies, Figure 1) and necessary (DN-AMPK studies, Figure 2) for fasting-induced effects on plasticity, AgRP neuron activation, and consequently hunger.

AMPK Phosphorylates PAK2 and Regulates its Activity in Neurons

We then considered AMPK targets that could regulate synaptic plasticity. A recent unbiased screen for $\alpha 2$ AMPK substrates identified p21-activated protein kinase (specifically the PAK2 isoform) (Banko et al., 2011), a known post-synaptic driver of excitatory synaptic plasticity (Hayashi et al., 2004; Kreis and Barnier, 2009; Penzes et al., 2003). PAKs are serine/threonine kinases regulated by GTPases of the Rac1 and Cdc42 family (Bokoch, 2003). The group 1 members of PAKs (PAKs 1, 2 and 3) are typified by a common N-terminal autoinhibitory domain (AID) and are highly homologous throughout (Bokoch, 2003). AMPK phosphorylates serine 20 of PAK2, and this appears to be necessary for AMPK-induced phosphorylation of the PAK2 substrate, myosin regulatory light chain (MRLC) (Banko et al., 2011). Of note, a phosphorylation site mapping program (<http://scansite.mit.edu>) strongly suggests that AMPK also phosphorylates PAK1 (on serine-21), but likely not PAK3 (on serine-20), which lacks an AMPK phosphorylation consensus motif (Banko et al., 2011). We performed RT-PCR on dissociated, single AgRP neurons and detected *Pak1*, *Pak2* and *Pak3* mRNAs, respectively, in 100%, 50% and 90% of AgRP neurons (Figure 3A). We focused our efforts on PAK2 because of the availability of reagents that readily detect its serine-20 phosphorylation, and prior work establishing that it is a downstream target of AMPK (Banko et al., 2011).

In the arcuate nucleus, fasting, which increases AMPK activity (Figure 3B), increased serine-20 phosphorylation of PAK2 (Figure 3C), and also threonine-508/505 phosphorylation of the PAK target, LIM kinase 2 (LIMK2) (Figure 3D). Of note, this fasting-induced increase in LIMK phosphorylation occurred specifically in AgRP neurons (Figure 3E). Importantly, PAK2 co-precipitates with $\alpha 2$ AMPK from protein lysates of the arcuate nucleus indicating that the two interact in cells within the arcuate nucleus (Figure

3F). We next injected into the mediobasal hypothalamus an adenovirus expressing, independently of cre, HA-tagged CA- γ 1 AMPK (Minokoshi et al., 2004). As shown in Figure 3G, CA- γ 1 AMPK in the hypothalamus increased phosphorylation of PAK2 and LIMK2. We further constructed an AAV viral vector expressing DN-AMPK and mCherry independently of cre and similarly injected it into the mediobasal hypothalamus (Figure 3H). As shown in Figure 3I–K, hypothalamic expression of DN-AMPK significantly attenuated fasting-induced phosphorylation of LIMK2, as evidenced by either immunofluorescence (Figure 3I, 3J) or western blotting (Figure 3K). Thus, increased AMPK activity is required for fasting-induced phosphorylation of the major PAK target, LIMK2. Using the immortalized hypothalamic cell line, GT1-7 (Mellon et al., 1990), we confirmed that two known activators of AMPK, reduced energy state (glucose starvation) and a cell permeable AMP analogue (AICAR), increased serine-20 phosphorylation of PAK2 (Figure 3L) and phosphorylation of acetyl CoA carboxylase on the well-known AMPK phosphorylation site. Likewise, expression of CA-AMPK also increased serine-20 phosphorylation (Figure 3M). Finally, AMPK activation by AICAR increased phosphorylation of the PAK2 target, LIMK2 (Figure 3N, “Empty” lanes), and overexpression of wild-type PAK2 greatly augmented this effect (Figure 3N, PAK2^{WT} lanes). Importantly, this augmentation was not seen following overexpression of a phospho-defective S20A mutant of PAK2 (Figure 3N, PAK2^{S20A} lanes). In total, these studies and those of Banko *et al.* (Banko et al., 2011) demonstrate that AMPK phosphorylates serine 20 on PAK2, that this is associated with increased phosphorylation of the PAK2 targets LIMK2 (this study) and MRLC (Banko et al., 2011), and that the ability of serine 20 to be phosphorylated by AMPK is necessary for AMPK-induced increased activity of PAK2 on LIMK2 (this study) and MRLC (Banko et al., 2011). Furthermore, our study demonstrates that AMPK regulation of PAK2 occurs in neurons. Of note, given the sequence homology between PAK1 and PAK2, such AMPK regulation may also occur for PAK1, which was not assessed in the current study due to unavailability of antibodies against serine-21 phosphorylated PAK.

Inhibition of PAKs Blocks Fasting- and AMPK-Mediated Plasticity in AgRP Neurons

Since all three PAKs are expressed in AgRP neurons (Figure 3A) and since PAK1, in addition to PAK2, could mediate the effects of AMPK on synaptic plasticity in AgRP neurons, we generated a cre-dependent AAV co-expressing EGFP and the autoinhibitory domain (AID) of PAK1 (DN-PAK). Of note, overexpressed DN-PAK binds to the catalytic domain of all three group 1 PAKs, preventing their activation (Hayashi et al., 2004). Hence, DN-PAK will inhibit all three PAKs in AgRP neurons. This DN-PAK virus was then stereotaxically injected into the arcuate nucleus of *AgRP-IRES-Cre* mice (Figure 4A, 4B). Expression of AAV-DIO-DN-PAK, as indicated by EGFP fluorescence, occurred in a pattern consistent with AgRP neurons (Figure 4C). To assess effects of PAK inhibition on synaptic plasticity, we injected AAV-DIO-DN-PAK into the arcuate nucleus of *AgRP-IRES-Cre* mice and assessed various parameters, in the fasted state, in DN-PAK-expressing neurons. Control AgRP neurons for these studies were from uninjected fasted *Npy-hrGFP* mice. Of note, PAK inhibition of AgRP neurons in fasted mice decreased dendritic spines (Figure 4D, 4E) and greatly reduced the frequency of mEPSCs (Figure 4F, 4G), but not their amplitude (Figure 4H). In addition, PAK inhibition decreased the activity of AgRP neurons as judged by their hyperpolarization (Figure 4I, 4J) and decreased firing rate (Figure 4K). To determine if PAK

activity was required for AMPK's effects on plasticity, we injected one side of the ARC with AAV-DIO-CA-AMPK alone and the other with a 1:1 mix of both AAV-DIO-CA-AMPK and AAV-DIO-DN-PAK (Figure 4L, 4M). Importantly, in *ad libitum* fed *Agrp-IRES-Cre* mice, the ability of CA-AMPK to increase mEPSC frequency (Figure 4O left bar, CA-AMPK alone, as previously seen in Figure 1O) was blocked by simultaneous inhibition of PAK (Figure 4O right bar, CA-AMPK + DN-PAK). Finally, in *Agrp-IRES-Cre* mice bilaterally injected with AAV-DIO-DN-PAK, body weight (Figure 4Q) and the amount of food eaten following a fast was significantly reduced (Figure 4R). These studies demonstrate that activation of group 1 PAKs is required for the stimulatory effects of AMPK on excitatory synaptic plasticity.

DISCUSSION

In the present study, we demonstrate the following: 1) AMPK activity in AgRP neurons is increased by fasting; 2) this is both necessary and sufficient for fasting-induced spinogenesis and excitatory synaptic plasticity; 3) in neurons AMPK phosphorylates PAK and leads to increased phosphorylation of a downstream substrate of PAK (LIMK2); and 4) this activation of PAK by AMPK mediates fasting- and also AMPK-mediated excitatory plasticity. Upregulation of synaptic activity by this AMPK \rightarrow PAK pathway is likely consequential because chemogenetic activation of the excitatory neuronal inputs to AgRP neurons drives hunger (Krashes et al., 2014), and NMDAR deletion in AgRP neurons, which prevents fasting-induced synaptic plasticity, reduces hunger (Liu et al., 2012). Furthermore, stimulation of excitatory neurotransmission in AgRP neurons by CA-AMPK promotes hunger. Conversely, inhibition of glutamatergic neurotransmission by DN-AMPK or DN-PAK suppresses hunger. Thus, regulation of synaptic plasticity by the AMPK \rightarrow PAK pathway in AgRP neurons is important in controlling hunger. In total, these findings establish a signaling (AMPK \rightarrow PAK) and neurobiological basis (postsynaptic regulation of glutamatergic neurotransmission in AgRP neurons) for AMPK regulation of energy balance.

A prior study concluded that a site of action by which AMPK regulates state-dependent plasticity is presynaptic, i.e. within the excitatory afferent axon terminals (Yang et al., 2011). There are, however, differences between the two studies that are worth noting. First, the prior study largely examined ghrelin-stimulated plasticity while our study focused on fasting-induced plasticity. Second, the means of altering AMPK and timescales for observing effects are different; the prior study used AMPK pharmacologic activators (AICAR and ZMP) and an inhibitor (compound C) and looked at effects following addition of these drugs to brain slices, while our study used genetic tools (CA-AMPK and DN-AMPK) delivered directly to postsynaptic AgRP neurons *in vivo* and then looked at effects *ex vivo*. Third, the prior study inferred a presynaptic role for AMPK by excluding a postsynaptic role, while our study directly tested and demonstrated a postsynaptic role for AMPK. As our study focused on postsynaptic AMPK and did not address the role of presynaptic AMPK, our findings do not exclude an additional presynaptic site of action. That said, we believe postsynaptic regulation of plasticity is important for the following reasons: a) postsynaptic NMDA receptors on AgRP neurons are required for fasting-induced plasticity (Liu et al., 2012), b) PAK, a known postsynaptic regulator of spinogenesis and excitatory synaptic plasticity (Kreis and Barnier, 2009), is phosphorylated and activated by

AMPK ((Banko et al., 2011) and the present study), and c) by direct genetic manipulation of AMPK in postsynaptic AgRP neurons, we demonstrate that postsynaptic AMPK is both necessary and sufficient for fasting-induced plasticity.

How then does fasting activate AMPK in AgRP neurons? While AMPK is regulated by cellular energy status (Hardie et al., 2012), this would seem to be an unlikely regulator in this scenario. Alternatively, intracellular calcium, which is known to drive synaptic plasticity (Bloodgood and Sabatini, 2007), could be responsible. Prior studies have established that increased calcium and subsequent activation of CAMKK β , an upstream AMPK-kinase, can increase AMPK activity (Anderson et al., 2008; Hardie et al., 2012; Hawley et al., 2005; Kawashima et al., 2012; Mairet-Coello et al., 2013). In neurons, intracellular calcium is increased by NMDA receptor activation, neuronal firing and ghrelin, and these three manipulations have been shown to activate AMPK via CAMKK β (Anderson et al., 2008; Andersson et al., 2004; Andrews et al., 2008; Hardie et al., 2012; Lopez et al., 2008; Yang et al., 2011). In this context it is of interest that AgRP neurons abundantly express the receptor for the fasting-induced hormone ghrelin (Willesen et al., 1999; Zigman et al., 2006), and that fasting-induced synaptic plasticity in AgRP neurons requires functional NMDA receptors on AgRP neurons (Liu et al., 2012). With regards to the source of glutamate which would activate these NMDA receptors, we have found that AgRP neurons receive strong excitatory drive from the paraventricular nucleus (PVH) and that this input is important in activating AgRP neurons and causing hunger (Krashes et al., 2014). Taken together, this leads to the hypothesis that increased calcium, secondary to elevated ghrelin, NMDA receptor action and increased neuronal firing, activates CaMKK β and its downstream target AMPK, and that this is responsible for fasting-induced plasticity in AgRP neurons. Given the widespread expression of NMDA receptors, CaMKK β , AMPK and PAK, it is tempting to speculate that the AMPK \rightarrow PAK \rightarrow plasticity pathway reported here will operate in circuits both within and also beyond the hypothalamus. If true, this would have important implications for many processes where plasticity plays a key regulatory role, one example being learning and memory.

Finally, it is possible that other targets in addition to PAK may be involved in AMPK-mediated synaptic plasticity. In this light, mitochondrial homeostasis and perhaps also mitochondrial distribution are of interest since they can affect neuronal activity (Dietrich et al., 2013; Li et al., 2004; Schneeberger et al., 2013) and can be regulated by AMPK (Toyama et al., 2016). If such pathways do indeed play a role, they appear to require PAK as PAK inhibition prevents AMPK-mediated synaptic plasticity (Figure 4O).

EXPERIMENTAL PROCEDURES

AAV viral expression

AAV viruses were packaged at BCH Viral Core or UNC Viral Core and stereotaxically injected into the arcuate nucleus of *AgRP-IRES-Cre* mice. See Supplemental Information for the detail.

Electrophysiology and two-photon imaging

Whole-cell patch-clamp recordings were obtained from fluorescent protein-identified AgRP neurons in acute coronal slices. Cells were filled with Alexa Fluor594 (10–20 μ M) and imaged using a home-built two-photon laser-scanning microscope (810–840 nm). See Supplemental Information for the detail.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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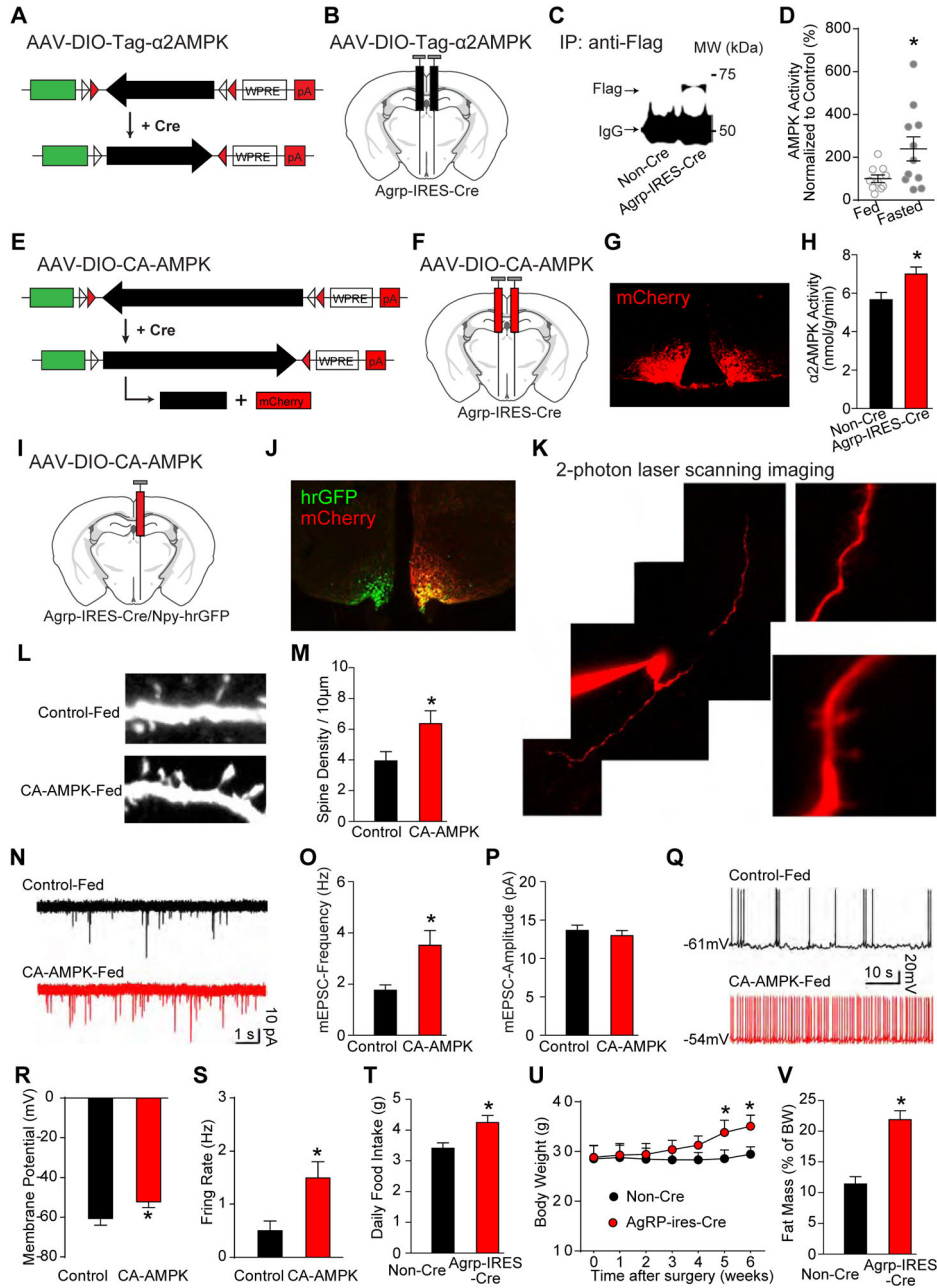


Figure 1. AMPK stimulates excitatory synaptogenesis in AgRP neurons

(A–D) Schematics of AAV-DIO-Tag- α 2AMPK (A) and stereotaxic injection (B), immunoprecipitation from arcuate lysates of fed mice (C), and AgRP neuron α 2AMPK activity immunoprecipitated with the anti-Flag antibody from the arcuate nucleus of fed and fasted *AgRP-IRES-Cre* mice (D) ($n_{fed}=10$ and $n_{fasted}=11$).

(E–H) Schematics of constitutively active AAV-DIO-CA-AMPK (E) and stereotaxic injection (F), immunofluorescence of mCherry (G), and arcuate α 2AMPK activity immunoprecipitated with anti- α 2AMPK antibody from *ad libitum* fed mice (H) ($n=8$).

(I–S) Following unilateral injection of AAV-DIO-CA-AMPK (I), immunofluorescence (J), example of two-photon imaging of an AgRP neuron (K), examples and summary of dendritic spines (L and M), mEPSCs (N–P), and firing properties (Q–S) are shown (n=10 neurons from 3 mice).

(T–V) Following bilateral injection of AAV-DIO-CA-AMPK, daily food intake (T), body weight (U), and body fat mass (V) (n=8).

Data are mean \pm SEM and * indicates $p < 0.05$ with unpaired two-tailed student's t-test.

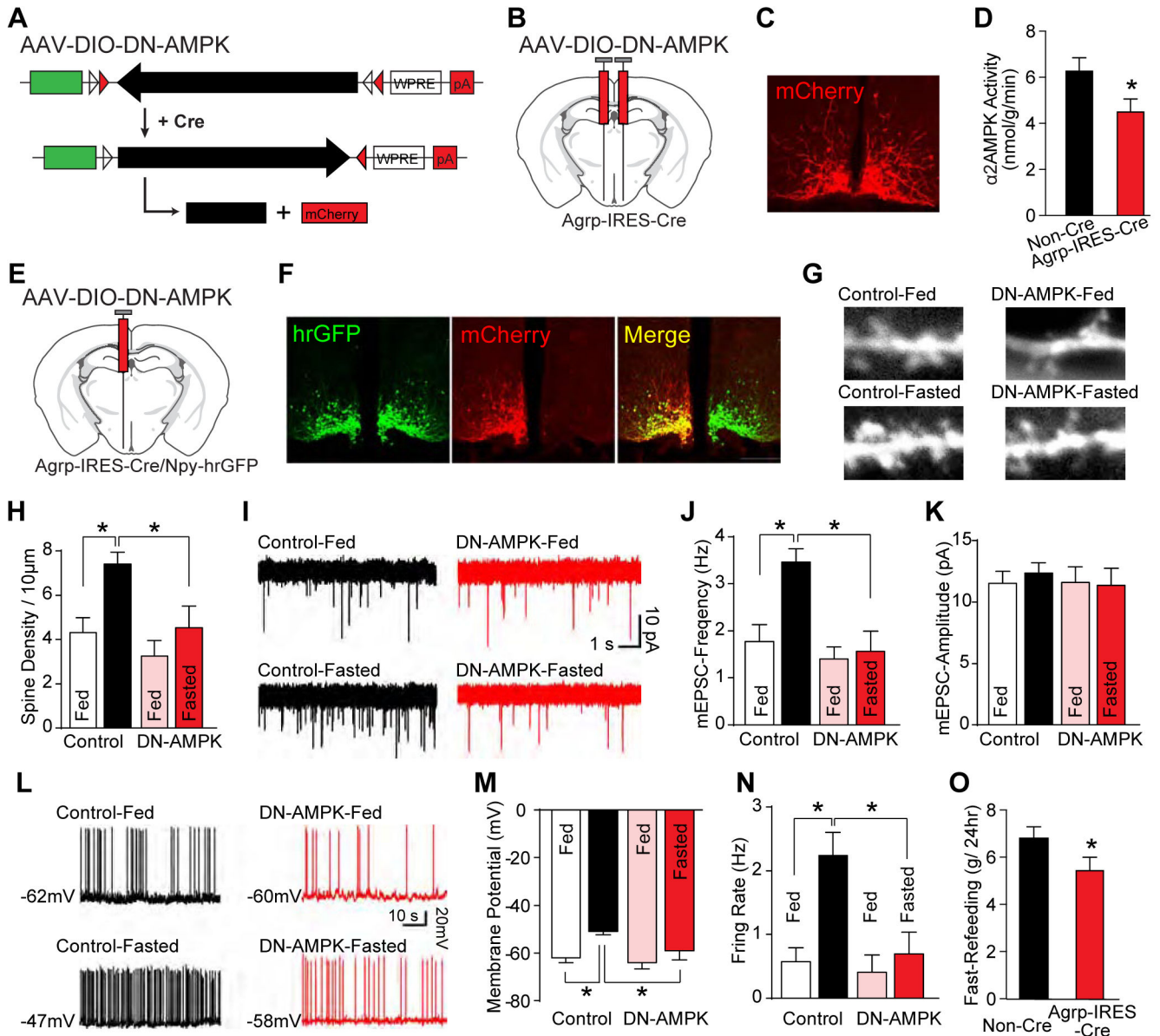


Figure 2. AMPK is required for fasting-induced synaptic plasticity in AgRP neurons

(A–D) Schematics of dominant negative AAV-DIO-DN-AMPK (A) and stereotaxic injection (B), immunofluorescence of mCherry (C), and arcuate $\alpha 2$ AMPK kinase activity from *ad libitum* fed mice (D) (n=8).

(E–N) Following unilateral injection of AAV-DIO-DN-AMPK (E), immunofluorescence (F), examples and summary of dendritic spines (G and H), mEPSCs (I–K), and firing properties (L–N) are shown ($n_{fed}=9$ and $n_{fasted}=11$ neurons from 3 mice per group) in fed or fasted mice.

(O) Following bilateral injection of AAV-DIO-DN-AMPK, food eaten following 24-hr fasting (n=8).

Data are mean \pm SEM and * indicates $p < 0.05$ with unpaired two-tailed student's t-test (D and O) and with unpaired one-way ANOVA test (H, J, K, M, and N).

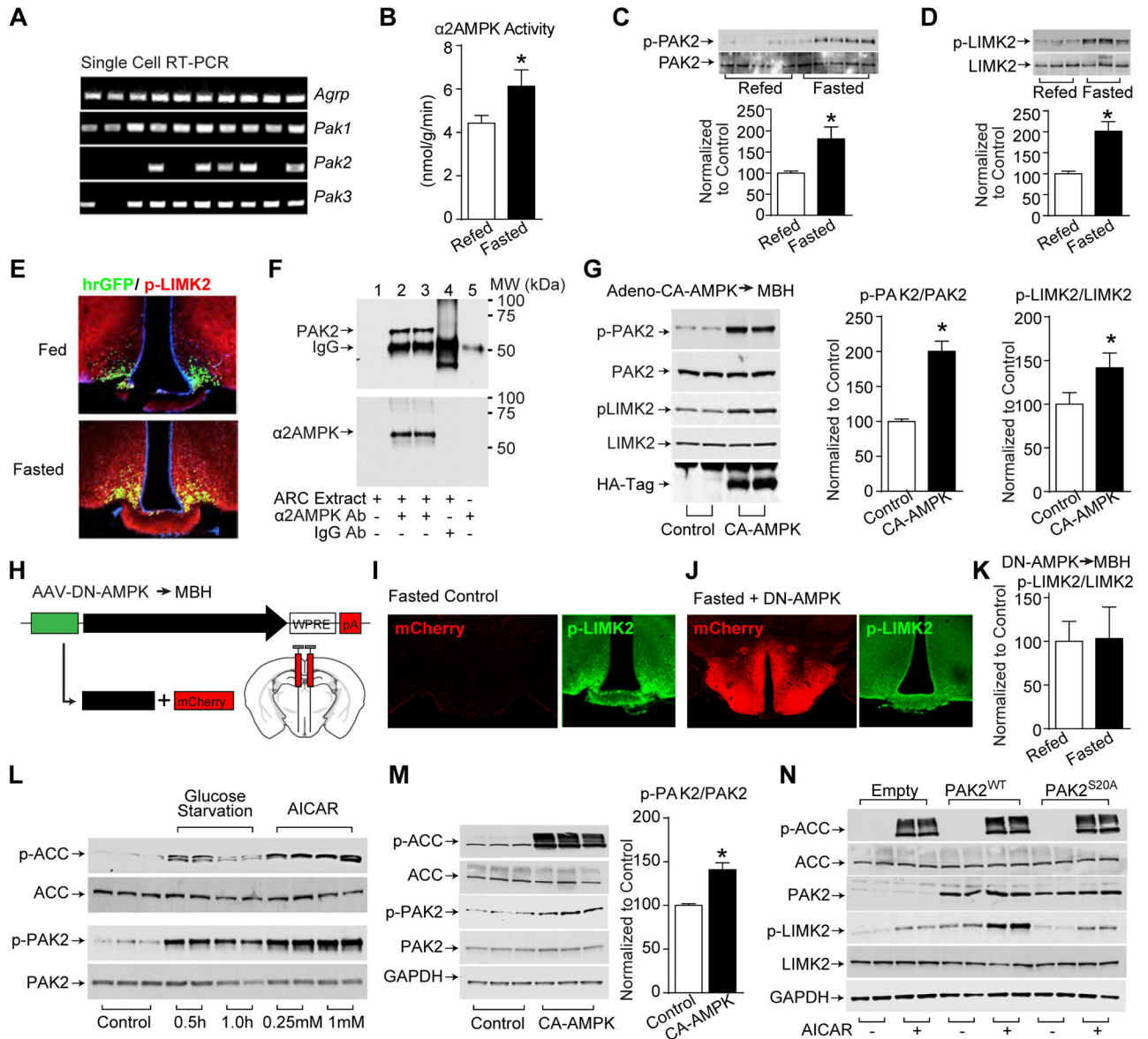


Figure 3. AMPK phosphorylates and stimulates PAK signaling

(A) Single cell RT-PCR in AgRP neurons

(B–D) Arcuate $\alpha 2$ AMPK activity (B) and total and phosphorylated PAK2 (Ser²⁰) (C) and LIMK2 (Thr⁵⁰⁵) (D) in arcuate lysates from fasted and 6-hr refed wildtype mice ($n_{refed}=9$ and $n_{fasted}=8$).

(E) Immunofluorescence of arcuate p-Thr⁵⁰⁵LIMK2 from fed and 24-hr fasted *Npy-hrGFP* mice.

(F) Immunoprecipitation of PAK2 and $\alpha 2$ AMPK from arcuate lysates of fed wildtype mice.

(G) Phosphorylation of PAK2 (Ser²⁰) and LIMK2 (Thr⁵⁰⁵) in the arcuate of fed wildtype mice following bilateral injection of HA_{tag}-CA-AMPK adenovirus ($n=5$).

(H–K) Schematics of cre-independent AAV-DN-AMPK and stereotaxic injection into mediobasal hypothalamus (MBH) (H), immunofluorescence of mCherry (red) and p-

Thr⁵⁰⁵LIMK2 (green) from fasted non-viral infected control mice (I) and AAV-DN-AMPK injected mice (J), and the ratio of total and phosphorylated LIMK2 (Thr⁵⁰⁵) in the arcuate lysates as detected with western blot from fasted and 6-hr refed mice following AAV-DN-AMPK injection (K)(n=8).

(L–N) Total and phosphorylated ACC (Ser⁷⁹), PAK2 (Ser²⁰) and LIMK2 (Thr⁵⁰⁵) in GT1-7 cells following glucose starvation or AICAR treatment (L), or transfection of CA-AMPK (n=9) (M), or transfection of PAK2^{WT} and PAK2^{S20A} mutants with 1 mM AICAR treatment (N). Proteins are normalized to GAPDH.

Data are mean ± SEM and * indicates p<0.05 with unpaired two-tailed student's t-test.

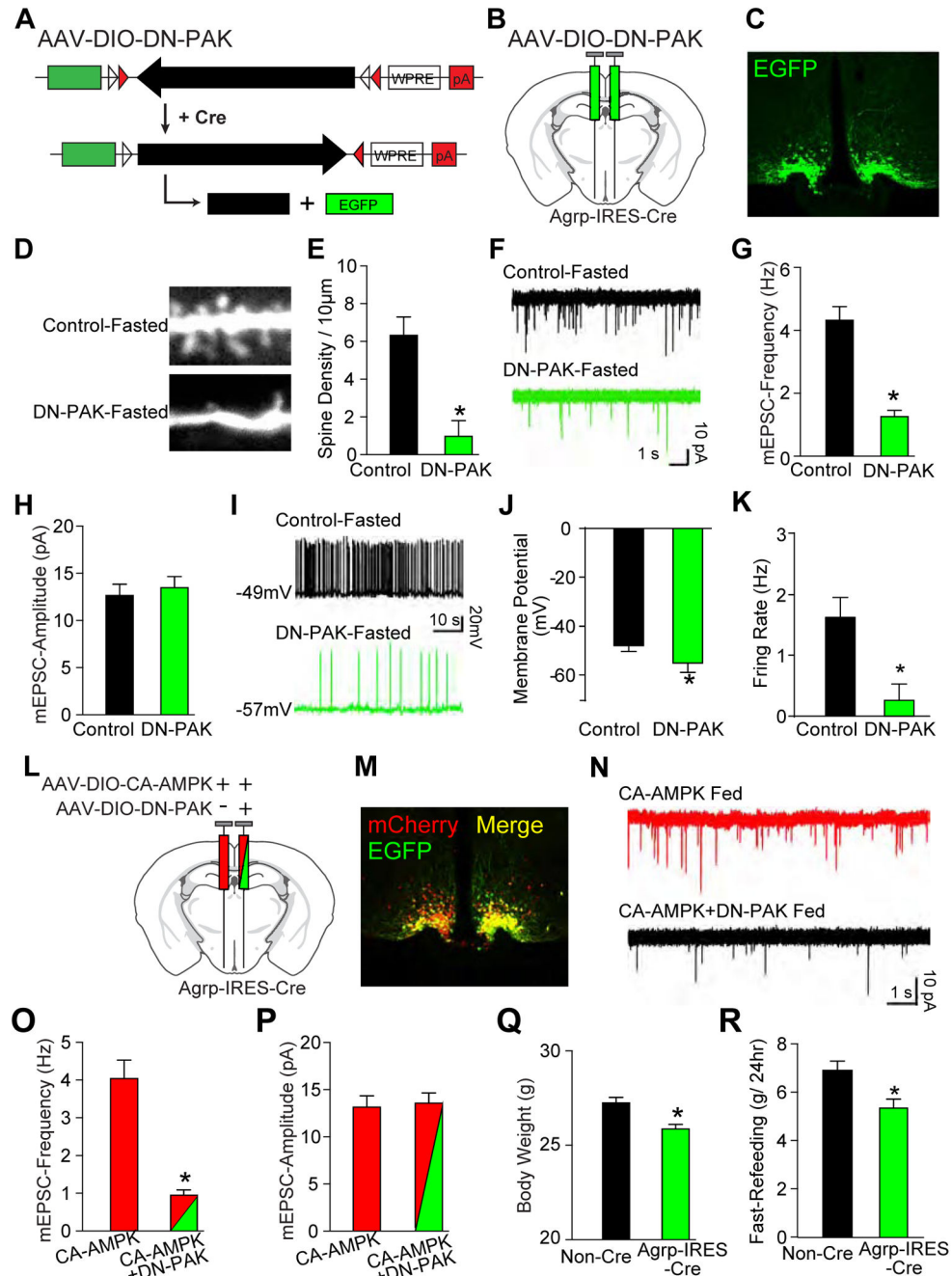


Figure 4. PAK is required for fasting- and AMPK-stimulated synaptic plasticity

(A–C) Schematics of dominant negative AAV-DIO-DN-PAK (A) and stereotaxic injection (B), and immunofluorescence of EGFP (C) in *AgRP-IRES-Cre* mice.

(D–K) Examples and summary of dendritic spines (D and E), mEPSCs (F–H), and firing properties (I–K) in 24-hr fasted *Npy-hrGFP* control and AAV-DIO-DN-PAK virus-injected *AgRP-IRES-Cre* mice.

(L–P) Schematic of AAV-DIO-CA-AMPK and AAV-DIO-DN-PAK viral injection (L), immunofluorescence (M), and example and summary of mEPSCs (N–P).

(Q and R) Body weight (Q) and food eaten following 24-hr fasting (R) from mice bilaterally injected with AAV-DIO-DN-PAK (n=8).

Data are mean \pm SEM (n=10 neurons from 3 mice per group in E, G, H, J, K, O, and P) and

* indicates $p < 0.05$ with unpaired two-tailed student's t-test.

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