Published in final edited form as:
Cell Metab. 2011 March 2; 13(3): 331–339. doi:10.1016/j.cmet.2011.01.016.

Induction of Leptin Resistance by Activation of cAMP-Epac Signaling

Makoto Fukuda[1,*], **Kevin W. Williams**[1], **Laurent Gautron**[1], and **Joel K. Elmquist**[1]
[1]Division of Hypothalamic Research, Departments of Internal Medicine and Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA

SUMMARY

Leptin regulates energy balance and glucose homeostasis. Shortly after leptin was identified, it was established that obesity is commonly associated with leptin resistance, though the molecular mechanisms remain to be identified. To explore potential mechanisms of leptin resistance, we employed organotypic brain slices to identify candidate signaling pathways that negatively regulate leptin sensitivity. We found that elevation of adenosine 3′, 5′-monophosphate (cAMP) levels impairs multiple signaling cascades activated by leptin within the hypothalamus. Notably, this effect is independent of protein kinase A activation. In contrast, activation of Epac, a cAMP-regulated guanine nucleotide exchange factor for the small G protein Rap1, was sufficient to impair leptin signaling with concomitant induction of SOCS-3 expression. Epac activation also blunted leptin-induced depolarization of hypothalamic POMC neurons. Finally, central infusion of an Epac activator blunted the anorexigenic actions of leptin. Thus, activation of hypothalamic cAMP-Epac pathway is sufficient to induce multiple indices of leptin resistance.

INTRODUCTION

Obesity arises when energy intake chronically exceeds energy expenditure. Obesity is associated with several comorbidities, including type 2 diabetes mellitus, several types of cancer, and cardiovascular disease (Gaede et al., 2008; Stamler et al., 1993; Van Gaal et al., 2006). Reduction in body weight has a beneficial impact on a number of metabolic and cardiovascular risk factors. Thus, development of effective strategies to fight obesity will reduce the incidence of a myriad of diseases. Leptin is a hormone that plays a central role in the regulation of energy balance and glucose homeostasis via activation of leptin receptors, particularly within the central nervous system (Bjørbaek and Kahn, 2004; Flier, 2006; Friedman, 2004; Zhang et al., 1994). Leptin administration decreases food intake, reduces body weight, and increases systemic insulin sensitivity when administrated to lean humans and animals. However, nearly all forms of obesity are associated with higher levels of circulating leptin (Considine et al., 1996; Frederich et al., 1995; Maffei et al., 1996). Thus, obese humans and animals display a decreased response to endogenous and exogenous leptin. This has been demonstrated by several measures, including attenuated anorectic responses, reduction of both signal transducers and activators of transcription (STAT) 3 phosphorylation, and neuro-peptide release after leptin administration (Bjørbaek and Kahn, 2004; Enriori et al., 2007; Flier, 2006; Friedman, 2004; Münzberg et al., 2004; Zhang et al.,

*Correspondence: makoto.fukuda@utsouthwestern.edu.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at doi:10.1016/j. cmet.2011.01.016.

1994). Thus, understanding the molecular mechanisms underlying and developing strategies to combat “leptin resistance” is a major goal of obesity research.

Cellular leptin resistance may be mediated by negative regulatory pathways of leptin receptor signaling. Indeed, recent studies have identified molecules that act as negative regulators of leptin signaling. These include suppressor of cytokine signaling-3 (SOCS-3) (Bjørbaek et al., 1999; Howard et al., 2004; Mori et al., 2004), protein tyrosine phosphatase 1B (PTP1B) (Banno et al., 2010; Bence et al., 2006; Cook and Unger, 2002; Zabolotny et al., 2002), and inflammatory signals such as IKKβ/NFκB and ER stress (Ozcan et al., 2009; Zhang et al., 2008). However, the signaling networks that confer central leptin resistance remain to be fully established. Therefore, delineation of cellular signaling networks responsible for leptin sensitivity is a high priority.

Accumulating evidence suggests that adenosine 3′, 5′-monophosphate (cAMP) generally suppresses proinflammatory cytokine signaling (Serezani et al., 2008). Numerous studies have demonstrated that cAMP plays a role as a downregulator of IL-6 signaling which uses JAK-STAT3 signaling (Bousquet et al., 2001; Delgado and Ganea, 2000; Fasshauer et al., 2002; Gasperini et al., 2002; Sands et al., 2006). Previous studies showed that leptin reduces cAMP levels through activation of phosphodiesterase 3B in pancreatic beta cells (Zhao et al., 1998) and in the brain (Sahu and Metlakunta, 2005; Zhao, 2005; Zhao et al., 2002). Leptin and its receptor are structurally and functionally related to the proinflammatory cytokine IL-6 cytokine family (Tartaglia, 1997). Thus, we hypothesized that cAMP-related signal might interfere with leptin signaling pathways and could be involved in central leptin resistance.

Classically, cAMP acts through cAMP-dependent protein kinase (PKA) (Daniel et al., 1998). However, it is unsettled whether or not PKA mediates the inhibitory effects of cAMP on the JAK-STAT3 pathway (Bousquet et al., 2001; Gasperini et al., 2002; Sands et al., 2006). cAMP also acts through Epac (exchange protein directly activated by cAMP), the guanine nucleotide-exchange factor for the small GTPase Rap1(de Rooij et al., 1998; Gloerich and Bos, 2010). Notably, Epac-mediated activation of Rap1 induces SOCS-3 expression in endothelial cells (Sands et al., 2006). However, the molecular pathway linking cAMP to the JAK-STAT3 pathway has not yet been established.

To facilitate the identification of possible signaling pathways that contribute to leptin resistance, we established an in vitro system of leptin action in the hypothalamus. We used an organotypic slice culture system, which enabled a direct assessment and manipulation of candidate molecular pathways inducing leptin resistance within hypothalamic neurons. By employing this system, we investigated whether activation of cAMP-dependent pathways induced leptin resistance in our hypothalamic slice model.

RESULTS AND DISCUSSION

Signaling from the Leptin Receptor in Organotypic Hypothalamic Slices

Organotypic slices of mouse hypothalamus were prepared and then maintained at an air-media interface on Millicell-CM filters in MEM base medium for 10 days. We first validated the model system by assessing the effects of leptin treatment of the slices. We found that leptin induced robust phosphorylation of STAT3 (Figures 1A and 1B). In contrast, no STAT3 phosphorylation was observed in the saline-treated slices (Figures 1 A and 1B), as shown by both immunohistochemistry and western blot analysis with an anti-phospho-STAT3 antibody. Notably, leptin-induced phosphorylation of STAT3 was only seen in hypothalamic nuclei such as arcuate nucleus (Arc) and the ventromedial hypothalamus (VMH), known to express leptin receptors (Elmquist et al., 2005; Scott et al.,

2009) (Figure 1A). Treatment of organotypic slices with leptin also led to phosphorylation of S6K (Figure 1B), another mediator of leptin action (Cota et al., 2006). We confirmed that leptin-induced STAT3 phosphorylation is mediated via the leptin receptor, by using slices prepared from the leptin receptor null (*Lepr*$^{neo/neo}$ mice) mice (Figures 1D and 1E) (Coppari et al., 2005). Collectively, these results demonstrate that the in vitro system is a model of leptin-induced STAT 3 and S6K activation. We also tested whether this model system was able to recapitulate leptin-induced leptin resistance (Myers et al., 2010). We found that pretreatment of slices with higher levels of leptin (120 nM for 6 hr) strongly impaired leptin-induced pSTAT3 (Figure S1 available online). This indicates that an in vitro system can mimic leptin-induced leptin resistance.

Cyclic AMP Impairs Leptin Signaling in Hypothalamic Neurons

To investigate whether cAMP impairs the leptin signaling pathways in the hypothalamus, we utilized pharmacological reagents to modulate cellular cAMP levels in slices. Interestingly, we found that treatment of the slices with both forskolin (an adenylate cyclase activator; Fsk [20 μM]) plus low level of leptin (0.5 nM)) (Fsk/Lep) had a potent inhibitory effect on leptin-induced STAT3 phosphorylation in the whole hypothalamus (Figures 2A–2D) and in the arcuate nucleus (Figure 2G). This was in contrast to the robust STAT3 phosphorylation observed in control slices (Figure 2A). Forskolin alone clearly blunted leptin-induced STAT3 phosphorylation (Figure 2B), and this effect was enhanced by the presence of low levels of leptin (0.5 nM) (Figure 2B). Treatment of the slices with the low dose of leptin (0.5 nM) alone had little inhibitory effect on leptin-STAT3 signaling as this dose did not elicit STAT3 phosphorylation at either 30 min or 6 hr (Figure 2B).

To further investigate potential signaling pathways involved, we used two different types of phosphodiesterase (PDE) inhibitors known to elevate intracellular cAMP levels: IBMX, a nonspecific inhibitor of PDE, and cilostamide, a selective inhibitor for PDE3. Both PDE inhibitors enhanced the inhibitory effects of forskolin on leptin-induced STAT3 phosphorylation (Figure 2C). We also found that Fsk/Lep also dampened the leptin-induced phosphorylation of S6 Kinase (Figure 2D), a response that is also impaired in the high-fat diet-induced obese rodents (Cota et al., 2008). Notably, Fsk/Lep treatment increased hypothalamic SOCS-3 and PTP1B at the levels of protein and messenger RNA (mRNA) (Figures 2E and 2F), both of which have been demonstrated to contribute to leptin resistance (Bence et al., 2006; Bjørbaek et al., 1999; Cook and Unger, 2002; Howard et al., 2004; Mori et al., 2004; Zabolotny et al., 2002).

Cyclic AMP-Induced Inhibitory Effect Is Independent of PKA

We next dissected potential downstream pathways mediating the inhibitory effects of Fsk/Lep on signaling from the leptin receptor. Classically, cAMP exerts many of its effects through protein kinase A (PKA) (Daniel et al., 1998). Thus, we tested whether the inhibitory action of cAMP is mediated in a PKA-dependent manner. Treatment of slices with an inhibitor of PKA, H89, had no effect on cAMP inhibition of leptin-induced phosphorylation of STAT3 and S6K (Figure 2H). Fsk/Lep also induced hypothalamic SOCS-3 in the presence of H 89, a PKA inhibitor (Figure 2I). These results suggest that elevations of cAMP negatively regulate leptin signaling in a PKA-independent manner.

Activation of Epac Blunts Leptin Signaling in the Hypothalamus

In addition to the PKA pathway, increased cAMP also activates an alternative pathway via Epac, a guanine nucleotide exchange factor, which activates a small G protein Rap1 (de Rooij et al., 1998). We found that Fsk/Lep treatment activated endogenous Rap1 in the hypothalamus (Figure S3). Thus, we next examined whether activation of the Epac-Rap1 pathway is sufficient to evoke an inhibitory effect on leptin receptor signaling. We used a

hydrolysis-resistant Epac activator [8-(4-chlorophenylthio)-2′-O-methyladenosine-3′, 5′-cyclic monophosphorothioate, 8-pCPT-2′-O-Me-cAMP] that selectively binds and activates Epac (Enserink et al., 2002). Treatment of the slices with 8-pCPT-2′-O-Me-cAMP plus low level of leptin (8-pCPT-2′-O-Me-cAMP/Lep) impaired leptin-induced phosphorylation of STAT3 (Figures 3A and 3B). Either leptin or 8-pCPT-2′-O-Me-cAMP alone had little effect on leptin-dependent pSTAT3 phosphorylation (Figure 3C). Epac activation also blunted the ability of leptin to modulate other cellular signaling of leptin: phosphorylation of S6K (Figures 3D and 3E) within the hypothalamus. Further, we found that 8-pCPT-2′-O-Me-cAMP induced SOCS-3 and PTP1B (Figures 3F and 3G). This induction occurred in a dose-dependent manner in the presence of low level of leptin, which alone had no effect on induction of either protein (Figure 3H). Collectively, these data suggest that activation of cAMP-Epac/Rap1 impairs hypothalamic leptin receptor signaling.

Cyclic AMP Does Not Interfere with CNTF Phosphorylation of STAT3 in the Hypothalamus

Recent studies have shown that ciliary neurotrophic factor (CNTF) and leptin have similar anorectic effects (Gloaguen et al., 1997) by modulating similar intracellular signaling cascades. In addition, CNTF can activate leptin-like signaling pathways and can reduce body weight in leptin-resistant obesity (Gloaguen et al., 1997; Lambert et al., 2001). To determine whether the cAMP-Epac pathway is specifically involved in leptin resistance, we assessed whether CNTF induction of STAT3 phosphorylation was blunted by activation of Epac. We found that CNTF caused STAT3 phosphorylation in hypothalamic slices even after Fsk/Lep pretreatment of the slices (Figure S4A). cAMP signaling has been reported to interfere with interleukin 6 (IL-6)-STAT3 signaling in different cell lines (Bousquet et al., 2001; Delgado and Ganea, 2000; Fasshauer et al., 2002; Gasperini et al., 2002; Sands et al., 2006). Thus, we tested whether elevation of cAMP impaired IL6-induced STAT3 phosphorylation in our system. As expected, Fsk/Lep pretreatment blocked IL-6 dependent STAT3 phosphorylation (Figure S4B).

Activation of the cAMP-Epac Pathway Impairs Leptin-Induced Depolarization of POMC Neurons

We also employed electrophysiological approach to assess the potential inhibitory effects of cAMP-Epac signaling on leptin's cellular actions. We assessed the ability of leptin to directly activate pro-opiomelanocortin (POMC) neurons, which are identified targets of leptin. In order to identify POMC neurons for whole-cell patch-clamp recordings, we used POMC-GFP mice (Parton et al., 2007; Ramadori et al., 2008). Whole-cell patch-clamp recordings were performed to assess the effects of leptin on membrane potential. In agreement with previous reports (Cowley et al., 2001; Hill et al., 2008; Williams et al., 2010), leptin caused rapid depolarization from rest in 8 of 12 POMC neurons in organotypic slices (6.4 ± 0.6 mV; resting membrane potential, −51.7 ± 1.3 mV; n = 8; Figures 4A and 4B). We next used organotypic slices from POMC-GFP mice that were pretreated for 6 hr with either Fsk/Lep or 8-pCPT-2′-O-Me-cAMP/Lep. We found that pretreatment with Fsk/Lep prevented the leptin induced depolarization in all POMC neurons examined (0.8 ± 0.3 mV, n = 13; resting membrane potential, −51.3 ± 2.3 mV; n = 13; Figure 4A and 4B). Similarly, leptin failed to depolarize POMC neurons of slices pretreated with 8-pCPT-2′-O-Me-cAMP/Lep (0.6 ± 0.6 mV; resting membrane potential, −50.3 ± 1.0 mV; n = 12; Figures 3A and 3B). Notably, the low dose of leptin alone failed to inhibit the leptin-induced depolarization of POMC neurons (5.8 ± 0.6mV; resting membrane potential, −52.2 ± 1.5mV; Figures 4A and 4B). Additionally, in the presence of tetrodotoxin, which blocks action potential-mediated synaptic transmission, Fsk/Lep still prevented leptin-induced depolarization of POMC neurons (0.5 ± 0.5mV; resting membrane potential, −49.9 ± 1.3mV; Figures 4A and 4B). Collectively, these data suggest that activation of cAMP-Epac

signaling directly desensitizes POMC neurons to leptin, which is independent of action potential-mediated synaptic transmission.

Activation of Brain Epac-Rap1 Pathway Causes Central Leptin Resistance In Vivo

To provide further insights to the functional significance of the Epac pathway as it relates to leptin signaling in vivo, we tested whether activation of the Epac pathway impairs the ability of exogenous leptin to inhibit food intake. We performed intracerebroventricular (ICV) infusions of a selective Epac activator (8-pCPT-2′-O-Me-cAMP) in chow fed lean mice. We first identified a dose of 8-pCPT-2′-O-Me-cAMP (5 μg) that did not alter food intake. We next evaluated the ability of ICV leptin injections to suppress food intake following an ICV pretreatment with this dose of the Epac activator. As expected, leptin treatment markedly reduced food intake in mice pretreated with vehicle (Figure 4C). In contrast, in the mice pretreated with the Epac activator leptin did not significantly reduce food intake 4 hr after leptin injection (Figure 4C). These results suggest that the acute anorexic responses to exogenous leptin are blunted by prior central infusions of an activator of the Epac-Rap1 pathway. Finally, we assessed Rap1 activity in the hypothalamus of obese mice after chronic exposure to a high-fat diet. We found that hypothalamic Rap1 activity is increased in the mice fed on high fat diet for 4 weeks (Figure 4D), suggesting that the hypothalamic Epac-Rap1 pathway is activated in the mice exposed to high fat.

Collectively, our in vitro and in vivo results support the model that activation of the cAMP-Epac pathway induces cellular leptin resistance within hypothalamic neurons, a critical site of leptin action. Activation of the cAMP-Epac pathway blunted signaling pathways downstream from the leptin receptor, including JAK-STAT3, mTOR-S6K, ERK, and AMPK. Thus, signaling systems downstream of the leptin receptor are negatively regulated by the cAMP-Epac pathway. Notably, activation of Epac induced SOCS-3 and PTP1B, two negative regulators of leptin signaling in vivo (Banno et al., 2010; Bence et al., 2006; Bjørbaek et al., 1999; Cook and Unger, 2002; Howard et al., 2004; Mori et al., 2004; Zabolotny et al., 2002). Our study is also consistent with a previous report that cilostamide, an inhibitor for phosphodiesterase 3B, inhibited leptin-induced suppression of food intake and STAT3 phosphorylation (Zhao et al., 2002). Taken together, our findings provide evidence linking the cAMP-Epac pathway to central leptin resistance, as seen in obesity.

One implication of our results is that leptin resistance may be induced by a broad range of extracellular stimuli that can activate the cAMP-Epac-Rap1 pathway. For example, any G protein-coupled receptors that act through either Gs or Gi would be a potential target to perturb intracellular cAMP levels (Neves et al., 2002). Thus, we tested whether α-MSH, an agonist of melanocortin receptors MC3R and MC4R impairs leptin-induced pSTAT3, since MC3R and MC4R activate Gs and are expressed in POMC and NPY neurons (Cone, 2005). However, we did not detect an inhibitory effect of α-MSH pretreatment on subsequent leptin signaling in our slices (data not shown). Thus, to date we have not found a “physiological” regulator that drives both activation of the pathway and leptin resistance. However, our findings support the notion that cAMP-Epac signaling may contribute leptin resistance in obesity, as we found elevations of Epac signaling in the hypothalamus of the mice fed with high-fat diet. Clearly, further studies are required to determine whether the Epac-Rap1 pathway is required to maintain leptin sensitivity in vivo. Nonetheless, perturbation of Epac-Rap1 signaling may contribute to the pathophysiology of leptin resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors gratefully acknowledge Michelle Choi and Danielle Lauzon for technical assistance, Claudia Vianna and Yong Xu for comments on the manuscript, and Philipp E. Scherer for interpretive assistance. This work was supported by National Institutes of Health grants PL1 DK081182 and UL1 RR024923; by R01DK53301, R01MH61583, RL1DK081185, (to J.K.E.), and by 1F32DK077487 and K01 DK087780 (to K.W.W.); by the American Diabetes Association and a Smith Family Foundation Pinnacle Program Project Award to J.K.E.; and by Scientist Development Grant from the American Heart Association to M.F.

References

Banno R, Zimmer D, De Jonghe BC, Atienza M, Rak K, Yang W, Bence KK. PTP1B and SHP2 in POMC neurons reciprocally regulate energy balance in mice. J Clin Invest. 2010; 120:720–734. [PubMed: 20160350]

Bence KK, Delibegovic M, Xue B, Gorgun CZ, Hotamisligil GS, Neel BG, Kahn BB. Neuronal PTP1B regulates body weight, adiposity and leptin action. Nat Med. 2006; 12:917–924. [PubMed: 16845389]

Bjørbaek C, Kahn BB. Leptin signaling in the central nervous system and the periphery. Recent Prog Horm Res. 2004; 59:305–331. [PubMed: 14749508]

Bjørbaek C, El-Haschimi K, Frantz JD, Flier JS. The role of SOCS-3 in leptin signaling and leptin resistance. J Biol Chem. 1999; 274:30059–30065. [PubMed: 10514492]

Bousquet C, Chesnokova V, Kariagina A, Ferrand A, Melmed S. cAMP neuropeptide agonists induce pituitary suppressor of cytokine signaling-3: novel negative feedback mechanism for corticotroph cytokine action. Mol Endocrinol. 2001; 15:1880–1890. [PubMed: 11682619]

Cone RD. Anatomy and regulation of the central melanocortin system. Nat Neurosci. 2005; 8:571–578. [PubMed: 15856065]

Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ, Bauer TL, et al. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N Engl J Med. 1996; 334:292–295. [PubMed: 8532024]

Cook WS, Unger RH. Protein tyrosine phosphatase 1B: a potential leptin resistance factor of obesity. Dev Cell. 2002; 2:385–387. [PubMed: 11970889]

Coppari R, Ichinose M, Lee CE, Pullen AE, Kenny CD, McGovern RA, Tang V, Liu SM, Ludwig T, Chua SC Jr, et al. The hypothalamic arcuate nucleus: a key site for mediating leptin's effects on glucose homeostasis and locomotor activity. Cell Metab. 2005; 1:63–72. [PubMed: 16054045]

Cota D, Proulx K, Smith KA, Kozma SC, Thomas G, Woods SC, Seeley RJ. Hypothalamic mTOR signaling regulates food intake. Science. 2006; 312:927–930. [PubMed: 16690869]

Cota D, Matter EK, Woods SC, Seeley RJ. The role of hypothalamic mammalian target of rapamycin complex 1 signaling in diet-induced obesity. J Neurosci. 2008; 28:7202–7208. [PubMed: 18614690]

Cowley MA, Smart JL, Rubinstein M, Cerdán MG, Diano S, Horvath TL, Cone RD, Low MJ. Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. Nature. 2001; 411:480–484. [PubMed: 11373681]

Daniel PB, Walker WH, Habener JF. Cyclic AMP signaling and gene regulation. Annu Rev Nutr. 1998; 18:353–383. [PubMed: 9706229]

de Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A, Bos JL. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. Nature. 1998; 396:474–477. [PubMed: 9853756]

Delgado M, Ganea D. Inhibition of IFN-gamma-induced janus kinase-1-STAT1 activation in macrophages by vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. J Immunol. 2000; 165:3051–3057. [PubMed: 10975815]

Elmquist JK, Coppari R, Balthasar N, Ichinose M, Lowell BB. Identifying hypothalamic pathways controlling food intake, body weight, and glucose homeostasis. J Comp Neurol. 2005; 493:63–71. [PubMed: 16254991]

Enriori PJ, Evans AE, Sinnayah P, Jobst EE, Tonelli-Lemos L, Billes SK, Glavas MM, Grayson BE, Perello M, Nillni EA, et al. Diet-induced obesity causes severe but reversible leptin resistance in arcuate melanocortin neurons. Cell Metab. 2007; 5:181–194. [PubMed: 17339026]

Enserink JM, Christensen AE, de Rooij J, van Triest M, Schwede F, Genieser HG, Døskeland SO, Blank JL, Bos JL. A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. Nat Cell Biol. 2002; 4:901–906. [PubMed: 12402047]

Fasshauer M, Klein J, Lossner U, Paschke R. Isoproterenol is a positive regulator of the suppressor of cytokine signaling-3 gene expression in 3T3-L1 adipocytes. J Endocrinol. 2002; 175:727–733. [PubMed: 12475383]

Flier JS. Neuroscience. Regulating energy balance: the substrate strikes back. Science. 2006; 312:861–864. [PubMed: 16690851]

Frederich RC, Hamann A, Anderson S, Löllmann B, Lowell BB, Flier JS. Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. Nat Med. 1995; 1:1311–1314. [PubMed: 7489415]

Friedman JM. Modern science versus the stigma of obesity. Nat Med. 2004; 10:563–569. [PubMed: 15170194]

Gaede P, Lund-Andersen H, Parving HH, Pedersen O. Effect of a multifactorial intervention on mortality in type 2 diabetes. N Engl J Med. 2008; 358:580–591. [PubMed: 18256393]

Gasperini S, Crepaldi L, Calzetti F, Gatto L, Berlato C, Bazzoni F, Yoshimura A, Cassatella MA. Interleukin-10 and cAMP-elevating agents cooperate to induce suppressor of cytokine signaling-3 via a protein kinase A-independent signal. Eur Cytokine Netw. 2002; 13:47–53. [PubMed: 11956020]

Gloaguen I, Costa P, Demartis A, Lazzaro D, Di Marco A, Graziani R, Paonessa G, Chen F, Rosenblum CI, Van der Ploeg LH, et al. Ciliary neurotrophic factor corrects obesity and diabetes associated with leptin deficiency and resistance. Proc Natl Acad Sci USA. 1997; 94:6456–6461. [PubMed: 9177239]

Gloerich M, Bos JL. Epac: defining a new mechanism for cAMP action. Annu Rev Pharmacol Toxicol. 2010; 50:355–375. [PubMed: 20055708]

Hill JW, Williams KW, Ye C, Luo J, Balthasar N, Coppari R, Cowley MA, Cantley LC, Lowell BB, Elmquist JK. Acute effects of leptin require PI3K signaling in hypothalamic proopiomelanocortin neurons in mice. J Clin Invest. 2008; 118:1796–1805. [PubMed: 18382766]

Howard JK, Cave BJ, Oksanen LJ, Tzameli I, Bjørbaek C, Flier JS. Enhanced leptin sensitivity and attenuation of diet-induced obesity in mice with haploinsufficiency of Socs3. Nat Med. 2004; 10:734–738. [PubMed: 15220914]

Lambert PD, Anderson KD, Sleeman MW, Wong V, Tan J, Hijarunguru A, Corcoran TL, Murray JD, Thabet KE, Yancopoulos GD, Wiegand SJ. Ciliary neurotrophic factor activates leptin-like pathways and reduces body fat, without cachexia or rebound weight gain, even in leptin-resistant obesity. Proc Natl Acad Sci USA. 2001; 98:4652–4657. [PubMed: 11259650]

Maffei M, Stoffel M, Barone M, Moon B, Dammerman M, Ravussin E, Bogardus C, Ludwig DS, Flier JS, Talley M, et al. Absence of mutations in the human OB gene in obese/diabetic subjects. Diabetes. 1996; 45:679–682. [PubMed: 8621022]

Mori H, Hanada R, Hanada T, Aki D, Mashima R, Nishinakamura H, Torisu T, Chien KR, Yasukawa H, Yoshimura A. Socs3 deficiency in the brain elevates leptin sensitivity and confers resistance to diet-induced obesity. Nat Med. 2004; 10:739–743. [PubMed: 15208705]

Münzberg H, Flier JS, Bjørbaek C. Region-specific leptin resistance within the hypothalamus of diet-induced obese mice. Endocrinology. 2004; 145:4880–4889. [PubMed: 15271881]

Myers MG Jr, Leibel RL, Seeley RJ, Schwartz MW. Obesity and leptin resistance: distinguishing cause from effect. Trends Endocrinol Metab. 2010; 21:643–651. [PubMed: 20846876]

Neves SR, Ram PT, Iyengar R. G protein pathways. Science. 2002; 296:1636–1639. [PubMed: 12040175]

Ozcan L, Ergin AS, Lu A, Chung J, Sarkar S, Nie D, Myers MG Jr, Ozcan U. Endoplasmic reticulum stress plays a central role in development of leptin resistance. Cell Metab. 2009; 9:35–51. [PubMed: 19117545]

Parton LE, Ye CP, Coppari R, Enriori PJ, Choi B, Zhang CY, Xu C, Vianna CR, Balthasar N, Lee CE, et al. Glucose sensing by POMC neurons regulates glucose homeostasis and is impaired in obesity. Nature. 2007; 449:228–232. [PubMed: 17728716]

Ramadori G, Lee CE, Bookout AL, Lee S, Williams KW, Anderson J, Elmquist JK, Coppari R. Brain SIRT1: anatomical distribution and regulation by energy availability. J Neurosci. 2008; 28:9989–9996. [PubMed: 18829956]

Sahu A, Metlakunta AS. Hypothalamic phosphatidylinositol 3-kinase-phosphodiesterase 3B-cyclic AMP pathway of leptin signalling is impaired following chronic central leptin infusion. J Neuroendocrinol. 2005; 17:720–726. [PubMed: 16219000]

Sands WA, Woolson HD, Milne GR, Rutherford C, Palmer TM. Exchange protein activated by cyclic AMP (Epac)-mediated induction of suppressor of cytokine signaling 3 (SOCS-3) in vascular endothelial cells. Mol Cell Biol. 2006; 26:6333–6346. [PubMed: 16914720]

Scott MM, Lachey JL, Sternson SM, Lee CE, Elias CF, Friedman JM, Elmquist JK. Leptin targets in the mouse brain. J Comp Neurol. 2009; 514:518–532. [PubMed: 19350671]

Serezani CH, Ballinger MN, Aronoff DM, Peters-Golden M. Cyclic AMP: master regulator of innate immune cell function. Am J Respir Cell Mol Biol. 2008; 39:127–132. [PubMed: 18323530]

Stamler J, Vaccaro O, Neaton JD, Wentworth D. Diabetes, other risk factors, and 12-yr cardiovascular mortality for men screened in the Multiple Risk Factor Intervention Trial. Diabetes Care. 1993; 16:434–444. [PubMed: 8432214]

Tartaglia LA. The leptin receptor. J Biol Chem. 1997; 272:6093–6096. [PubMed: 9102398]

Van Gaal LF, Mertens IL, De Block CE. Mechanisms linking obesity with cardiovascular disease. Nature. 2006; 444:875–880. [PubMed: 17167476]

Williams KW, Margatho LO, Lee CE, Choi M, Lee S, Scott MM, Elias CF, Elmquist JK. Segregation of acute leptin and insulin effects in distinct populations of arcuate proopiomelanocortin neurons. J Neurosci. 2010; 30:2472–2479. [PubMed: 20164331]

Zabolotny JM, Bence-Hanulec KK, Stricker-Krongrad A, Haj F, Wang Y, Minokoshi Y, Kim YB, Elmquist JK, Tartaglia LA, Kahn BB, Neel BG. PTP1B regulates leptin signal transduction in vivo. Dev Cell. 2002; 2:489–495. [PubMed: 11970898]

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. Nature. 1994; 372:425–432. [PubMed: 7984236]

Zhang X, Zhang G, Zhang H, Karin M, Bai H, Cai D. Hypothalamic IKKbeta/NF-kappaB and ER stress link overnutrition to energy imbalance and obesity. Cell. 2008; 135:61–73. [PubMed: 18854155]

Zhao AZ. Control of food intake through regulation of cAMP. Curr Top Dev Biol. 2005; 67:207–224. [PubMed: 15949535]

Zhao AZ, Bornfeldt KE, Beavo JA. Leptin inhibits insulin secretion by activation of phosphodiesterase 3B. J Clin Invest. 1998; 102:869–873. [PubMed: 9727054]

Zhao AZ, Huan JN, Gupta S, Pal R, Sahu A. A phosphatidylinositol 3-kinase phosphodiesterase 3B-cyclic AMP pathway in hypothalamic action of leptin on feeding. Nat Neurosci. 2002; 5:727–728. [PubMed: 12101402]

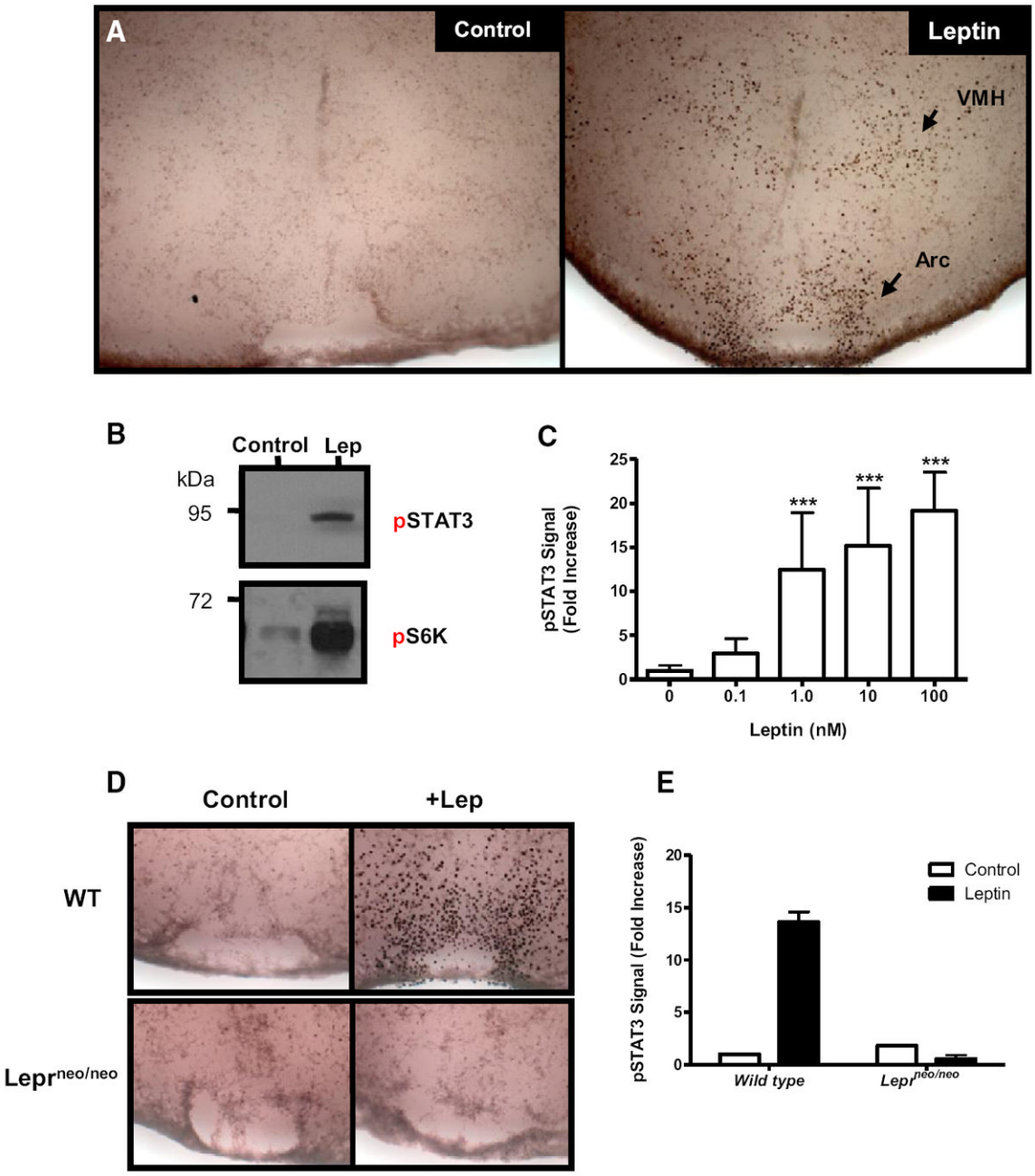

Figure 1. Leptin Signaling in Organotypic Hypothalamic Slices

(A) Leptin-induced pSTAT3 in the slices. The slices were prepared from mice and maintained for 10 days. The slices were treated with either leptin (100 nM) or saline for 30 min and fixed with 4% formalin and subjected to immunohistochemistry with anti-pSTAT3 antibodies followed by DAB staining. The presence of pSTAT3-labeled cells is evident in the arcuate nucleus (Arc) and the ventromedial hypothalamic nucleus (VMH) of the slices treated with leptin.

(B) Western blotting of hypothalamic pSTAT3 by leptin. The slices treated with leptin or saline for 30 min were extracted and subjected to western blot with antibodies against pSTAT3 and pS6K.

(C) Dose-dependent phosphorylation of STAT3 by leptin. The slices were incubated with an increasing dose of leptin for 30 min and then stained with an anti-pSTAT3 antibody as in (A). pSTAT3 signal within the Arc was measured. Results were plotted as the fold increase relative to the control (0 nM). Values are presented as mean ± standard error of the mean (SEM); ***$p < 0.001$, compared to 0 nM for t test, *$p < 0.05$ for one-way ANOVA.
(D) Absent of pSTAT3 by leptin in the leptin receptor null mice. The slices prepared from wild-type mice or leptin receptor knockout mice (Coppari et al., 2005) were treated with leptin (100 nM) or saline for 30 min and then stained with anti-pSTAT3 antibodies.
(E) Quantification of (D). Values are presented as mean ± SEM; **$p < 0.01$ for two-way ANOVA.
See also Figure S1.

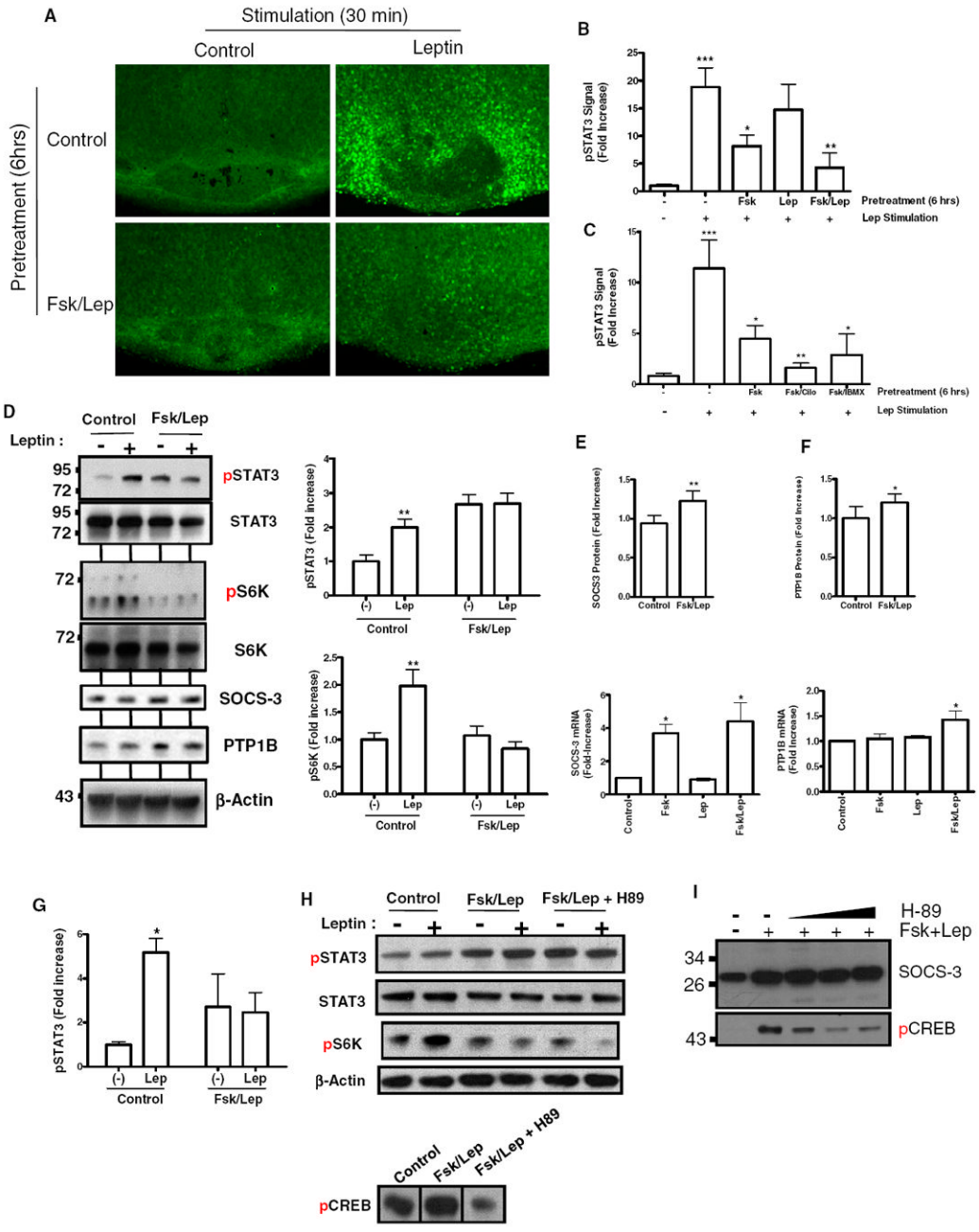

Figure 2. Inhibitory Effect of cAMP on Hypothalamic Leptin Signaling Pathways

(A) Inhibitory effect of Fsk/Lep on leptin-induced pSTAT3. The organotypic slices were pretreated with DMSO/saline (Control) or forskolin plus low levels of leptin (Fsk/Lep [20 μM/0.5 nM]) for 6 hr and then stimulated with or without leptin (100 nM) for 30 min. The slices were fixed and stained with an anti-pSTAT3 antibody (pY705).

(B) Inhibitory effect of Fsk is enhanced by the presence of low levels of leptin. The slices that were preincubated with saline, forskolin, or low levels of leptin or with both Fsk and low levels of leptin for 6 hr were stimulated with leptin (100 nM for 30 min). pSTAT3 immunostaining signal was quantified as described in Figure 1B. Values are presented as mean ± SEM. Results were analyzed with one-way ANOVA followed by Tukey's multiple

comparison test. Control versus Lep stimulation, ***p < 0.001; compared to Lep stimulation, **p < 0.01, 0.01 < *p < 0.05.

(C) Phosphodiesterase inhibitors enhanced the inhibitory effects of forskolin on leptin-induced STAT3 phosphorylation. Treatment of cilostamide (25 μM) or IBMX (25 μM) with Fsk (20 μM) had a stronger inhibitory effect on the leptin-induced STAT3 phosphorylation than Fsk treatment alone. The experiment was performed as in (B) instead of using low levels of leptin. Values are presented as mean ± SEM. Results were analyzed with one-way ANOVA followed by Tukey's multiple comparison test. Control versus Lep stimulation, ***p < 0.001; compared to Lep stimulation, 0.01 < *p < 0.05, **p < 0.01.

(D) The effect of Fsk/Lep on hypothalamic lepin signaling pathways. Left: The slices that were preincubated with either saline or Fsk/Lep (20 μM/0.5 nM for 6 hr) were stimulated with or without leptin (100 nM for 30 min). The hypothalami were cut out and then extracted and subjected to western blot with antibodies against pSTAT3, pS6K, SOCS-3, and PTP1B. Right: Quantification by image analysis of hypothalamic STAT3 and S6K phosphorylation. Values are presented as mean ± SEM. For pstat3, 11 separate experiments, **p (–) versus Lep, p = 0.0015 for two-way ANOVA; for pS6K, six different experiments, **p (–) versus Lep, p = 0.0002 for two-way ANOVA.

(E) Fsk/Lep induction of SOCS3. Top: Quantification by image analysis of hypothalamic SOCS-3 protein. Sixteen separate experiments, 0.001 < **p < 0.01 for Wilcoxon signed rank test. Bottom: Relative levels of hypothalamic *Socs-3* mRNA measured by real-time PCR; p = 0.0031 for one way ANOVA, 0.001 < **p < 0.01, *p < 0.05 for Tukey's multiple comparison test.

(F) Fsk/Lep induction of PTP1B. Top: Quantification by image analysis of hypothalamic PTP1B protein. Ten separate experiments were performed. 0.01 < *p < 0.05 for Wilcoxon signed rank test. Bottom: Relative levels of hypothalamic *ptp1b* mRNA measured by real-time PCR; *p < 0.05 for t test.

(G) Quantification of STAT3 phosphorylation in the arcuate nucleus. Eight arcuate nuclei were collected per group. Proteins were extracted and subjected to western blot with anti-pSTAT3 antibodies; p = 0.0288 for two-way ANOVA, *p < 0.05 for t test.

(H) Fsk/Lep's inhibitory effect is independent of PKA. Treatment with Fsk/lep blunted leptin signaling in the presence of H-89, an inhibitor for PKA.

(I) Fsk/Lep induction of SOCS3 occurred in the presence or absence of varying dosages of H-89.

See also Figure S2.

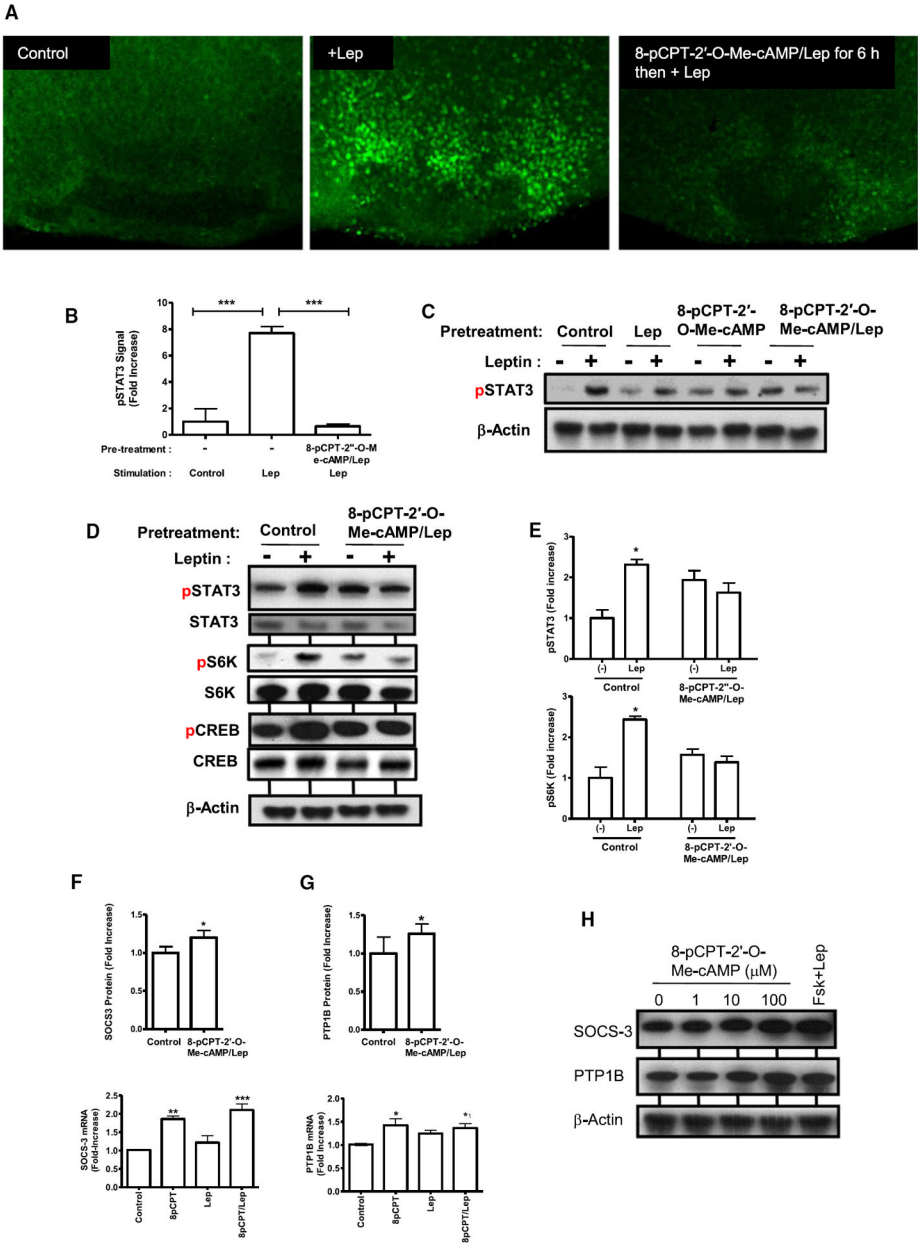

Figure 3. Activation of Epac Sufficiently Blunts Hypothalamic Leptin Signaling Pathways

(A) Inhibitory effect of an Epac agonist (8-pCPT-2′-O-Me-cAMP)/Lep on leptin-induced pSTAT3. An Epac agonist (100 μM) with a low level of leptin (0.5 nM) for 6 hr impaired leptin- (100 nM for 30 min) induced STAT3 phosphorylation in the hypothalamus.

(B) Quantification of pSTAT3 signal in (A). pSTAT3 immunostaining signal was quantified as described in Figure 1B. Values are presented as mean ± SEM; ***$p < 0.001$, compared to 0 nM for t test.

(C) Treatment of 8-pCPT-2′-O-Me-cAMP (100 μM) plus a low level of leptin (0.5 nM) had a stronger inhibitory effect on the leptin-induced STAT3 phosphorylation than 8-pCPT-2′-O-Me-cAMP treatment alone.

(D) The effect of an Epac agonist/Lep on hypothalamic lepin signaling pathways. Treatment of slices with 8-pCPT-2′-O-Me-cAMP (100 μM) plus a low level of leptin (0. nM) dampened hypothalamic STAT3 and S6K phosphorylation. Importantly, CREB

phosphorylation was not increased in the slices treated with 8-pCPT-2′-O-Me-cAMP (100 μM) plus a low level of leptin.

(E) Quantification of hypothalamic STAT3 and S6K phosphorylation. Values are presented as mean ± SEM. For pSTAT3, four separate experiments, **p (–) versus Lep, p = 0.001 for two-way ANOVA; for pS6K, three different experiments, **p (–) versus Lep, p = 0.0128 for two-way ANOVA.

(F) Quantification of hypothalamic SOCS-3 protein (top) and relative levels of hypothalamic *Socs-3* mRNA measured by real-time PCR (bottom). For SOCS-3 protein, six separate experiments, *p < 0.05 for Wilcoxon signed rank test. For mRNA, four experiments; p = 0.0004 for one-way ANOVA, **p < 0.01 and ***p < 0.001 for Tukey's multiple comparison test.

(G) Quantification of hypothalamic PTP1B. Top: Six separate experiments; *p < 0.05 for Wilcoxon signed rank test. Bottom: Four experiments; p = 0.0364 for one way ANOVA, *p < 0.05 for Tukey's multiple comparison test.

(H) SOCS3 and PTP1B were increased dose dependently of the Epac agonist (8-pCPT-2′-O-Me-cAMP, 1-100 μM) in the presence of leptin (0.5 nM), which alone had no effect on induction of either protein.

See also Figure S3.

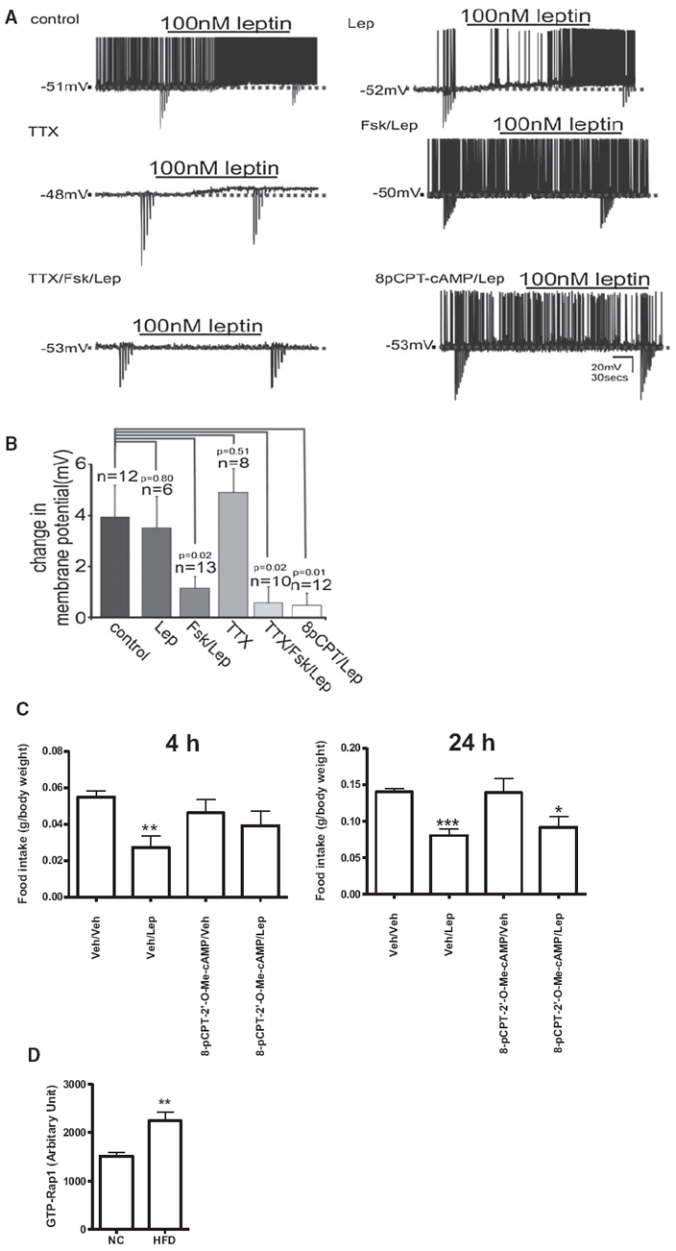

Figure 4. Activation of the cAMP-Epac Pathway Impairs the Leptin-Induced Depolarization of POMC Neurons

(A) Current-clamp recordings demonstrated that activation of Epac signaling occluded the leptin-induced depolarization of POMC neurons from rest. Leptin-induced (100 nM) depolarization of POMC neurons of organotypic slices that were pretreated for 6 hr with either saline (control, top left), a low level of leptin alone (0.03 nM, top right), TTX (0.5 μM, middle left), Fsk/Lep (20 μM/0.03 nM, middle right), TTX/Fsk/Lep (0.5 μM /20 μM/ 0.03 nM, bottom left) or Epac agonist (8-pCPT-2′-O-Me-cAMP)/Lep (100 μM/0.03 nM, bottom right).

(B) Leptin-induced responses of identified POMC neurons pretreated with the indicated reagents. p values compared with the WT are shown. Values are presented as mean ± SEM. Comparisons between two groups were made by unpaired Student's t test. $p < 0.05$ was considered to be statistically significant.

(C) Activation of Epac induces central leptin resistance. Male C57BL/6 mice were randomly assigned to four weight-matched groups (26.09 ± 1.59 g for Veh/Veh, 26.34 ± 1.96 g for Veh/Lep, 26.03 ± 2.01 g for 8-pCPT-2′-O-Me-cAMP/Veh, or 26.54 ± 2.10 g for 8-pCPT-2′-O-Me-cAMP/Lep, $p = 0.8938$ for one-way ANOVA). The mice were first injected ICV into the lateral ventricle with vehicle or 5 μg 8-pCPT-2′-O-Me-cAMP followed by 4 hr later by second injection of either vehicle or leptin (5 μg). The cumulative food intake was measured for 4 hr and 24 hr after the second injection. Values are presented as mean ± SEM. Results were analyzed with one-way ANOVA followed by Tukey's multiple comparison test; $p = 0.0062$ (4 hr) and $p < 0.0001$ (24 hr) for one-way ANOVA; ***$p < 0.001$ and **$p < 0.01$ compared to Veh/Veh, *$p < 0.05$ compared to 8-pCPT-2′-O-Me-cAMP/Veh.
(D) Exposure to a high-fat diet for 4 weeks increases Rap1 activity in the hypothalamus. Hypothalamic Rap1 activity was assessed in the mice either fed with high-fat diet or normal chow for 4 weeks. Values are presented as mean ± SEM; **$p < 0.01$, compared to NC for t test.