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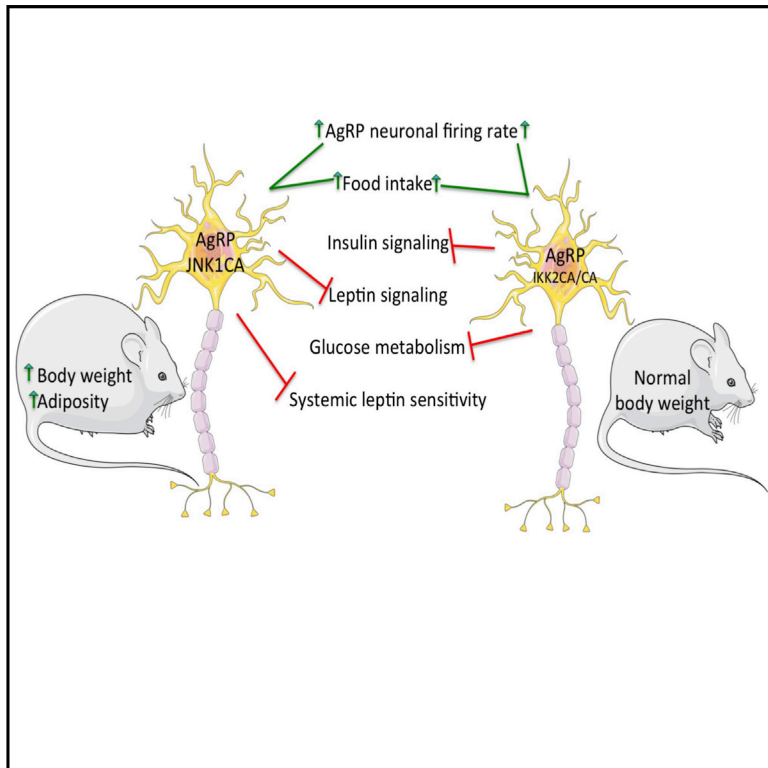
Distinct Roles for JNK and IKK Activation in Agouti-Related Peptide Neurons in the Development of Obesity and Insulin Resistance

Graphical Abstract



Highlights

Inflammatory signaling in AgRP neurons promotes obesity and insulin resistance

JNK and IKK activations in AgRP neurons have distinct metabolic consequences

JNK activation in AgRP neurons causes cellular and systemic leptin resistance

IKK activation in AgRP neurons leads to cellular and systemic insulin resistance

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In Brief

Inflammatory signaling in the CNS is crucial in the development of obesity-associated insulin and leptin resistance. Tsaousidou et al. demonstrate that constitutive JNK activation in agouti-related peptide (AgRP)-expressing neurons is sufficient to induce weight gain and leptin resistance in mice, whereas IKK2 activation fails to cause obesity but blunts insulin signaling and impairs systemic glucose homeostasis. The data reveal distinct effects of c-Jun N-terminal kinase (JNK)- and inhibitor of nuclear factor kappa-B kinase (IKK)-dependent signaling in AgRP neurons, which cooperate in the manifestation of the metabolic syndrome.

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Distinct Roles for JNK and IKK Activation in Agouti-Related Peptide Neurons in the Development of Obesity and Insulin Resistance

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SUMMARY

Activation of c-Jun N-terminal kinase 1 (JNK1)- and inhibitor of nuclear factor kappa-B kinase 2 (IKK2)-dependent signaling plays a crucial role in the development of obesity-associated insulin and leptin resistance not only in peripheral tissues but also in the CNS. Here, we demonstrate that constitutive JNK activation in agouti-related peptide (AgRP)-expressing neurons of the hypothalamus is sufficient to induce weight gain and adiposity in mice as a consequence of hyperphagia. JNK activation increases spontaneous action potential firing of AgRP cells and causes both neuronal and systemic leptin resistance. Similarly, activation of IKK2 signaling in AgRP neurons also increases firing of these cells but fails to cause obesity and leptin resistance. In contrast to JNK activation, IKK2 activation blunts insulin signaling in AgRP neurons and impairs systemic glucose homeostasis. Collectively, these experiments reveal both overlapping and nonredundant effects of JNK- and IKK-dependent signaling in AgRP neurons, which cooperate in the manifestation of the metabolic syndrome.

INTRODUCTION

Inflammatory processes play a crucial role during the development of obesity and diabetes mellitus (Hotamisligil, 2008). In adipose tissue, obesity leads to the recruitment of immune cells, which release cytokines such as tumor necrosis factor and interleukin-6 (Hotamisligil, 2008). These and other cytokines are able to activate inflammatory signaling cascades such as c-Jun N-terminal kinase 1 (JNK1) and inhibitor of nuclear factor kappa-B kinase 2 (IKK2) (Li and Verma, 2002; Bogoyevitch, 2006) in insulin target tissues, thereby promoting the development of insulin resistance. The critical role of these kinases in obesity-induced insulin resistance has been defined through the generation of haploinsufficient conditional IKK2 knockout mice as well as hepatocyte- and myeloid-cell-specific IKK2-deficient mice, which are protected against systemic insulin resistance and glucose intolerance (Yuan et al., 2001; Arkan et al., 2005; Cai et al., 2005). Similarly, conventional JNK1, but not JNK2, knockout mice are protected against obesity and glucose intolerance (Hirosumi et al., 2002). In addition to obesity-associated activation of cytokine signaling, obesity can overload the cellular protein-folding capacity, leading to activation of the ER stress response, which in turn activates JNK1 and IKK2 (Ozcan et al., 2004, 2006). Moreover, nutrient components such as saturated fatty acids can activate both JNK1 and IKK2 signaling through the engagement of Toll-like receptor (TLR)-dependent signaling or as a consequence of excessive ceramide synthesis (Shi et al., 2006; Holland et al., 2007). Taken together, activation of these two kinases represents a hallmark in the development of insulin resistance in peripheral tissues.

In the CNS, the onset of hypothalamic leptin and insulin resistance is a key event in the development of obesity and impaired glucose homeostasis (Vogt and Brüning, 2013; Plum et al., 2006a; Belgardt and Brüning, 2010). Both the adipose-tissue-derived hormone leptin and the pancreatic hormone insulin act on hypothalamic neurons to decrease food intake and hepatic glucose production and, further, to increase energy expenditure (Obici et al., 2002a, b; Belgardt and Brüning, 2010; Ring and Zeltser, 2010). Several hypothalamic neurons are directly regulated by these hormones, with the role of pro-opiomelanocortin (POMC) and the agouti-related peptide (AgRP) neurons being best defined. Acute ablation of POMC

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neurons induces hyperphagia, whereas ablation of AgRP neurons leads to anorexia (Gropp et al., 2005; Luquet et al., 2005). Similarly, optogenetic activation of POMC neurons reduces food intake and body weight, whereas activation of AgRP evokes voracious feeding (Aponte et al., 2011). Accordingly, leptin and insulin receptor signaling in POMC or AgRP neurons is necessary for normal regulation of body weight and glucose homeostasis (Balthasar et al., 2004; Kitamura et al., 2006; van de Wall et al., 2008; Belgardt et al., 2008; Xu et al., 2007). Importantly, lack of insulin signaling in AgRP neurons is sufficient to deregulate hepatic glucose production (Könner et al., 2007; Lin et al., 2010), whereas combined leptin and insulin receptor deficiency in POMC neurons induces systemic insulin resistance (Hill et al., 2010). Of note, both POMC and AgRP neurons are intimately involved in the normal regulation of energy homeostasis not only in rodents but also in humans (Coll et al., 2007).

Whereas these and other investigations have defined neuronal populations directly targeted by insulin and leptin for the regulation of energy homeostasis and glucose metabolism, multiple studies have revealed the development of insulin and leptin resistance in the CNS of obese animals and human patients (Münzberg et al., 2004; Enriori et al., 2007; Hallschmid et al., 2008; Belgardt and Brüning, 2010).

Both ER stress and increased proinflammatory cytokine expression have been detected in the hypothalami of obese mice and rats (De Souza et al., 2005; Ozcan et al., 2009). Likewise, neuronal ablation of the TLR adaptor molecule MyD88 is sufficient to maintain hypothalamic leptin sensitivity despite acute palmitate treatment or in chronically obese mice (Kleinridders et al., 2009), and hypothalamic IKK2 and JNK1 activation has been reported in obese mice and rats (De Souza et al., 2005; Zhang et al., 2008; Belgardt et al., 2010). Conversely, mice with CNS-wide ablation of IKK2 were protected from high-fat diet (HFD)-induced obesity and glucose intolerance (Zhang et al., 2008), and we and others could demonstrate that JNK1 ablation in the CNS is sufficient to improve hypothalamic and systemic insulin sensitivity upon HFD consumption (Belgardt et al., 2010; Sabio et al., 2010; Unger et al., 2010).

However, whereas both neuronal JNK1 and IKK2 inhibition protects from HFD-associated pathologies, it has not yet been demonstrated in which hypothalamic neuronal population JNK1 and/or IKK2 action deregulates energy and/or glucose homeostasis and whether neuron-restricted JNK1 and/or IKK2 activation are sufficient to alter energy and glucose homeostasis. In the present study, we demonstrate that activation of JNK signaling specifically in AgRP neurons results in increased body weight and adiposity by progressively impairing neuronal leptin signaling and systemic leptin sensitivity. On the other hand, IKK2 activation promotes insulin resistance in AgRP neurons and ultimately also in the periphery without affecting energy homeostasis. Thus, we reveal that activation of both IKK and JNK signaling in AgRP neurons is sufficient to promote the development of hallmarks of the metabolic syndrome, but we also identify distinct and nonoverlapping roles for both inflammatory signaling mediators in AgRP neurons in this process.

RESULTS

Activation of JNK Signaling in AgRP Neurons Occurs in Obesity and Increases Firing of These Cells

To assess whether JNK activation occurs in AgRP neurons during obesity development, we employed a reporter mouse strain that allows for genetic identification of AgRP/neuropeptide Y (NPY)-coexpressing neurons via expression of humanized *Renilla* GFP under the control of the mouse *Npy* promoter (NPY^{GFP}) and fed 8-week-old mice with normal chow diet (NCD) ($NCD{:}NPY^{GFP}$) or HFD for 10 days ($HFD{:}NPY^{GFP}$). Immunostaining for phosphorylated c-Jun—as a readout for JNK activation and a crucial regulator of hypothalamic inflammation (De Souza et al., 2005; Belgardt et al., 2010; Sabio et al., 2010; Unger et al., 2010)—in combination with GFP revealed an ~3-fold increase of p-c-Jun immunoreactivity in GFP-positive neurons in NPY^{GFP} mice after HFD consumption compared to the NCD-fed NPY^{GFP} mice (Figures 1A and 1B). These data indicate that, indeed early during the course of HFD feeding, JNK is activated in AgRP neurons.

To address whether JNK activation per se in AgRP neurons, as observed upon obesity development and HFD consumption (Figures 1A and 1B), is sufficient to induce metabolic abnormalities, we sought to generate a genetic mouse model for cell-type-specific JNK activation. Constitutive activation of JNK has previously been achieved by expression of a constitutively active version of the upstream kinase JNKK2/MKK7 (MKK7D; Wang et al., 1998) or a fusion protein consisting of JNKK2/MKK7 and JNK1 (JNKK2-JNK1; Zheng et al., 1999). Thus, we generated mice allowing for the conditional expression of a MKK7D-JNK1 fusion protein upon Cre-recombinase-induced excision of a transcriptional Stop cassette ($R26Stop^{FL}JNK1CA$ mice) from the ROSA26 locus (Pal et al., 2013). In order to activate JNK signaling in AgRP neurons, we crossed $R26Stop^{FL}JNK1CA$ mice with mice expressing the Cre-recombinase in these cells ($AgRPCre^{+/-}$; Tong et al., 2008).

To investigate the efficiency of this transgenic approach to activate JNK, we generated reporter mice expressing GFP in AgRP neurons by crossing the $AgRP^{Cre}$ with the Z/EG mice, in the presence or absence of JNK1CA expression. Immunostaining for phosphorylated c-Jun —as a read-out for JNK activation—in combination with GFP revealed that less than 20% of GFP^{+}-AgRP neurons exhibited p-c-Jun immunoreactivity in lean control animals, whereas in $AgRP^{JNK1CA;GFP}$ mice, more than 70% of GFP^{+}-AgRP neurons showed detectable phosphorylation of c-Jun (Figures 1C and 1D), in fact, to similar degree as observed upon HFD feeding. Importantly, constitutive JNK activation in AgRP neurons did not affect the total number of GFP^{+}-AgRP cells, although JNK1 had been implicated in a cell-type-specific regulation of cell death and survival (Bogoyevitch, 2006; Figure 1E). Taken together, these results indicate that the $AgRP^{JNK1CA}$ mice are a suitable model to investigate the physiological consequences of chronically activated JNK1 signaling in AgRP neurons without altering AgRP neuron viability.

To determine the effect of activated JNK signaling on the cellular properties of AgRP neurons, we first investigated the electrophysiological parameters of AgRP neurons. Here, we employed the NPY^{GFP} reporter mouse strain and crossed it to $AgRP^{JNK1CA}$ and the respective control mice. Perforated patch-clamp recordings of genetically identified AgRP/NPY

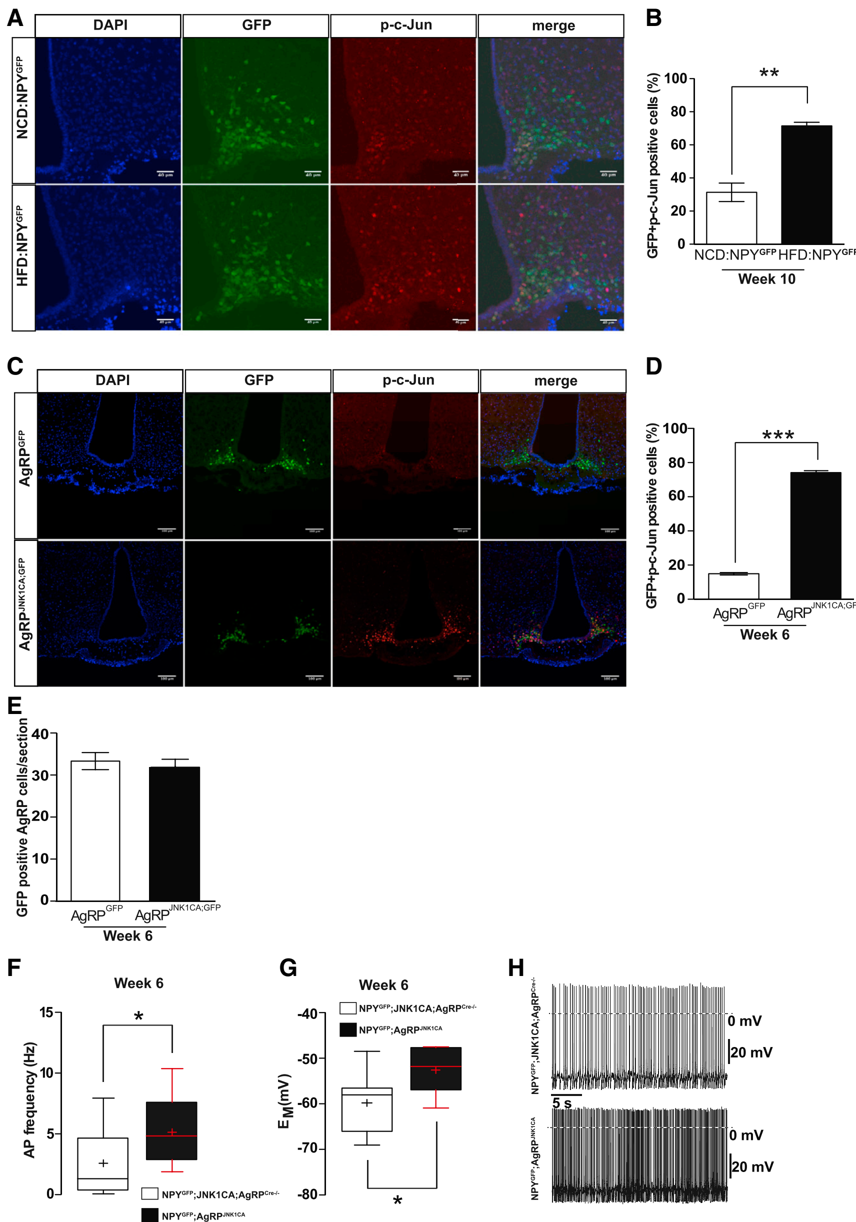

Figure 1. Activation of JNK Signaling in AgRP Neurons Occurs in Obesity and Increases Firing of These Cells

(A and B) High-fat diet consumption for 10 days is sufficient to trigger c-Jun phosphorylation in AgRP neurons of NPY^{GFP} reporter mice. Immunostaining for phospho-c-Jun and GFP performed in arcuate nuclei of NPY^{GFP} reporter mice at the age of 10 weeks fed with normal chow diets (NCD) or high-fat diet for 10 days (HFD). The number of p-c-Jun and GFP double-positive neurons was significantly increased in NPY^{GFP} mice fed with HFD (HFD:NPY^{GFP}; n = 3) compared to NPY^{GFP} mice fed with normal chow diet (NCD:NPY^{GFP}; n = 3). The scale bar represents 40 μm.

(C and D) Expression of the JNK1CA protein in AgRP neurons leads to cell-specific phosphorylation of the JNK target c-Jun in vivo. AgRP neurons were visualized by immunostaining for GFP in AgRP^{GFP} and AgRP$^{JNK1CA;GFP}$ reporter animals. Immunostaining for phospho-c-Jun revealed few AgRP neurons positive for p-c-Jun in control reporter animals, whereas the majority of AgRP neurons in AgRP$^{JNK1CA;GFP}$ mice showed clear immunoreactivity for p-c-Jun. Quantification of p-c-Jun and GFP-positive AgRP neurons is depicted in (D) in control AgRP^{GFP} (n = 3) and AgRP$^{JNK1CA;GFP}$ (n = 3) reporter mice. The scale bar represents 100 μm.

(E) Chronic JNK1 activation does not affect AgRP neuron numbers. All GFP^{+} AgRP neurons were counted in control AgRP^{GFP} (n = 3) and AgRP$^{JNK1CA;GFP}$ (n = 3) reporter mice. No difference in neuron counts per slide was detected between genotypes.

(F) Spontaneous action potential frequencies of NPY^{GFP};JNK1CA;AgRP$^{Cre-/-}$ (n = 3 mice; n = 13 AgRP neurons) and NPY^{GFP};AgRP^{JNK1CA} (n = 3 mice; n = 9 AgRP neurons). AP, action potential.

(G) Membrane potentials of NPY^{GFP};JNK1CA; AgRP$^{Cre-/-}$ (n = 3 mice; n = 13 AgRP neurons) and NPY^{GFP}/AgRP^{JNK1CA} (n = 3 mice; n = 8 AgRP neurons).

(H) Representative recordings of spontaneous action potential frequencies of NPY^{GFP};JNK1CA; AgRP$^{Cre-/-}$ and NPY^{GFP}/AgRP^{JNK1CA} mice at the age of 6 weeks.

Displayed values are means ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001. See also Figure S1.

neurons revealed that activation of JNK signaling in AgRP neurons of NPY^{GFP};AgRP^{JNK1CA} mice increased the spontaneous firing frequency of these cells and depolarized the membrane potential compared to AgRP/NPY neurons of lean control littermates (NPY^{GFP};JNK1CA;AgRP$^{Cre-/-}$; Figures 1F–1H). We did not observe any differences in other basic electrophysiological properties, such as input resistance, cell capacitance, and spike-frequency adaptation (Figure S1).

JNK Activation in AgRP Neurons Causes Cellular and Systemic Leptin Resistance

Apart from regulating neuronal firing of AgRP neurons, leptin exerts its regulatory role on feeding behavior through Stat3-dependent regulation of neuropeptide expression, including that of AgRP and NPY (Bates and Myers, 2003). Thus, we directly assessed leptin's ability to activate STAT3 phosphorylation in AgRP neurons of AgRP^{JNK1CA} and control mice. To this end, we employed mice, which allow for Cre-dependent expression of β-galactosidase (Plum et al., 2006b), and crossed these animals with AgRP^{JNK1CA} mice, yielding both control mice with genetically marked AgRP neurons (AgRP^{LacZ} mice) as well as AgRP$^{JNK1CA;LacZ}$ mice. Whereas in vivo leptin stimulation markedly induced Stat3 phosphorylation in AgRP neurons of 10-week-old control mice, leptin's ability to induce pSTAT3 immunoreactivity in AgRP neurons of AgRP$^{JNK1CA;LacZ}$ mice was largely reduced, demonstrated both as the percentage of pSTAT3 immunoreactive LacZ-positive cells per section (Figures 2A and 2B) and as intensity of the pSTAT3 immunoreactivity in

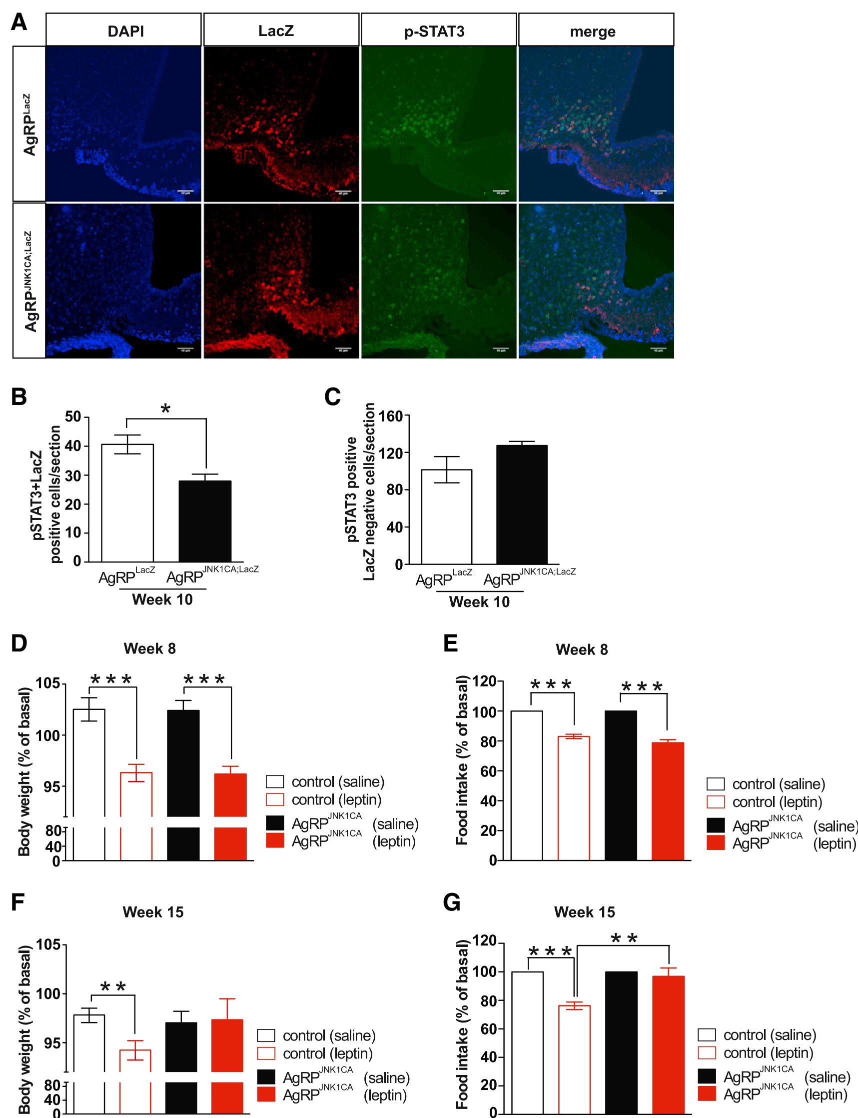

Figure 2. JNK1 Activation Causes AgRP-Neuron-Specific and Subsequently Systemic Leptin Resistance

(A) Representative pSTAT3 immunostaining of AgRP^{LacZ} and AgRP$^{JNK1CA;LacZ}$ after fasting for 16 hr and intraperitoneal leptin stimulation (1 mg/kg) in 10-week-old animals. The scale bar represents 40 μm.

(B) Average number of pSTAT3 and LacZ immunoreactive cells per section of control AgRP^{LacZ} (n = 3) and AgRP$^{JNK1CA;LacZ}$ (n = 3) mice.

(C) Average number pSTAT3 immunoreactive non-AgRP (LacZ-negative) cells after leptin stimulation is indistinguishable between AgRP^{LacZ} (n = 3) and AgRP$^{JNK1CA;LacZ}$ (n = 3) mice. The same images as for (B) were used for quantification.

(D) Changes in body weight after intraperitoneal leptin treatment in control (n = 7) and AgRP^{JNK1CA} (n = 7) mice at 8 weeks of age. Data represent percentage of basal body weight (the body weight in the beginning of each treatment) after a 3-day (72 hr) treatment with twice-daily injections (12 hr apart) of saline followed by a 3-day treatment with twice-daily injections of 2 mg/kg leptin.

(E) Changes in food intake after intraperitoneal leptin treatment in control (n = 7) and AgRP^{JNK1CA} (n = 7) mice at 8 weeks of age. Data represent daily food intake after a 3-day (72 hr) treatment with twice-daily injections (12 hr apart) of saline followed by a 3-day treatment with twice-daily injections of 2 mg/kg leptin.

(F) As in (D), but at 15 weeks of age of control (n = 10) and AgRP^{JNK1CA} (n = 4) mice.

(G) As in (E), but at 15 weeks of age of control (n = 10) and AgRP^{JNK1CA} (n = 4) mice.

Displayed values are means ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. See also Figure S2.

LacZ-positive cells (Figure S2A; for detailed information please see Supplemental Experimental Procedures). Thus, activating JNK signaling in AgRP neurons induces leptin resistance in these cells. This effect is AgRP-cell-specific, because pSTAT3 immunoreactivity after leptin stimulation is not affected in non-AgRP cells (Figures 2C and S2B).

To investigate systemic leptin sensitivity, control and AgRP^{JNK1CA} mice were injected twice daily for 3 consecutive days with saline followed by twice-daily injections for 3 consecutive days with 2 mg/kg leptin. Leptin treatment significantly reduced food intake and body weight in both control and AgRP^{JNK1CA} mice at 8 weeks of age (Figures 2D and 2E). Importantly, at the age of 15 weeks, leptin treatment failed to suppress food intake and to reduce body weight in AgRP^{JNK1CA} mice, whereas control mice still responded to the food-intake- and body-weight-reducing action of leptin (Figures 2F and 2G). Of note, leptin resistance at this age occurred in weight-matched animals to rule out a potential confounding effect of increased body weight between the two genotypes (Figures S2D and S2E). Collectively, these experiments reveal that chronic activation of JNK signaling in AgRP neurons is sufficient to initially cause cell-autonomous and subsequently systemic leptin resistance.

Activation of JNK Signaling in AgRP Neurons Causes Obesity

To address the effect of activating JNK signaling in AgRP neurons on energy homeostasis, we monitored the body weight of male control and AgRP^{JNK1CA} mice. Whereas body weight did not differ between genotypes at the beginning of our analysis, AgRP^{JNK1CA} mice displayed progressively increased body weight starting at the age of 5 weeks, which continued until the end of our study at 28 weeks of age (Figure 3A). Moreover, analysis of body composition revealed increased adipose tissue mass in AgRP^{JNK1CA} compared to control mice (Figure 3B), which was associated with adipocyte hyperplasia (Figures 3C and 3D).

In light of the increased body weight and adiposity of AgRP^{JNK1CA} mice, we aimed to identify the mechanism of how AgRP-neuron-specific activation of JNK signaling caused a

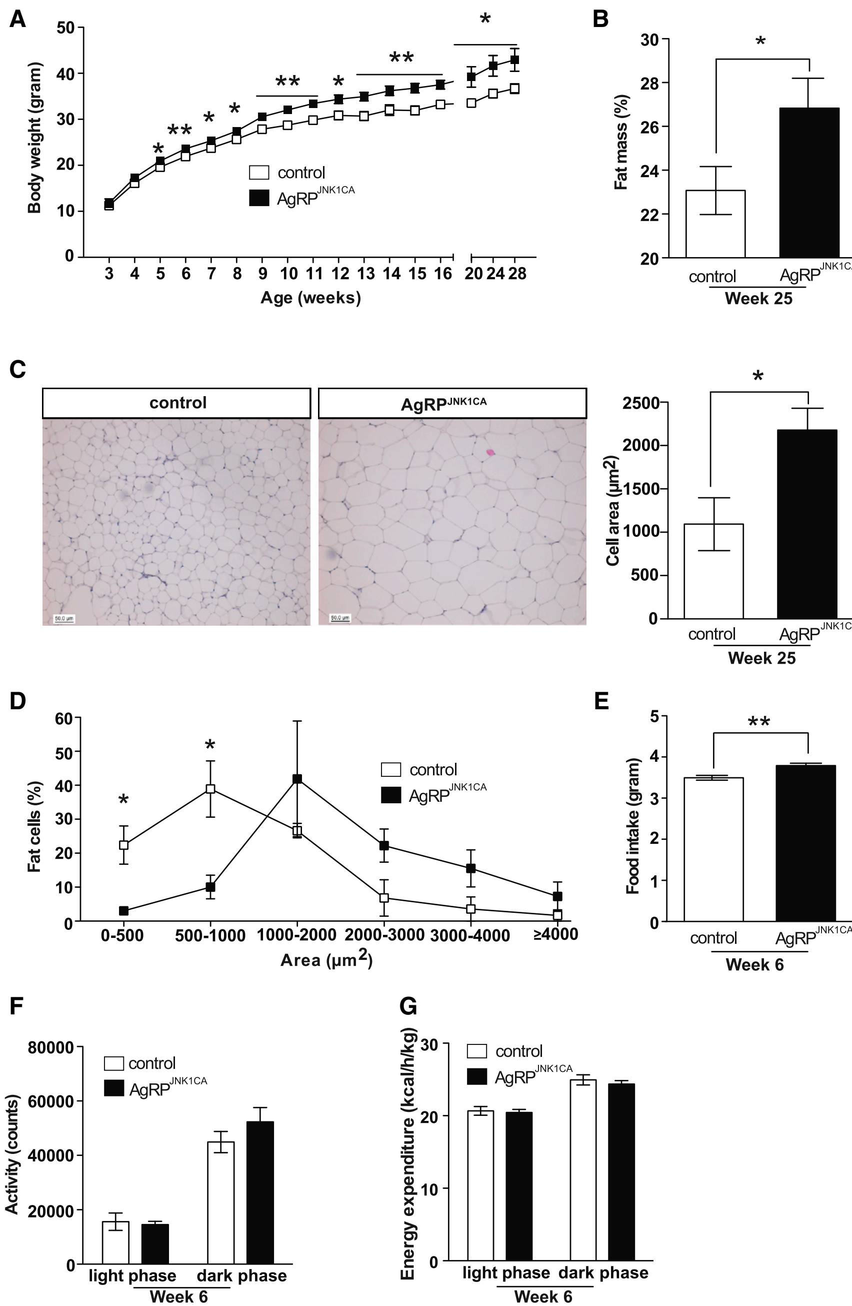

Figure 3. Chronic JNK1 Signaling in AgRP Neurons Results in Increased Body Weight and Adiposity

(A) Average body weight of control (n = 20) and AgRP^{JNK1CA} mice (n = 20) on normal diet.

(B) Average body fat as measured by nuclear magnetic resonance analysis of control (n = 12) and AgRP^{JNK1CA} mice (n = 14) on normal diet at the age of 25 weeks.

(C) Representative pictures of epididymal adipose tissue paraffin sections. The scale bar represents 50 µm. Average fat cell area of control (n = 3) and AgRP^{JNK1CA} mice (n = 4) at the age of 25 weeks.

(D) Fat cell area distribution of control (n = 3) and AgRP^{JNK1CA} mice (n = 4) at the age of 25 weeks.

(E) Average ad libitum food intake at the age of 6 weeks of control (n = 12) and AgRP^{JNK1CA} mice (n = 12).

(F) Locomotor activity at the age of 6 weeks of control (n = 7) and AgRP^{JNK1CA} mice (n = 7).

(G) Energy expenditure at the age of 6 weeks of control (n = 7) and AgRP^{JNK1CA} mice (n = 7).

Displayed values are means ± SEM. *$p < 0.05$; **$p < 0.01$.

positive energy balance. These analyses revealed that daily food intake was significantly increased upon activation of JNK signaling in AgRP cells (Figure 3E), whereas locomotor activity and energy expenditure remained unaltered in AgRP^{JNK1CA} mice compared to control mice (Figures 3F and 3G). Thus, activation of JNK signaling in AgRP neurons is sufficient to promote hyperphagia and obesity development in mice.

Unaltered Glucose Homeostasis in AgRP^{JNK1CA} Mice

Insulin action in AgRP neurons is critical for the control of hepatic glucose output (Könner et al., 2007; Lin et al., 2010). Insulin activates the phosphatidylinositol 3-kinase (PI3K) cascade in AgRP neurons, which catalyzes the generation of phosphatidylinositol 3,4,5 triphosphate (PIP3) from phosphatidylinositol 4,5 diphosphate (PIP2). To examine whether JNK activation leads to the development of insulin resistance, we investigated the effect of constitutive JNK activation in AgRP neurons on insulin's ability to activate PI3K signaling in these cells by immunostaining for PIP3 in genetically marked β-galactosidase-positive AgRP neurons of mice expressing LacZ in AgRP neurons in the presence or absence of JNK1CA expression (AgRP$^{JNK1CA;LacZ}$ and AgRP^{LacZ}, respectively). In both AgRP^{JNK1CA} and control mice, insulin treatment resulted in comparable PIP3 formation in AgRP neurons, indicating that activation of JNK signaling did not cause neuronal insulin resistance in this cell type (Figures 4A and 4B). Basal levels of immunoreactive PIP3 were also indistinguishable between AgRP^{LacZ} and AgRP$^{JNK1CA;LacZ}$ mice (Figure 4C). Consistent with unaltered insulin action in AgRP neurons, systemic glucose homeostasis remained unaffected in AgRP^{JNK1CA} compared to control mice as assessed by insulin and glucose tolerance tests (Figures 4D and 4E and the body weights in Figure S3). Thus, these experiments revealed the development of leptin, but not insulin resistance, as a consequence of JNK activation in AgRP neurons.

Activation of IKK2 Signaling Increases the Firing Rate of AgRP Neurons

Because activation of JNK signaling in AgRP neurons was sufficient to induce obesity and leptin resistance, but neither caused

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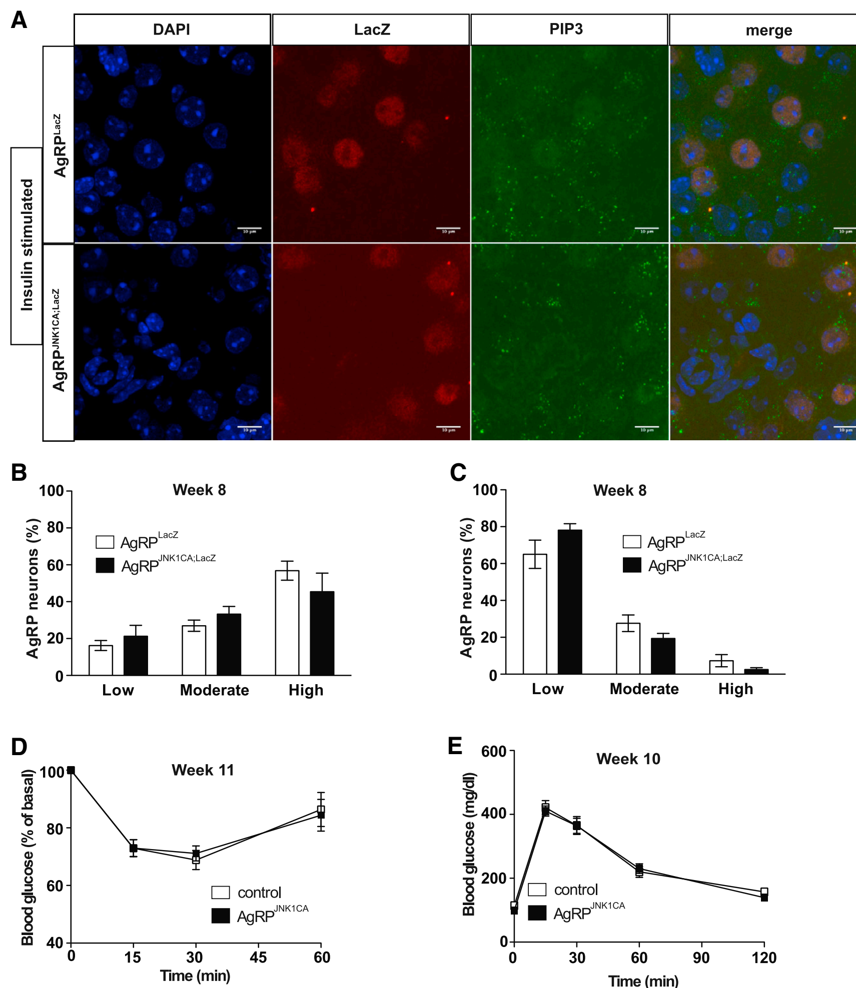

Figure 4. Unaltered AgRP-Neuronal Insulin Sensitivity and Glucose Homeostasis in AgRP^{JNK1CA} Mice

(A) Representative PIP3 immunostaining of AgRP^{LacZ} and AgRP$^{JNK1CA;LacZ}$ after intravenous insulin stimulation with 5 U of insulin. The scale bar represents 10 μm.

(B) Quantification of PIP3 levels in AgRP neurons of AgRP^{LacZ} (n = 5 mice; n = 460 AgRP neurons) and AgRP$^{JNK1CA;LacZ}$ (n = 3 mice; n = 148 AgRP neurons) mice after fasting for 16 hr and insulin stimulation (in the vena cava inferior) with 5 U of insulin for 10 min.

(C) Quantification of PIP3 levels in AgRP neurons of AgRP^{LacZ} (n = 3 mice; n = 234 AgRP neurons) and AgRP$^{JNK1CA;LacZ}$ (n = 3 mice; n = 346 AgRP neurons) mice in basal state (saline injection in the vena cava inferior) after fasting for 16 hr.

(D) Blood glucose levels as percentage of the initial blood glucose during an insulin tolerance test of control (n = 14) and AgRP^{JNK1CA} mice (n = 18).

(E) Blood glucose levels during glucose tolerance test of control (n = 9) and AgRP^{JNK1CA} (n = 11) mice. The mice were fasted for 6 hr before the injection with 20% glucose (10 ml/kg).

Displayed values are means. See also Figure S3.

neuronal insulin resistance nor impaired systemic glucose metabolism, we sought to investigate whether chronic activation of IKK2 signaling in AgRP neurons would impact on the regulation of energy homeostasis and/or glucose homeostasis. Therefore, we generated mice expressing a constitutive active IKK2 mutant (IKK2CA) (Sasaki et al., 2006) specifically in AgRP neurons (AgRP^{Cre}R26Stop$^{FL/WT}$IKK2EE, referred to as AgRP^{IKK2CA} mice). Given that, in this mouse model, IKK2EE expression is driven by the endogenous ROSA-26 promoter, which exhibits lower expression than the construct employed for expressing the JNK1CA variant, we generated homozygous mice expressing two copies of IKK2CA (AgRP$^{IKK2CA/CA}$) to achieve a more-robust expression of the IKK2CA variant.

To test the functionality of this approach, we employed pIκBα immunostaining as a readout for IKK2 activation in AgRP neurons of reporter mice expressing β-galactosidase in AgRP neurons upon Cre-mediated expression of LacZ from the ROSA26 locus. Because both the β-galactosidase reporter as well as the IKK2CA allele were expressed from the ROSA allele, we compared the degree of pIκBα immunoreactivity in control AgRP^{LacZ} mice and AgRP$^{IKK2CA;LacZ}$ mice, which express only one copy of the IKK2CA allele. Whereas approximately 45% of β-galactosidase-positive neurons displayed pIκBα immunoreactivity in control AgRP^{LacZ} mice, AgRP-specific constitutive activation of IKK2 resulted in pIκBα immunoreactivity in approximately 70% of β-galactosidase-positive neurons in AgRP$^{IKK2CA;LacZ}$ mice (Figures 5A and 5B). Constitutive IKK2 activation in AgRP neurons did not affect the total number of LacZ^{+}-AgRP cells (Figure 5C). Collectively, these experiments reveal successful activation of IKK2 signaling in AgRP neurons in mice, already in those that express only one copy of the IKK2CA allele.

Next, we performed electrophysiological recordings of genetically marked NPY^{GFP} neurons of control NPY^{GFP};IKK2CA/CA; AgRP$^{Cre-/-}$ or NPY^{GFP};AgRP$^{IKK2CA/CA}$ mice. Similar to what we had observed in NPY^{GFP};AgRP^{JNK1CA} mice, activation of IKK2 signaling in AgRP neurons increased their firing rate compared to AgRP neurons of control mice (Figures 4D–4F). Again, other basic electrophysiological properties remained unaltered (Figure S1). These experiments indicate that activation of both major inflammatory mediators JNK1 and IKK2 propagate increased activity of orexigenic AgRP/NPY neurons in the arcuate nucleus in a cell-autonomous manner.

Activation of IKK2 Signaling in AgRP Neurons Does Not Affect Leptin Sensitivity or Body Weight

In contrast to what we observed in AgRP^{JNK1CA} mice, leptin-mediated suppression of food intake was retained in AgRP$^{IKK2CA/CA}$ mice to the same degree as in control animals even at the age of 15 weeks, when AgRP^{JNK1CA} mice develop profound systemic

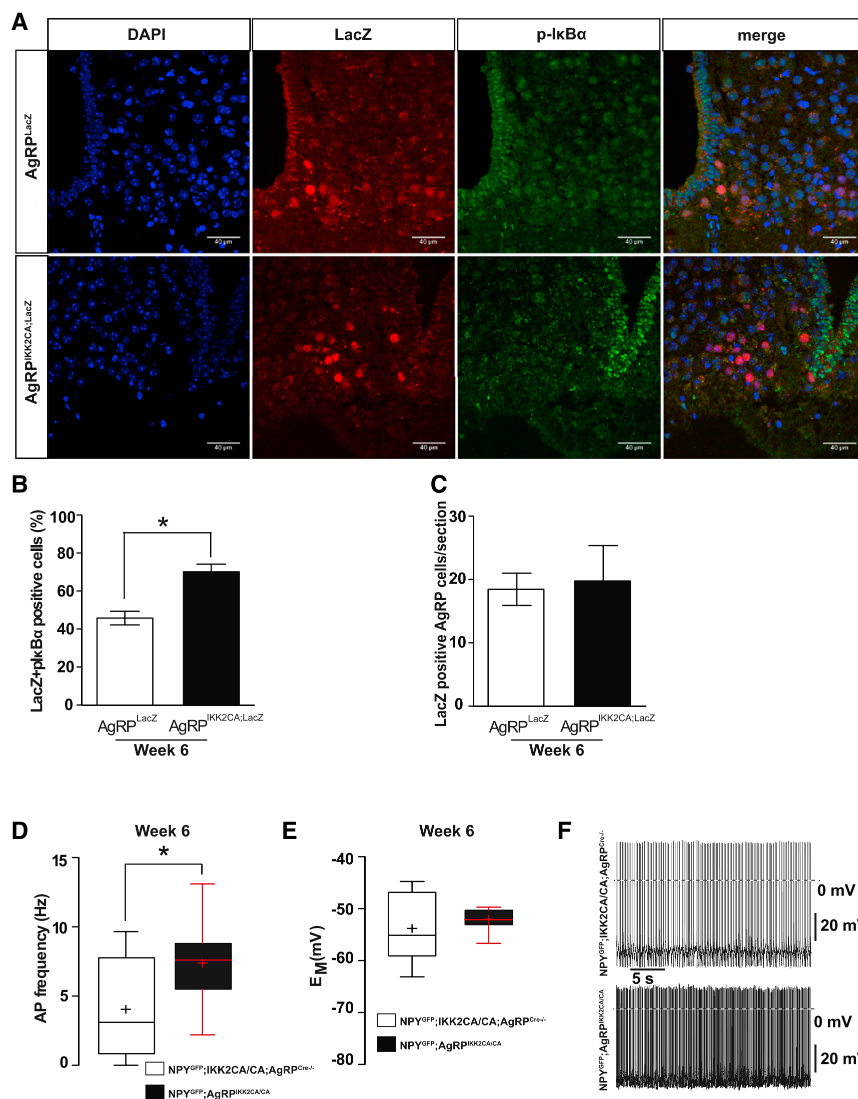

Figure 5. Activation of IKK2 Signaling Increases the Firing Rate of AgRP Neurons

(A and B) Expression of the IKK2CA protein in AgRP neurons leads to cell-specific phosphorylation of the IKK2 target IκBα in vivo. AgRP neurons were visualized by immunostaining for β-galactosidase in AgRP^{LacZ} and AgRP$^{IKK2CA;LacZ}$ reporter animals. Immunostaining for phospho-IκBα revealed 45% of the AgRP neurons positive in control reporter animals, whereas 70% of AgRP neurons in AgRP$^{IKK2CA;LacZ}$ mice showed clear immunoreactivity for p-IκBα. Quantification of p-IκBα-positive and β-galactosidase-positive AgRP neurons is depicted in (B) in control AgRP^{LacZ} (n = 3) and AgRP$^{IKK2CA;LacZ}$ (n = 3) reporter mice. The scale bar represents 50 μm.

(C) Chronic IKK2 activation does not affect AgRP neuron numbers. β-galactosidase-positive AgRP neurons were counted in control AgRP^{LacZ} (n = 3) and AgRP$^{IKK2CA;LacZ}$ (n = 3) reporter mice. No difference in neuron counts per slide was detected between genotypes.

(D) Spontaneous action potential frequencies of NPY^{GFP};IKK2CA/CA;AgRP$^{Cre-/-}$ (n = 3 mice; n = 17 AgRP neurons) and NPY^{GFP};AgRP$^{IKK2CA/CA}$ (n = 3 mice; n = 11 AgRP neurons).

(E) Membrane potentials of NPY^{GFP};IKK2CA/CA;AgRP$^{Cre-/-}$ (n = 3 mice; n = 17 AgRP neurons) and NPY^{GFP};AgRP$^{IKK2CA/CA}$ (n = 3 mice; n = 11 AgRP neurons).

(F) Representative recordings of spontaneous action potential frequencies of NPY^{GFP};IKK2CA/CA;AgRP$^{Cre-/-}$ and NPY^{GFP};AgRP$^{IKK2CA/CA}$ mice at the age of 6 weeks. Displayed values are means ± SEM. *p < 0.05.

leptin resistance (Figures 6A and 6B). Taken together, activating IKK2 signaling in AgRP neurons does not interfere with body weight maintenance or leptin sensitivity, in contrast to what is observed upon activation of JNK signaling in the same neurons.

Upon validation of the functionality of the constitutively active IKK2 variant expressed in AgRP neurons, we performed a metabolic characterization of AgRP$^{IKK2CA/CA}$ and their respective control mice. In contrast to what we had observed in AgRP^{JNK1CA} mice, AgRP$^{IKK2CA/CA}$ mice did not develop increased body weight or adiposity (Figures 6C and 6D) despite a slightly elevated food intake compared to controls (Figure 6E). Similarly, locomotor activity and energy expenditure remained unaltered between genotypes (Figures 6F and 6G).

Impaired Glucose Homeostasis in AgRP$^{IKK2CA/CA}$ Mice

Despite unaltered body weight and adiposity, AgRP$^{IKK2CA/CA}$ mice displayed impaired insulin sensitivity and mild glucose intolerance as compared to control mice (Figures 7A and 7B). Because glucose homeostasis critically depends on the ability of insulin to activate PI3K in AgRP neurons, leading to subsequent membrane hyperpolarization of these neurons, we investigated the ability of insulin to activate PI3K signaling in AgRP neurons of control AgRP^{LacZ} mice and AgRP$^{IKK2CA;LacZ}$ mice. Whereas intravenous insulin stimulation induced high levels of immunoreactive PIP3 in approximately 60% of AgRP neurons in control mice, this proportion was significantly reduced to 40% in mice expressing only one IKK2CA allele (Figures 7C and 7D). Of note, at basal levels, the amount of PIP3 formation was also indistinguishable between AgRP$^{IKK2CA;LacZ}$ and control mice (Figure 7E). Thus, activating IKK2 signaling in AgRP neurons causes cell-autonomous insulin resistance and subsequently impairs systemic insulin sensitivity in the absence of altered body weight regulation.

DISCUSSION

Hypothalamic inflammation is recognized as a critical feature of diet-induced neuronal leptin and insulin resistance (Zhang et al., 2008; Belgardt et al., 2010; Thaler et al., 2012). Whereas hypothalamic activation of inflammatory pathways including that of JNK- and IKK2-mediated signaling had been reported early upon high-fat feeding of mice (De Souza et al., 2005), the

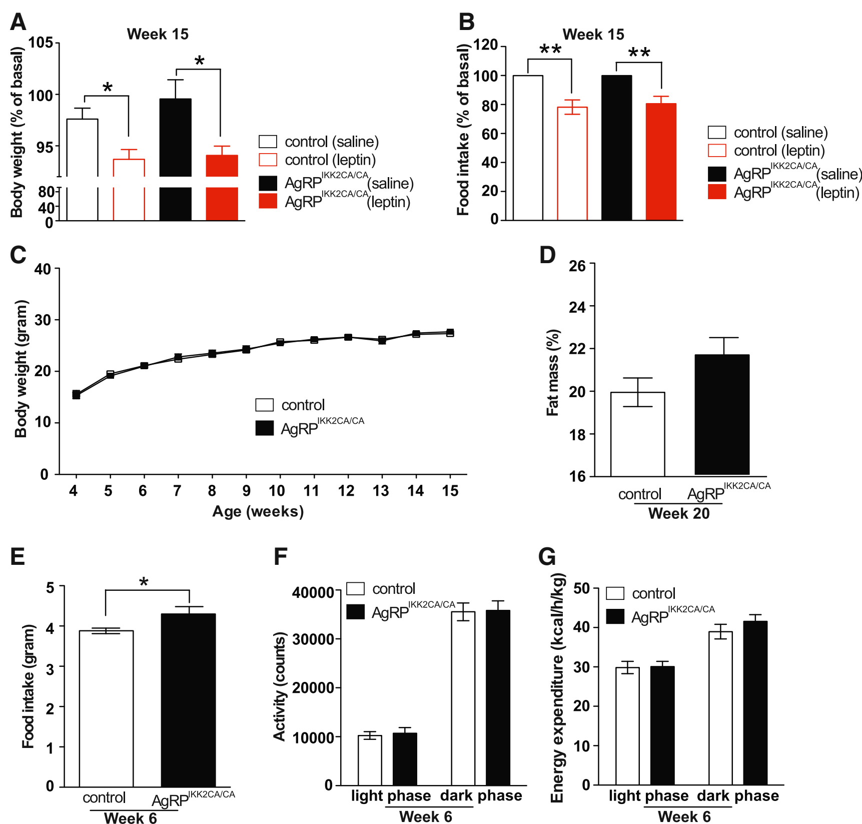

Figure 6. Chronic Activation of IKK2 Signaling in AgRP Neurons Does Not Affect Body Weight and Leptin Sensitivity

(A) Changes in body weight after intraperitoneal leptin treatment in control (n = 7) and AgRP^{JNK1CA} (n = 7) mice at 15 weeks of age. Data represent percentage of basal body weight (body weight in the beginning of each treatment) after a 3-day (72 hr) treatment with twice-daily injections (12 hr apart) of saline followed by a 3-day treatment with twice-daily injections of 2 mg/kg leptin.

(B) Changes in food intake after intraperitoneal leptin treatment in control (n = 5) and AgRP^{JNK1CA} (n = 5) mice at 15 weeks of age. Data represent daily food intake after a 3-day (72 hr) treatment with twice-daily injections (12 hr apart) of saline followed by a 3-day treatment with twice-daily injections of 2 mg/kg leptin.

(C) Average body weight of control (n = 20) and AgRP$^{IKK2CA/CA}$ mice (n = 20) on normal chow diet.

(D) Body fat as measured by nuclear magnetic resonance analysis of control mice (n = 8) and AgRP$^{IKK2CA/CA}$ mice (n = 7) on normal chow diet at the age of 20 weeks.

(E) Average ad libitum food intake of control (n = 10) and AgRP$^{IKK2CA/CA}$ mice (n = 7) at the age of 6 weeks.

(F) Locomotor activity of control (n = 20) and AgRP$^{IKK2CA/CA}$ mice (n = 19).

(G) Energy expenditure at the age of 6 weeks of control (n = 20) and AgRP$^{IKK2CA/CA}$ mice (n = 19) at the age of 6 weeks.

Displayed values are means ± SEM. *p < 0.05; **p < 0.01.

functional significance of this phenomenon remained unclear, because it was conceivable that hypothalamic inflammation could contribute to the development of CNS insulin and/or leptin resistance or maybe even represent a homeostatic, compensatory mechanism to counteract the consequences of obesity. Nevertheless, recent experiments demonstrating that attenuation of neuronal IKK2, JNK1, TLR, and ER stress signaling protect from the development of diet-induced obesity (Zhang et al., 2008; Kleinridders et al., 2009; Belgardt et al., 2010) indicate that hypothalamic inflammation at least chronically contributes to the manifestation of the metabolic syndrome.

However, the contribution of different cell types that reside in the hypothalamus, such as astrocytes, microglia, stem cells, endothelial cells, and neurons, to the initiation and manifestation of hypothalamic inflammation is only partially understood (García-Cáceres et al., 2013; Gao et al., 2014; Purkayastha and Cai, 2013; Gosselin and Rivest, 2008). Moreover, due to the diversity and partial functional antagonism of distinct hypothalamic neurons, we sought to first clarify the role of inflammatory signaling in AgRP neurons of the arcuate nucleus in the manifestation of the metabolic syndrome. To this end, the present study clearly reveals that activation of JNK and IKK2 in these cells is sufficient to initiate key features of the metabolic syndrome, even in the absence of additional environmental triggers, such as high-fat feeding. At the same time, our results demonstrate that activation of inflammatory signaling pathways in neurons is a crucial determinant in the pathogenesis of obesity and impaired insulin sensitivity, even in the absence of altered microglia function.

Interestingly, our experiments reveal common and distinct consequences of AgRP-neuron-restricted JNK versus IKK2 activation. Activation of either inflammatory signaling branch results in increased firing of AgRP neurons. In fact, both JNK and IKK2 have been demonstrated to regulate glutamatergic signaling and synapse maturation, raising the possibility that both inflammatory kinases promote AgRP neuron excitability via similar or distinct pathways (Thomas et al., 2008; Bockhart et al., 2009; Ahn and Choe, 2010; Schmeisser et al., 2012). Interestingly, despite the fact that, in both models, AgRP firing is increased by ∼2-fold (Figure S1I), only the AgRP^{JNK1CA} mice develop obesity. As optogenetic stimulation has clearly defined that robustly increasing AgRP neuron firing evokes voracious feeding, our experiments reveal that slight increases in AgRP firing per se do not cause obesity. Despite increased firing at young age, as detected in our animals, compensatory mechanisms might normalize electrical activity of these cells in long term despite increased JNK and IKK2 activity. Ultimately, we cannot rule out that yet unidentified cellular heterogeneity of AgRP neurons—as observed for POMC neurons—may contribute to these differential outcomes (Williams et al., 2010).

Despite the similar alterations of neuronal firing upon JNK and IKK2 activation, only activating JNK in AgRP neurons promotes initially cellular and subsequently systemic leptin resistance.

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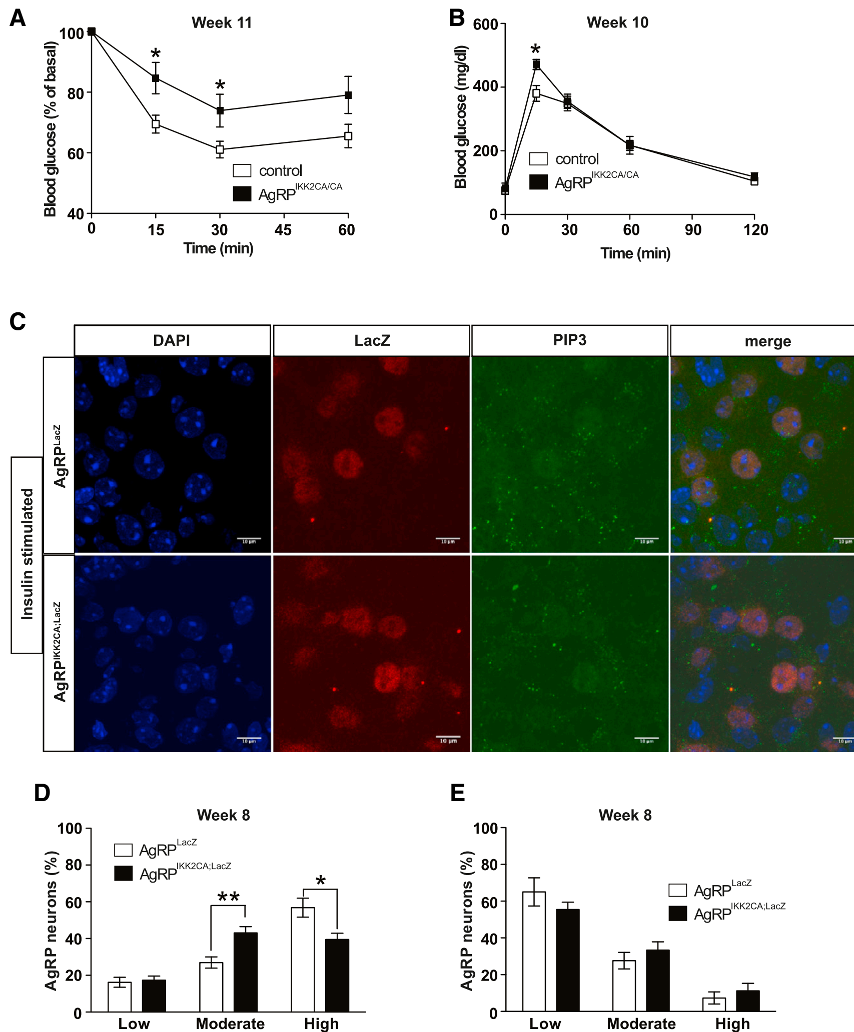

Figure 7. Impaired Glucose Homeostasis in AgRP$^{IKK2CA/CA}$ Mice

(A) Blood glucose levels as percentage of the initial blood glucose during an insulin tolerance test of control (n = 21) and AgRP$^{IKK2CA/CA}$ (n = 20).

(B) Blood glucose levels during glucose tolerance test of control (n = 8) and AgRP$^{IKK2CA/CA}$ (n = 7). The mice were fasted for 6 hr before the experiment.

(C) Representative PIP3 immunostaining of AgRP^{LacZ} (same as depicted in Figure 4A) and AgRP$^{IKK2CA;LacZ}$ mice after intravenous insulin stimulation with 5 U of insulin. The scale bar represents 10 μm.

(D) Quantification of PIP3 levels in AgRP neurons of AgRP^{LacZ} (n = 5 mice; n = 460 AgRP neurons; same as depicted in Figure 4B) and AgRP$^{IKK2CA;LacZ}$ (n = 5 mice; n = 357 AgRP neurons) mice after fasting for 16 hr and insulin stimulation (in the vena cava inferior) with 5 U of insulin for 10 min.

(E) Quantification of PIP3 levels in AgRP neurons of AgRP^{LacZ} (n = 3 mice; n = 234 AgRP neurons; same as depicted in Figure 4C) and AgRP$^{IKK2CA;LacZ}$ (n = 3 mice; n = 256 AgRP neurons) mice in basal state (saline injection in the vena cava inferior) after fasting for 16 hr.

Displayed values are means ± SEM. *p < 0.05; **p < 0.01.

These findings are consistent with reports that mice with targeted disruption of leptin receptor signaling in the AgRP neurons develop hyperphagia and obesity (van de Wall et al., 2008). Moreover, AgRP neurons have been reported to be one of the first hypothalamic neuronal populations to sense changes in plasma metabolic signals and develop cellular leptin resistance (Olofsson et al., 2013), possibly as a consequence of activating inflammatory signaling cascades, i.e., JNK. Indeed, it has been demonstrated that JNK activation can promote SOCS-3 expression, a well-characterized inhibitor of leptin action (Qin et al., 2007; Bjørbaek et al., 1999; Howard et al., 2004). On the other hand, JNK activation in AgRP neurons fails to promote cell-autonomous and systemic insulin resistance, despite the fact that JNK-dependent serine phosphorylation of IRS-1 has been proposed to cause insulin resistance, at least in vitro (Aguirre et al., 2002). However, mice with a mutation of IRS-1 serine 307 to alanine, which prevents this phosphorylation, are surprisingly more insulin resistant under high-fat diet conditions than their control littermates (Copps et al., 2010). These results indicate that JNK activation does not necessarily result in attenuation of insulin signaling via IRS-1 ser307 phosphorylation in vivo, consistent with what we observe in AgRP neurons.

On the other hand, activation of IKK2 in AgRP neurons causes cell-autonomous and systemic insulin resistance in the absence of leptin resistance and obesity. In fact, this observation is consistent with the notion that disruption of insulin signaling in the AgRP neurons impairs systemic insulin sensitivity through impairing insulin's ability to suppress hepatic glucose production but without affecting body weight regulation (Könner et al., 2007). In contrast, previous studies had shown that ablation of IKK2 from the CNS was sufficient to largely reduce the development of obesity and leptin resistance upon high-fat diet feeding (Zhang et al., 2008). This points to the possibility that IKK2 acts in other neurons to promote weight gain and leptin resistance, potentially through its action on hypothalamic neurogenesis (Li et al., 2012).

In summary, our results indicate that both JNK1 and IKK2 inflammatory pathways are sufficient to impair aspects of energy and glucose metabolism when activated in AgRP neurons, clearly assigning a functional role of these signaling pathways in this cell type to the manifestation of the metabolic syndrome.

EXPERIMENTAL PROCEDURES

Animal Care

All animal procedures were conducted in compliance with protocols approved by local government authorities (Bezirksregierung Köln) and were in accordance with NIH guidelines. Mice were housed in groups of 3–5 at

22°C–24°C using a 12 hr light/12 hr dark cycle. Animals were fed normal chow diet (Teklad Global Rodent 2018; Harlan) containing 53.5% carbohydrates, 18.5% protein, and 5.5% fat (12% of calories from fat) or high-fat diet (only for the experiment of Figure 1A; HFD; C1057; Altromin) containing 32.7% carbohydrates, 20% protein, and 35.5% fat (55.2% of calories from fat). Animals had ad libitum access to water at all times, and food was only withdrawn if required for an experiment. Body weight was measured once a week.

Generation of Mice

Only male mice were used in these studies to avoid the effect of different stages of estrous cycle on glucose homeostasis. NPY^{GFP}, $R26Stop^{FL}$JNK1CA, $R26Stop^{FL}$IKK2CA, Z/EG, LacZ, and $AgRP^{Cre}$ mice have been described previously (Pal et al., 2013; Sasaki et al., 2006; Novak et al., 2000; Tong et al., 2008). All mouse strains had been crossed onto a C57B6/N background for at least three generations, and littermate controls were used at all times except in reporter animal breedings for the AgRP. Instead, we crossed $AgRP^{LacZ}$ transgenic mice with $R26Stop^{FL/FL}$IKK2CA.

Analytical Procedures

Blood glucose values were determined from whole venous blood using an automatic glucose monitor (Contour; Bayer). Serum hormones were measured as recently described using commercially available ELISAs (Belgardt et al., 2010).

Indirect Calorimetry, Physical Activity, and Food Intake

All measurements were performed in a PhenoMaster System (TSE systems), which allows measurement of metabolic performance and activity monitoring by an infrared light-beam frame. Mice were placed at room temperature (22°C–24°C) in 7.1 l chambers of the PhenoMaster open circuit calorimetry. Mice were allowed to acclimatize in the chambers for at least 24 hr. Food and water were provided ad libitum in the appropriate devices and measured by the built-in automated instruments. Locomotor activity and parameters of indirect calorimetry were measured for at least the following 48 hr. Presented data are average values obtained in these recordings.

Body Composition

Nuclear magnetic resonance was employed to determine body composition using the NMR Analyzer minispeq mq7.5 (Bruker Optik).

Perforated Patch Recordings

AgRP neurons were recorded at room temperature under current and voltage clamp in the perforated patch configuration using an EPC10 patch-clamp amplifier (HEKA). For detailed information, see the Supplemental Experimental Procedures.

Data Analysis and Statistical Methods

Data analysis was performed with Igor Pro 6 (Wavemetrics) and Graphpad Prism (version 5.0b; Graphpad Software). Numerical values are given as mean ± SE. Box plots are generated according to Tukey; means are reflected by "+" and medians by the dash, respectively. To determine differences in means, t tests were performed. A significance level of 0.05 was accepted for all tests. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$ versus controls.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.10.045.

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