Neuronal Rap1 regulates energy balance, glucose homeostasis, and leptin actions

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Abstract

The central nervous system (CNS) contributes to obesity and metabolic disease; however, the underlying neurobiological pathways remain to be fully established. Here we show that the small GTPase Rap1 is expressed in multiple hypothalamic nuclei that control whole-body metabolism and is activated in high-fat diet (HFD)-induced obesity. Genetic ablation of CNS Rap1 protects mice from dietary obesity, glucose imbalance, and insulin resistance in the periphery and from HFD-induced neuropathological changes in the hypothalamus, including diminished cellular leptin sensitivity and increased endoplasmic reticulum (ER) stress and inflammation. Further, pharmacological inhibition of CNS Rap1 signaling normalizes hypothalamic ER stress and inflammation, improves cellular leptin sensitivity, and reduces body weight in mice with dietary obesity. We also demonstrate that Rap1 mediates leptin resistance via interplay with ER stress. Thus, neuronal Rap1 critically regulates leptin sensitivity and mediates HFD-induced obesity and hypothalamic pathology and may represent a potential therapeutic target for obesity treatment.

Graphical abstract

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AUTHOR CONTRIBUTIONS
M.F. conceived the study. K.K. and M.F. designed the experiments. K.K., P.X., E.L.C., S.S.C. and A.N. performed the experiments. A.M. and Y.X. contributed reagents and intellectually assisted with the Rap1ΔCNS mice studies. K.K., P.X. and M.F. analyzed data and interpreted the results. The major part of the manuscript was written by M.F. with some help from K.K. All authors approved the final version of the manuscript.
The central nervous system (CNS) has been long established as robust homeostatic systems for the maintenance of normal body weight and euglycemia (Coll et al., 2007; Dietrich and Horvath, 2013; Morton et al., 2006; Myers and Olson, 2012; Ryan et al., 2012). The crucial role of the CNS in the development of obesity is also becoming increasingly apparent with recent discoveries of obesity-susceptibility genes that are often associated with CNS functions (Locke et al., 2015). Obesogenic conditions such as high-fat diet (HFD) feeding cause these CNS homeostatic systems to shift toward positive energy balance, which ultimately leads to obesity (Ryan et al., 2012). However, the neural pathways that actively respond to HFD feeding and mediate adiposity under overnutrition remain incompletely characterized.

HFD leads to multiple, profound neuropathological changes in hypothalamic nuclei that control body weight (Konner and Bruning, 2012; Morton et al., 2006; Myers et al., 2010; Ryan et al., 2012). Hypercaloric feeding rapidly induces neuronal resistance to the actions of leptin, a powerful adipocyte-derived satiety hormone that maintains normal body weight and euglycemia (Frederich et al., 1995; Konner and Bruning, 2012; Morton et al., 2006; Myers et al., 2010; Ryan et al., 2012). Although the detailed mechanisms are still unclear, cellular leptin signaling in the CNS is clearly impaired in rodent models of HFD-induced obesity (Myers et al., 2012; Ryan et al., 2012). Thus, defective intracellular leptin signaling in the CNS has been proposed as an underlying cellular mechanism for leptin resistance. Signaling molecules that directly inhibit leptin signaling, including suppressor of cytokine signaling-3 (SOCS-3) (Bjorbaek et al., 1998; Howard et al., 2004; Mori et al., 2004), protein tyrosine phosphatase 1B (PTP1B) (Bence et al., 2006; Cook and Unger, 2002; Zabolotny et al., 2002), and T-cell protein tyrosine phosphatase (TCPTP) (Loh et al., 2011), have been identified as crucial mediators of leptin resistance. All of these factors are upregulated in the hypothalamus by HFD-induced obesity (Bjorbaek et al., 1998; Cook and Unger, 2002; Loh et al., 2011; Zabolotny et al., 2002). Moreover, neuron-specific deletion of these inhibitors protects against HFD-induced obesity as well as leptin resistance and insulin resistance (Bence et al., 2006; Howard et al., 2004; Loh et al., 2011; Mori et al., 2004). Thus, SOCS-3
and tyrosine phosphatases collectively contribute to the development of HFD-induced obesity. Obesity induced by HFD is also associated with ER stress and inflammation in the CNS. Recent studies suggest that HFD-induced ER stress and inflammation in the CNS impair hypothalamic control of body weight and glucose balance (Coll et al., 2007; Dietrich and Horvath, 2013; Morton et al., 2006; Myers and Olson, 2012; Ryan et al., 2012). Hypothalamic ER stress and inflammation are markedly increased by overfeeding and in multiple obesity models (De Souza et al., 2005; Ozcan et al., 2009; Zhang et al., 2008b). Pharmacologic or genetic induction of ER stress and/or inflammation in the CNS upregulates SOCS-3, PTP1B, and TCPTP expression and causes leptin resistance and obesity (Cakir et al., 2013; Hosoi et al., 2008; Zhang et al., 2008b). On the contrary, manipulations that alleviate hypothalamic ER stress or reduce hypothalamic inflammation ameliorate cellular leptin resistance and obesity in animals (Kleinridders et al., 2009; Milanski et al., 2009; Ozcan et al., 2009; Schneeberger et al., 2013; Zhang et al., 2008b). Although HFD feeding clearly elicits hypothalamic dysfunction, promoting obesity, the underlying molecular signaling pathways are poorly understood.

The Ras-like small GTPase Rap1 is a crucial regulator of multiple cellular processes, including adhesion, polarity, and proliferation, in non-neuronal cells (Gloerich and Bos, 2011). In the CNS, Rap1 has diverse roles in an array of neuronal functions from neuronal excitability, synaptic plasticity, and neuronal polarity to memory and learning (Spilker and Kreutz, 2010). However, the contributions of CNS Rap1 to energy balance and glucose homeostasis are largely unknown. In the present study, we investigated the role of CNS Rap1 in the regulation of whole-body energy and glucose metabolism by producing and characterizing mice with targeted deletion of Rap1a and Rap1b, the genes encoding Rap1, selectively in forebrain neurons.

RESULTS

Loss of neuronal Rap1 protects against diet-induced obesity

We first investigated whether Rap1 is expressed in the hypothalamus. Consistent with previous work (Kim et al., 1990; Pan et al., 2008), quantitative real-time polymerase chain reaction (qRT-PCR) showed that Rap1a and Rap1b are expressed in various tissues, including the CNS, and that both mRNAs are abundant in the hypothalamus (Figure S1A). We further examined the hypothalamic distribution of Rap1 by immunohistochemistry analyses. Rap1 is expressed throughout the mediobasal hypothalamus, including in multiple nuclei that regulate whole-body metabolism such as the arcuate (ARC), ventromedial (VMH), and dorsomedial (DMH) nuclei (Figure S2A and Figure 1D). We then asked if CNS Rap1 is activated in HFD-induced obese mice. As shown in Figure S2B, the active (GTP-bound) form of Rap1 is increased in the brain of HFD-induced obese mice compared with lean control mice. Total Rap1 levels were not changed in response to HFD (Figure 7A). These results and a previous study showing that Rap1 activity is increased in lean mice after acute HFD feeding (Fukuda et al., 2011) strongly suggest that Rap1 is involved in the metabolic responses to HFD feeding.

We thus explored whether CNS Rap1 contributes to diet-induced obesity and associated metabolic disturbances. To produce mice Rap1-null in the forebrain (Rap1ΔCNS) (Pan et al.,
2008), we crossed double-floxed Rap1a and Rap1b (Rap1) mice to mice harboring the CaMKIIαCre driver, which express Cre recombinase in postnatal forebrain neurons in the CNS (Minichiello et al., 1999). We confirmed CNS-specific recombination of the floxed alleles (Figure 1A), forebrain (including hypothalamus) deletion of Rap1 mRNAs (Figure 1B), and selective depletion of Rap1 protein from forebrain regions including the hypothalamus (Figure 1C). Rap1 protein levels were also significantly reduced in multiple hypothalamic nuclei (Figure 1D).

Using this Rap1ΔCNS mouse model, we examined if Rap1 has a role in the CNS regulation of energy and glucose homeostasis in vivo. Male Rap1ΔCNS and control male mice (the double floxed mice of Rap1a and Rap1b) were placed on a HFD starting at 4 weeks of age to test if loss of Rap1 protects against diet-induced obesity. The HFD-fed male Rap1ΔCNS mice showed markedly reduced body weight gain (Figure 2A), significantly lower adiposity (Figure 2E), and reduced serum leptin levels (Figure 2I) compared with controls. In contrast, Rap1ΔCNS and control male mice fed a normocaloric chow diet exhibited similar body weight, adiposity and serum leptin (Figures 2C, G and K). Female Rap1ΔCNS mice under HFD also demonstrated lower body weight and adiposity than female controls (Figures 2B, 2D, 2F, 2H, 2I and 2L), suggesting no sexual dimorphism in Rap1 function. We used male mice only for subsequent experiments.

We then investigated the basis for the leaner phenotype of HFD-fed Rap1ΔCNS mice by directly assessing energy balance in open-circuit indirect calorimetry cages. Although body weight and adiposity of Rap1ΔCNS mice did not diverge from control animals after 2 weeks of HFD (Figure S3A and S3B), Rap1ΔCNS mice exhibited hypophagia (Figure 2M) associated with increased hypothalamic expression of anorexigenic neuropeptide POMC mRNA and decreased expression of orexigenic neuropeptide NPY and AgRP mRNAs (Figure 2S and Figure S5B). In contrast, no difference in energy expenditure (oxygen consumption, carbon dioxide production, locomotor activity, or thermogenesis) was observed between Rap1ΔCNS and control mice (Figures 2N–2Q). Notably, Rap1ΔCNS mice showed a lower respiratory quotient than controls, indicating the preferential use of fat as an energy source (Figure 2R). Thus, decreased food intake and preferential oxidation of fat as an energy substrate likely contributes to decreased adiposity in neuronal Rap1-null mice under hypercaloric feeding. In chow-fed lean mice, food intake (Figure S4A), energy expenditure (Figure S4B–4F) and mRNA levels of feeding-related hypothalamic neuropeptides (Figure S5A) did not differ significantly between genotypes. These findings suggest that CNS Rap1 plays a crucial role in mediating diet-induced body weight gain and adiposity.

**Improved glucose balance and peripheral insulin sensitivity in Rap1ΔCNS mice**

Consistent with the leaner body weight phenotype, Rap1ΔCNS mice displayed significantly lower levels of blood glucose and insulin than control animals under HFD feeding (Figures 3A and 3B), suggesting that mice lacking Rap1 in the CNS have increased peripheral insulin sensitivity. Indeed, HFD-fed Rap1ΔCNS mice showed enhanced glucose tolerance (Figure 3C) and improved insulin sensitivity (Figure 3D). This higher glucose-tolerant and insulin-sensitive phenotype was also observed in age- and weight-matched Rap1ΔCNS cohorts.
maintained on a normocaloric diet (Figures 3E–3H), suggesting that Rap1 deficiency in the CNS influences insulin/glucose balance regardless of body weight and adiposity. In further support of improved insulin sensitivity, insulin signaling was significantly enhanced in the liver, muscle, and fat of HFD-fed Rap1ΔCNS mice, as assessed by western blot analyses using phospho-specific antibodies to Akt and Gsk3β (Figure 3I), the central mediators of insulin signaling (Manning and Cantley, 2007). We also observed that hepatic insulin-dependent phosphorylation of Akt was significantly increased in normal chow-fed Rap1ΔCNS mice (Figure S6). In agreement with enhanced hepatic insulin sensitivity, hepatic expression levels of the gluconeogenic genes phosphoenolpyruvate carboxykinase (Pepck) and glucose-6-phosphatase (G6pc) were significantly reduced in HFD-fed Rap1ΔCNS mice compared with HFD-fed controls (Figure 3J). Collectively, these findings suggest that in addition to its role in body weight regulation, neuronal Rap1 regulates glucose balance and peripheral insulin sensitivity.

**Enhanced leptin sensitivity in neuronal Rap1-deficient mice**

Mice with genetic ablation of neuronal Rap1 exhibit traits suggestive of enhanced leptin sensitivity, including decreased circulating leptin levels (Figure 2I), hypophagia (Figure 2M), and altered levels of leptin-regulated hypothalamic neuropeptides (Figure 2S). We therefore tested whether Rap1 is required for the development of HFD-induced leptin resistance. Rap1ΔCNS and control mice were placed on HFD (60% fat) for 8 weeks, beginning at 2 months of age, to induce leptin resistance. We did not observe any significant difference in body weight (28.02 ± 0.71 g for Control vs. 27.61 ± 0.62 g for Rap1ΔCNS, n = 8/group, P > 0.05, t-test), fat mass (4.76 ± 0.45 g vs. 3.45 ± 0.42 g, n = 8/group, P > 0.05, t-test), or lean mass (20.82 ± 0.44 g vs. 21.72 ± 0.31 g, n = 8/group, P > 0.05, t-test) between the two groups after 8 weeks of HFD feeding (Figure S3C and S3D), probably because of the late-onset of HFD challenge. Using these age- and body weight-matched cohorts, we then assessed the anorectic response to leptin by injecting Rap1ΔCNS and control mice with leptin twice daily. Although control mice developed leptin resistance (Figures 4A and 4B), Rap1ΔCNS mice responded to leptin with body weight reduction and suppression of food intake (Figures 4A and 4B). Further, cellular leptin sensitivity, as demonstrated by leptin-induced phosphorylation of STAT3, a marker of activated leptin signaling (Bates et al., 2003; Gao et al., 2004; Metlakunta et al., 2008; Vaisse et al., 1996), was significantly enhanced in Rap1ΔCNS mice but absent in controls under HFD condition (Figure 4C). Also, hypothalamic Socs-3 and Tcptp were significantly lower in Rap1ΔCNS mice than in controls (Figure 4D). In addition to its effect under a HFD diet, Rap1 deficiency potently enhanced leptin actions under normal caloric conditions (Figure S7). Therefore, deletion of CNS Rap1 enhances cellular leptin signaling and protects against leptin resistance.

**ESI-05 reverses leptin resistance in HFD-induced obesity**

To assess the translational value of CNS Rap1, we investigated the effects of a well-established selective inhibitor of Epac2, ESI-05 (Rehmann, 2013; Tsalkova et al., 2012). Epac2 is one of the two members of exchange protein directly activated by cAMP (Epac) that serves as GTP/GDP exchange factors for Rap1. Epac2 is predominantly expressed throughout the brain and in the adrenal gland in humans (Kawasaki et al., 1998) and in mice (Figure S1B). We infused leptin, the selective Epac2 inhibitor ESI-05, or both into the brains...
of wild-type HFD-induced obese mice. Co-administration of ESI-05 markedly sensitized leptin-responsive neurons, as indicated by restoring leptin-induced suppression of food intake, reduction of body weight (Figures 5A and 5B), and phosphorylation of independent leptin signaling mediators STAT3 and S6K (Figures 5C and 5D). Notably, ESI-05 restored leptin sensitivity to a similar degree in normocaloric-fed lean mice receiving leptin alone (Figures 5A and 5B). To confirm Rap1 mediation of ESI-05 effects, we repeated these experiments in Rap1ΔCNS mice. Consistent with mediation by Rap1, ESI-05 did not enhance leptin sensitivity in Rap1ΔCNS mice (Figures 5E and 5F). Next, we investigated whether ESI-05 has this anti-obesity effect when centrally administered alone to HFD-induced hyperleptinemic and leptin-resistant obese mice. Central daily infusion of ESI-05 (0.2 nmol/brain/day) significantly reduced body weight and food intake of HFD-induced obese mice (Figures 5G and 5H). In contrast, the body weight of vehicle-treated control obese mice exhibited no changes during the course of the experiment (Figures 5G and 5H). Thus, chronic administration of ESI-05 alone is indeed able to decrease the body weight of HFD-induced obese mice (vehicle versus ESI-05, P < 0.05). Collectively, these findings demonstrate that Epac2 inhibition reverses leptin resistance and reduces body weight in HFD-induced obese mice.

**Rap1 is required to mediate leptin resistance conferred by chemically-induced ER stress**

We next sought to determine potential underlying mechanisms by which central Epac-Rap1 signaling contributes to leptin resistance. Cellular leptin resistance can be caused by multiple mechanisms that include ER stress and hyperleptinemia (Frederich et al., 1995; Konner and Bruning, 2012; Morton et al., 2006; Myers et al., 2010; Ozcan et al., 2009; Ryan et al., 2012; Zhang et al., 2008b), which prompted us to explore potential interactions between Epac signaling and putative leptin–resistance-inducing factors. First, we modeled leptin resistance by treating organotypic brain slices with pharmacological agents that induce cellular leptin resistance. Similar to previous observations (Fukuda et al., 2011; Williams et al., 2014), leptin-induced phosphorylation of STAT3 was blocked by treatment with ER stress inducers tunicamycin (TU), thapsigargin, and dithiothreitol (Figure 6A and 6B), whereas leptin stimulated STAT3 phosphorylation in controls (Figure 6A and 6B). Strikingly, pretreatment with ESI-05, a selective Epac2 inhibitor (Tsalkova et al., 2012) abolished ER stress-induced leptin resistance in slices (Figure 6A and 6B). ESI-05 also blocked cellular leptin resistance induced by forskolin, which activates Epac-Rap1 signaling (de Rooij et al., 1998; Fukuda et al., 2011) (Figure 6A and 6B). ESI-05 had negligible effects on leptin resistance resulting from treatment with high-dose leptin (mimicking hyperleptinemia) (Figure 6A and 6B). ESI-05 alone did not stimulate leptin-dependent STAT3 phosphorylation (Figure 6B). To further confirm the effect of ESI-05 in vivo, we chemically induced ER stress in the brain of lean C57BL/6 mice by the injection of TU. TU treatment increased GTP-bound (active) Rap1 in brain (Figure 6C). Inhibition of CNS Epac2 prevented hypothalamic leptin resistance and Sox3-3 induction triggered by centrally injected TU in mice (Figure 6D and 6E), confirming our ex vivo findings. Interestingly, other key factors involved in leptin resistance such as negative regulators of leptin signaling (PTP1B and TCPTP) and a positive regulator, SHP2 (Zhang et al., 2004) remained unaltered (Figure 6E). These findings suggest that Epac2 participates in ER–stress-induced leptin resistance.
Reciprocal connection between Rap1 and ER stress in the CNS under overnutrition

Because HFD-induced obese mice exhibited both increased CNS Rap1 activity (Figure 7A and Figure S2B) and ER stress (Ozcan et al., 2009; Won et al., 2009; Zhang et al., 2008b) (Figure 7B), we next investigated whether Rap1 is involved in cellular processes that mediate HFD-induced ER stress. To test this, we manipulated CNS Rap1 activity by either pharmacologic inhibition using ESI-05 or by brain-specific Rap1 deletion (in Rap1ΔCNS mice). Central delivery of ESI-05 into the brain of wild-type HFD-induced obese mice significantly suppressed elevated Rap1 activity in the CNS (Figure 7A). Treatment with ESI-05 also reversed the increased expression levels of ER stress marker genes ($Xbp1s$, $Chop$ and $Atf4$) and elevated $Il-6$ and $Socs-3$ in the hypothalamus of HFD-induced obese mice (Figure 7B). In addition, ESI-05 significantly reduced hypothalamic phosphorylation of the core components of the unfolded protein response (UPR), PERK, and eIF2α (Walter and Ron, 2011) (Figure 7C). These responses were almost completely recapitulated in Rap1ΔCNS mice. We challenged Rap1ΔCNS mice and age- and body weight-matched controls with HFD for 4 weeks and measured hypothalamic expression levels of ER stress markers and $Il-6$. After 4 weeks of HFD feeding, there were no significant differences in body weight and adiposity between the two groups. Nonetheless, qRT-PCR revealed significant increases in ER stress markers ($Chop$, $Edem$, $Atf4$, and $Grp94$) and pro-inflammatory cytokine $Il-6$ in the hypothalamus of control mice after HFD challenge, whereas HFD failed to upregulate the classical markers of UPR activation and $Il-6$ in Rap1ΔCNS mice (Figure 7D). This suggests that Rap1 deficiency in the CNS prevents HFD induction of ER stress and pro-inflammatory cytokine $Il-6$. In contrast, a reduction in ER stress with the chemical ER chaperone tauroursodeoxycholic acid (TUDCA) (Ozcan et al., 2006) restored Rap1 activity to normal levels and reduced ER stress markers (Figure 7E and 7F). $Socs-3$ was markedly reduced in the hypothalamus of TUDCA- (Figure 7G) and ESI-05– (Figure 7B) treated obese mice. These findings revealed a previously unrecognized mechanistic link between ER stress and Epac-Rap1 signaling in mediating hypothalamic leptin resistance.

DISCUSSION

Overnutrition is associated with reduced sensitivity to key metabolic hormones such as leptin and insulin, and insensitivity to these hormones is fundamental to the pathogenesis of metabolic disease. Although it is clear that the CNS participates in the biological responses to obesogenic conditions, the detailed neurobiological pathways remain unclear. In this study, we provide compelling genetic and pharmacological evidence that Rap1 in the CNS acts as a key component of the mechanistic pathway linking overnutrition to obesity and metabolic disorders. Mice with Rap1-deficiency in the CNS gained significantly less body weight and adiposity during HFD feeding compared with HFD-fed control mice and were resistant to HFD-induced systemic glucose imbalance, central leptin resistance, and peripheral insulin resistance. In contrast, Rap1ΔCNS mice have little effect on body weight, energy expenditure and hypothalamic regulation of feeding-related neuropeptides under normocaloric feeding. A specific role for Rap1 in metabolic dysfunction under a hypercaloric diet is further supported by current and previous findings that CNS Rap1 activity in HFD-fed mice is higher than in chow-fed ones. The decrease in adiposity in HFD-fed Rap1ΔCNS mice is likely due to hypophagia without changes in energy expenditure.
because motor activity and thermogenesis were unchanged. This unaltered energy expenditure in Rap1ΔCNS mice compared with controls is in accord with a previous study reporting that leptin does not actually enhance energy expenditure in mice but only prevents the decrease associated with leptin-induced hypophagia (Halaas et al., 1997). Additionally, Rap1ΔCNS mice have a decreased respiratory quotient, suggesting a preferential use of fat as an energy source. This may also contribute to the reduced adiposity gain during HFD feeding. Consistent with reduced feeding behavior, Rap1ΔCNS mice also have reduced mRNA levels of the orexigenic neuropeptides AgRP and NPY and increased mRNA encoding the anorexigenic neuropeptide POMC. As shown in Figure 1C and 1D, Rap1-expressing cells were retained in the hypothalamus of Rap1ΔCNS mice (Rap1a^+/−, Rap1b^+/−, CaMKIIα-Cre). This is consistent with previous reports showing that CaMKIIα expression in the forebrain is almost exclusively restricted to excitatory, glutamatergic neurons and absent from GABAergic neurons and non-neuronal cells (Liu and Murray, 2012). Thus, Rap1ΔCNS mice is a partial knockdown model of CNS Rap1 and could potentially underestimate the role of CNS Rap1 in controlling metabolism. Collectively, these results suggest that CNS Rap1 plays a critical role in the development of HFD-induced obesity, while suppression can protect against obesity and metabolic disruption by controlling food intake and maintaining leptin and insulin sensitivity.

In addition to its effect on energy balance, deletion of Rap1a and Rap1b from forebrain neurons resulted in markedly improved glucose tolerance, enhanced systemic insulin sensitivity, and increased cellular insulin signaling in skeletal muscle, adipose tissue, and liver under HFD feeding. Consistent with improved hepatic insulin sensitivity, Rap1ΔCNS mice exhibit reduced hepatic expression of the key gluconeogenic enzymes, PEPCK and G6pc. Improved systemic glucose balance appears to occur independently of Rap1 deletion-induced suppression of body weight and adiposity because age-, body weight- and adiposity-matched lean Rap1ΔCNS mice also displayed lower glucose levels and improved glucose and insulin tolerance under normocaloric diet feeding. Thus, these data suggest a previously unrecognized role for neuronal Rap1 in controlling peripheral insulin sensitivity without changes in body weight. The molecular mechanisms mediating this effect are unclear, but could involve greatly reduced hypothalamic SOCS-3 in neuronal Rap1-deficient mice, as selective SOCS-3 deletion in VMH SF1 neurons (Zhang et al., 2008a) or leptin receptor-expressing cells (Pedroso et al., 2014) improves glucose and insulin tolerance and enhances peripheral insulin sensitivity without affecting body weight. However, additional mechanisms are likely involved because Socs-3 expression alone did not induce glucose imbalance in mice when expressed in leptin-responsive neurons (Reed et al., 2010).

The robust effects of forebrain-specific Rap1 deletion are likely mediated by enhanced hypothalamic sensitivity to leptin. Rap1ΔCNS mice fed a HFD have lower serum leptin levels than HFD-fed control mice, implying enhanced leptin sensitivity. We further demonstrate that pharmacological inhibition of CNS Rap1 signaling also improves multiple indices of leptin sensitivity and protects mice against development of leptin resistance. Rap1ΔCNS mice failed to develop resistance to the anorectic and cellular actions of exogenous leptin under HFD feeding conditions. Notably, Rap1ΔCNS mice displayed enhanced leptin actions regardless of diet type, further supporting the primary role of Rap1 in controlling neuronal leptin sensitivity. Although leptin sensitivity was increased in Rap1ΔCNS mice under both
normal chow and HFD conditions, we observed that food intake and preferential oxidation of fat were only affected in mice under hypercaloric feeding. The lack of the effects during a normocaloric diet may be due to the lower concentration of serum leptin levels in chow-fed mice, which may not be high enough to induce reduced food intake and increased preferential oxidation of fat even in Rap1 deficient mice. Remarkably, reduced Rap1 activity in the CNS, either by genetic deletion or by pharmacologic inhibition, resulted in suppression of two direct endogenous inhibitors of cellular leptin signaling, SOCS-3 and TCPTP, as well as inhibitory mechanisms such as inflammatory signals and UPR pathways. Because these inhibitors and inhibitory pathways appear to limit cellular leptin signaling under overnutrition (Bence et al., 2006; Bjorbaek et al., 1998; Cook and Unger, 2002; De Souza et al., 2005; Howard et al., 2004; Loh et al., 2011; Mori et al., 2004; Ozcan et al., 2009; Zabolotny et al., 2002; Zhang et al., 2008b), reductions associated with Rap1 deficiency likely account, at least in part, for enhanced leptin sensitivity in Rap1ΔCNS mice. These results collectively support our hypothesis that neuronal Rap1 is a major regulator of leptin sensitivity and acts as a mediator of leptin resistance in obesity.

One of the most important questions arising from this study concerns how overnutrition leads to the activation of Rap1 in the CNS. Rap1 can be activated (converted from the GDP-to GTP-bound form) by at least five distinct classes of guanine nucleotide exchange factors (GEFs) (Spilker and Kreutz, 2010). Previous studies reported that the GEFs Epac1 and Epac2 attenuate cellular leptin signaling in cultured cells and brain slices (Fukuda et al., 2011; Sands et al., 2006). However, the biological significance of this effect remains controversial because while one study showed resistance to diet-induced obesity in Epac1-null mice (Yan et al., 2013), another reported augmented diet-induced obesity and glucose imbalance in the Epac1 global-knockout mice (Kai et al., 2013). In contrast to this conflicting data on the role of Epac1 in the control of whole-body energy and glucose balance, our findings suggest that brain Epac2 is likely involved in Rap1 activation and its effects on hypothalamic functions in HFD-induced obesity. Interestingly, Rap1 activity is elevated in the brain of HFD induced obese mice, compared to that of normal chow fed-lean mice, in the absence of changes in the total protein levels of Rap1 (Figure 7A), Epac1 and Epac2 (Figure S2C), indicating that HFD activation of Epac-Rap1 signaling seems to be mediated via post-translational modification on Epac. Considering that Epac is directly activated by cAMP, one implication of this result is that Rap1 could be activated via G protein-coupled receptors (GPCRs) that act through either Gs or Gi to modulate the cAMP–Epac pathway (Neves et al., 2002). Because CNS Rap1 activity is increased by HFD, it is interesting to speculate that a GPCR ligand is produced in response to HFD that links overnutrition to Rap1 activation in the CNS. Identification of such a circulating factor is a critical step for the development of agents that modulate CNS Rap1, possibly as pharmacological treatment for eating disorders, obesity, and metabolic diseases.

Our ex vivo studies revealed a previously unidentified link between Rap1 and ER stress, the relevance of which was substantiated in vivo. These findings imply that reciprocal interaction perpetuates CNS ER stress and Rap1 activation during overnutrition, subsequently leading to leptin resistance, and predict that inhibition of Epac-Rap1 signaling breaks this link. Suppression of CNS ER stress produces anti-obesity benefits (Hosoi et al., 2014; Liu et al., 2015; Ozcan et al., 2009); therefore, suppression of Epac-Rap1 signaling
might also produce benefits by enhancing leptin sensitivity and improving energy balance. Our data strongly support this view. Central administration of ESI-05 to HFD-induced obese mice significantly reversed ER stress, expression of pro-inflammatory IL-6, and upregulation of the endogenous leptin inhibitor SOCS-3 in the hypothalamus. The effect of ESI-05 is sufficiently robust to restore leptin sensitivity to that of healthy lean mice. ESI-05 treatment is associated with prolonged weight loss maintenance even after treatment cessation. This distinguishing property of ESI-05 makes it more attractive as a potential anti-obesity therapy. While ESI-05 alone showed its effect within a few days after the onset of the treatment (Fig. 5G and H), an acute anorectic effect was only observed with ESI-05 plus a supraphysiological dose of leptin (5 µg). This may be due to differences in the concentration of leptin in the brain. Cerebrospinal fluid leptin concentration (< 0.5 ng/ml) was reported to be one to two orders of magnitude lower than serum levels (Schwartz et al., 1996). In addition, increasing the concentration of exogenous leptin in the brain causes a dose- and time-dependent decrease in body weight and food intake (Halaas et al., 1997; Rahmouni et al., 2002). Therefore, we speculate that the exogenous leptin given to the brain sensitized by ESI-05 causes a more robust and immediate reduction in body weight and food intake, than endogenous leptin. Nevertheless, it is important to note that ESI-05 alone caused a body weight reduction in HFD-induced obese mice. Apart from its action in the brain, Epac2 was reported to be involved in insulin secretion in pancreatic beta cells (Song et al., 2013; Zhang et al., 2009). Further studies will thus need to clarify whether systemic inhibition of Epac2 affects whole body glucose balance. Additional evidence that Rap1 is the molecule that mediates the therapeutic benefit of ESI-05 comes from our experiments using Rap1ΔCNS mice. The lack of ESI-05 effects on leptin sensitivity in Rap1ΔCNS mice strongly suggests that the effect is via Rap1 and also rules out potential off-target effects of ESI-05. Most importantly, ESI-05 infusion alone causes weight loss in diet-induced obese and hyperleptinemic mice, likely by reversing leptin insensitivity associated with HFD-induced upregulation of SOCS-3. Collectively, these results demonstrate the potential of ESI-05 as a leptin sensitizer and provide insight into the promising, translational value of the Rap1 pathway.

EXPERIMENTAL PROCEDURES

Mice and Diets

Mice were used for all experiments. C57BL/6 mice were obtained from the Jackson Laboratory. Rap1alox/lox/Rap1blox/lox mice were provided by Dr. Alexei Morozov (Pan et al., 2008). All genetically modified mice were backcrossed to C57BL/6 (Jackson Laboratories) background more than six times. Rap1ΔCNS mice were generated in the following breeding strategy: double floxed male mice of Rap1a and Rap1b were crossed to the female CaMKIIα Cre driver (line 159) (Minichiello et al., 1999). We used only female CaMKIIICre to obtain cohorts since CaMKIIICre is expressed in testis (Figure 1A) (Minichiello et al., 1999) and the male germline can produce offspring that carries the Cre allele in all tissues. From these matings, we produced mice with deletion of Rap1a and Rap1b in Cre-expressing neurons and control mice with floxed Rap1a and Rap1b genes. All mice were maintained on a 12:12 hr light-dark cycle condition and temperature-controlled environment with ad libitum access to water and normal diet (Pico Lab 5V5R) or high-fat.

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diet (60% kcal fat; Research diet, D12492). Care of all animals and procedures conformed to the Guide for Care and Use of Laboratory Animals of the US National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine (AN-6076).

**Physiological Measurements**

Body weight was measured weekly. Blood samples were collected via saphenous vein from 4-hour-fasted mice. Serum blood was isolated after centrifugation (5000 × g for 10 min) at 4 °C and stored at –80 °C. Blood glucose was measured by using One Touch Ultra Blood Glucose Meter. Plasma leptin and insulin were analyzed with Milliplex MAP Mouse Metabolic Hormone Magnetic Bead Panel Kit. Glucose tolerance tests were performed on overnight fasted mice. D-glucose (1.5 g/kg) was injected intraperitoneally and blood glucose was measured at indicated time periods. Insulin tolerance tests were performed on 4-hour-fasted mice. Insulin (1 U/kg) was injected intraperitoneally and blood glucose was measured at indicated periods.

**Body Composition and Energy Expenditure Measurements**

Whole-body composition was measured using NMR imaging (EchoMRI). Body weight- and body composition-matched 5-week-old control and Rap1ΔCNS mice were fed on a high-fat diet. Two weeks later, metabolic assessment was performed at 7 weeks of age. Mice were first acclimatized to the metabolic cages and housed individually for 3 days before measurements were taken. Metabolic parameters, including O₂ consumption, CO₂ production, respiratory exchange ratio, heat production, ambulatory activity and food intake, were determined by using the Columbus Instruments Comprehensive Lab Animal Monitoring System (CLAMS).

**Leptin Sensitivity Test**

Mice were singly housed and acclimatized for 1 week prior to the study. Body weight- and body composition-matched 15-week-old control and Rap1ΔCNS mice were placed on high-fat diet for 8 weeks. Both groups were injected intraperitoneally with vehicle (Dulbecco’s PBS, dPBS, Sigma-Aldrich, D8537) twice a day (5 p.m. and 9 a.m.) for 4 consecutive days. Three days after the last vehicle treatment, mice were injected intraperitoneally with leptin (3 mg/kg, Harbor-UCLA Research and Education Institute) twice a day for 6 consecutive days. Food intake and body weight were measured daily. Similarly, we performed a leptin sensitivity test for ESI-05 treatment mice. I.c.v. surgery was carried out on mice fed a high-fat diet and then they were singly housed. One week after the i.c.v. surgery, the mice were injected with vehicle, leptin (2 µg/mouse), ESI-05 (0.2 nmol/mouse) or leptin/ESI-05 twice a day. Body weight and food intake were measured daily.

**Organotypic Brain Slice Culture**

Hypothalamic slices were made essentially as described before (Fukuda et al., 2011). Briefly, C57BL/6 mice pups, 8–11 days old, were decapitated, and the brains were quickly removed. Hypothalamic tissues were blocked and sectioned in depth of 250 µm on a vibratome (VT1000S, Leica) in chilled Gey’s Balanced Salt Solution (Invitrogen) enriched
with glucose (0.5%) and KCl (30 mM). The coronal slices containing the arcuate nucleus were then placed on Millicell-CM filters (Millipore, pore size 0.4 µm, diameter 30 mm), and then maintained at an air-media interface in MEM (Invitrogen) supplemented with heat-inactivated horse serum (25%, Invitrogen), glucose (32 mM) and GlutaMAX (2 mM, Invitrogen). Cultures were typically maintained for 10 days in standard medium, which was replaced three times a week. After 10 days, the slices were used for experiments.

Cannula Implantation and Treatments

Mice were anaesthetized with isoflurane and positioned in a stereotaxic frame. The skull was exposed and a 26-gauge single stainless steel guide cannula (C315GS-5-SPC, Plastics One, Roanoke, VA, USA) was implanted into the third cerebral ventricles (−0.9 mm from the bregma, ±0.5 mm lateral, −2.5 mm from the skull). The cannula was secured to the skull with screws and dental cement. After i.c.v. cannulation, the mice were housed singly and given at least 1 week to recover. On experimental days, the mice were infused with 1 µL of each solution: vehicle (dPBS or dimethyl sulfoxide), leptin (2 µg/mouse), ESI-05 (0.2 nmol/mouse, Axxora, BLG-M092-05), Leptin/ESI-05, Tunicamycin (TU, 10 µg/mouse, EMD Millipore, 654380), TU/ESI-05, or TUDCA (2.5 µg/mouse, EMD Millipore, 580549).

Detection of GTP-Rap1 by Rap1 Pull-down Assay

Rap1 pull-down assay was performed using the Active Rap1 Pull-Down and Detection Kit (Thermo Fisher Scientific) according to the manufacturer’s recommendation. After brain samples were dissected out, samples were then snap frozen and subsequently stored at −80°C. Proteins were extracted by the provided lysis/wash/buffer with protease cocktail inhibitor and then lysates were centrifuged at 16,000 × g for 15 min at 4 °C. The protein concentration was determined with the BCA protein assay reagent (Pierce, 23225) with bovine serum albumin as standard. Equal amounts of protein were subjected to affinity precipitation of GTP-Rap1 by using the Active Rap1 Pull-Down and Detection Kit. The amount of Rap1 was assessed by performing Western blotting with provided antibody (1:1,000). To assess the levels of total Rap1 or β-actin, cell extract was directly applied to Western blotting without pull-down assay.

Statistics

The data are presented as mean ± SEM. Statistical analyses were performed using GraphPad Prism for a two-tailed unpaired Student’s t test, or one- or two-way ANOVA followed by post hoc Tukey’s, Bonferroni’s or Sidak's tests. P < 0.05 was considered to be statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

Bates SH, Stearns WH, Dundon TA, Schubert M, Tso AW, Wang Y, Banks AS, Lavery HJ, Haq AK, Maratos-Flier E, et al. STAT3 signalling is required for leptin regulation of energy balance but not reproduction. Nature. 2003; 421:856–859. [PubMed: 12594516]

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]

Bjorbaek C, Elmqquist JK, Frantz JD, Shoelson SE, Flier JS. Identification of SOCS-3 as a potential mediator of central leptin resistance. Mol Cell. 1998; 1:619–625. [PubMed: 960946]

Cakir I, Cyr NE, Perello M, Litvinov BP, Romero A, Stuart RC, Nillni EA. Obesity induces hypothalamic endoplasmic reticulum stress and impairs proopiomelanocortin (POMC) post-translational processing. J Biol Chem. 2013; 288:17675–17688. [PubMed: 23640886]

Coll AP, Faroqui IS, O‘Rahilly S. The hormonal control of food intake. Cell. 2007; 129:251–262. [PubMed: 17448988]

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

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]

De Souza CT, Araujo EP, Bordin S, Ashimine R, Zollner RL, Boscher AC, Saad MJ, Velloso LA. Consumption of a fat-rich diet activates a proinflammatory response and induces insulin resistance in the hypothalamus. Endocrinology. 2005; 146:4192–4199. [PubMed: 16002529]

Dietrich MO, Horvath TL. Hypothalamic control of energy balance: insights into the role of synaptic plasticity. Trends in neurosciences. 2013; 36:65–73. [PubMed: 23318157]

Frederick RC, Hamann A, Anderson S, Lollmann 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]

Fukuda M, Williams Kevin W, Gautron L, Elmqquist Joel K. Induction of Leptin Resistance by Activation of cAMP-Epac Signaling. Cell metabolism. 2011; 13:331–339. [PubMed: 21356522]

Gao Q, Wolfgang MJ, Neschen S, Morino K, Horvath TL, Shulman GI, Fu XY. Disruption of neural signal transducer and activator of transcription 3 causes obesity, diabetes, infertility, and thermal dysregulation. Proc Natl Acad Sci U S A. 2004; 101:4661–4666. [PubMed: 15070774]

Gloerich M, Bos JL. Regulating Rap small G-proteins in time and space. Trends in cell biology. 2011; 21:615–623. [PubMed: 21820312]

Halaas JL, Boozier C, Blair-West J, Fidahusein N, Denton DA, Friedman JM. Physiological response to long-term peripheral and central leptin infusion in lean and obese mice. Proc Natl Acad Sci U S A. 1997; 94:8878–8883. [PubMed: 9238071]

Hosoi T, Sasaki M, Miyahara T, Hashimoto C, Matsuo S, Yoshii M, Ozawa K. Endoplasmic reticulum stress induces leptin resistance. Mol Pharmacol. 2008; 74:1610–1619. [PubMed: 18755873]

Hosoi T, Yamaguchi R, Noji K, Matsuo S, Baba S, Toyoda K, Suezawa K, Kamei K, Tsukahara T, Tanaka T, Oda S. Endoplasmic reticulum stress induces leptin resistance. Mol Pharmacol. 2008; 74:1610–1619. [PubMed: 18755873]

Howard JK, Cave BJ, Oksanen LJ, Tzameli I, Bjorbaek 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]

Kai AK, Lam AK, Chen Y, Tai AC, Zhang X, Lai AK, Yeung PK, Tam S, Wang J, Lam KS, et al. Exchange protein activated by cAMP 1 (Epac1)-deficient mice develop beta-cell dysfunction and metabolic syndrome. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2013; 27:4122–4135. [PubMed: 23825225]
Kawasaki H, Springett GM, Mochizuki N, Toki S, Nakaya M, Matsuda M, Housman DE, Graybiel AM. A family of cAMP-binding proteins that directly activate Rap1. Science. 1998; 282:2275–2279. [PubMed: 9856955]

Kim S, Mizoguchi A, Kikuchi A, Takai Y. Tissue and subcellular distributions of the smg-21/rap1/Krev-1 proteins which are partly distinct from those of c-ras p21s. Mol Cell Biol. 1990; 10:2645–2652. [PubMed: 2111441]

Kleinridders A, Schichten D, Konner AC, Belgardt BF, Mauer J, Okamura T, Wunderlich FT, Medzhitov R, Bruning JC. MyD88 signaling in the CNS is required for development of fatty acid-induced leptin resistance and diet-induced obesity. Cell Metab. 2009; 10:249–259. [PubMed: 19808018]

Konner AC, Bruning JC. Selective insulin and leptin resistance in metabolic disorders. Cell Metab. 2012; 16:144–152. [PubMed: 22883229]

Liu J, Lee J, Salazar Hernandez MA, Mazitschek R, Ozcan U. Treatment of obesity with celastrol. Cell. 2015; 161:999–1011. [PubMed: 26000480]

Liu XB, Murray KD. Neuronal excitability and calcium/calmodulin-dependent protein kinase type II: location, location, location. Epilepsia. 2012; 53(Suppl 1):45–52. [PubMed: 2262808]

Locke AE, Kahali B, Berndt SI, Justice AE, Pers TH, Day FR, Powell C, Vedantam S, Buchkovich ML, Yang J, et al. Genetic studies of body mass index yield new insights for obesity biology. Nature. 2015; 518:197–206. [PubMed: 25673413]

Loh K, Fukushima A, Zhang X, Galic S, Briggs D, Enriori PJ, Simonds S, Wiede F, Reichenbach A, Hauser C, et al. Elevated hypothalamic TCPTP in obesity contributes to cellular leptin resistance. Cell Metab. 2011; 14:684–699. [PubMed: 22000926]

Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. Cell. 2007; 129:1261–1274. [PubMed: 17604717]

Metlakunta AS, Sahu M, Sahu A. Hypothalamic phosphatidylinositol 3-kinase pathway of leptin signaling is impaired during the development of diet-induced obesity in FVB/N mice. Endocrinology. 2008; 149:1121–1128. [PubMed: 18048492]

Milanski M, Degasperi G, Coope A, Morari J, Denis R, Cintra DE, Tsukumo DM, Anhe G, Amaral ME, Takahashi HK, et al. Saturated fatty acids produce an inflammatory response predominantly through the activation of TLR4 signaling in hypothalamus: implications for the pathogenesis of obesity. J Neurosci. 2009; 29:359–370. [PubMed: 19144836]

Minichiello L, Korte M, Wolfer D, Kuhn R, Unsicker K, Cestari V, Rossi-Arnaud C, Lipp HP, Bonhoeffer T, Klein R. Essential role for TrkB receptors in hippocampus-mediated learning. Neuron. 1999; 24:401–414. [PubMed: 10571233]

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]

Morton GJ, Cummings DE, Baskin DG, Barsh GS, Schwartz MW. Central nervous system control of food intake and body weight. Nature. 2006; 443:289–295. [PubMed: 16988703]

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

Myers MG Jr, Leibl RL, Seeley RJ, Schwartz MW. Obesity and leptin resistance: distinguishing cause from effect. Trends Endocrinol Metab. 2012

Myers MG Jr, Olson DP. Central nervous system control of metabolism. Nature. 2012; 491:357–363. [PubMed: 23151578]

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

Ozcan U, 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]

Ozcan U, Yilmaz E, Ozcan L, Furushashi M, Vaillancourt E, Smith RO, Gorgun CZ, Hotamisligil GS. Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. Science. 2006; 313:1137–1140. [PubMed: 16931765]
Pan BX, Vautier F, Ito W, Bolshakov VY, Morozov A. Enhanced cortico-amygdala efficacy and suppressed fear in absence of Rap1. J Neurosci. 2008; 28:2089–2098. [PubMed: 18305243]

Pedroso JA, Buongiorgio DC, Cardinali LI, Furigo IC, Ramos-Lobo AM, Tirapegui J, Elias CF, Donato J Jr. Inactivation of SOCS3 in leptin receptor-expressing cells protects mice from diet-induced insulin resistance but does not prevent obesity. Molecular metabolism. 2014; 3:608–618. [PubMed: 25161884]

Rahmouni K, Haynes WG, Morgan DA, Mark AL. Selective resistance to central neural administration of leptin in agouti obese mice. Hypertension. 2002; 39:486–490. [PubMed: 11882595]

Reed AS, Unger EK, Olofsson LE, Piper ML, Myers MG Jr, Xu AW. Functional role of suppressor of cytokine signaling 3 upregulation in hypothalamic leptin resistance and long-term energy homeostasis. Diabetes. 2010; 59:894–906. [PubMed: 2068134]

Rehmann H. Epac-inhibitors: facts and artefacts. Scientific reports. 2013; 3:3032. [PubMed: 24149987]

Ryan KK, Woods SC, Seeley RJ. Central nervous system mechanisms linking the consumption of palatable high-fat diets to the defense of greater adiposity. Cell Metab. 2012; 15:137–149. [PubMed: 22244528]

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]

Schneeberger M, Dietrich MO, Sebastian D, Imbernon M, Castano C, Garcia A, Esteban Y, Gonzalez-Franquesa A, Rodriguez IC, Borotolozzi A, et al. Mitofusin 2 in POMC neurons connects ER stress with leptin resistance and energy imbalance. Cell. 2013; 155:172–187. [PubMed: 24074867]

Schwartz MW, Peskind E, Raskind M, Boyko EJ, Porte D Jr. Cerebrospinal fluid leptin levels: relationship to plasma levels and to adiposity in humans. Nat Med. 1996; 2:589–593. [PubMed: 8616722]

Song WJ, Mondal P, Li Y, Lee SE, Hussain MA. Pancreatic beta-cell response to increased metabolic demand and to pharmacologic secretagogues requires EPAC2A. Diabetes. 2013; 62:2796–2807. [PubMed: 23578994]

Spilker C, Kreutz MR. RapGAPs in brain: multipurpose players in neuronal Rap signalling. The European journal of neuroscience. 2010; 32:1–9. [PubMed: 20576033]

Tsalkova T, Mei FC, Li S, Chepurny OG, Leech CA, Liu T, Holz GG, Woods VL Jr, Cheng X. Isoform-specific antagonists of exchange proteins directly activated by cAMP. Proc Natl Acad Sci U S A. 2012; 109:18613–18618. [PubMed: 23091014]

Vaisse C, Halaas JL, Horvath CM, Darnell JE Jr, Stoffel M, Friedman JM. Leptin activation of Stat3 in the hypothalamus of wild-type and ob/ob mice but not db/db mice. Nat Genet. 1996; 14:95–97. [PubMed: 8782827]

Walter P, Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. Science. 2011; 334:1081–1086. [PubMed: 22116877]

Williams KW, Liu T, Kong X, Fukuda M, Deng Y, Berglund ED, Deng Z, Gao Y, Liu T, Sohn JW, et al. Xbp1s in Pmc Neurons Connects ER Stress with Energy Balance and Glucose Homeostasis. Cell Metab. 2014

Won JC, Jang PG, Namkoong C, Koh EH, Kim SK, Park JY, Lee KU, Kim MS. Central administration of an endoplasmic reticulum stress inducer inhibits the anorexigenic effects of leptin and insulin. Obesity (Silver Spring, Md). 2009; 17:1861–1865.

Yan J, Mei FC, Cheng H, Lao DH, Hu Y, Wei J, Patrikeev I, Hao D, Stutz SJ, Dineley KT, et al. Enhanced leptin sensitivity, reduced adiposity, and improved glucose homeostasis in mice lacking exchange protein directly activated by cyclic AMP isoform 1. Mol Cell Biol. 2013; 33:918–926. [PubMed: 23263987]

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

Zhang CL, Katoh M, Shibasaki T, Minami K, Sunaga Y, Takahashi H, Yokoi N, Iwasaaki M, Miki T, Seino S. The cAMP sensor Epac2 is a direct target of antidiabetic sulfonylurea drugs. Science. 2009; 325:607–610. [PubMed: 19644119]
Zhang EE, Chapeau E, Hagihara K, Feng GS. Neuronal Shp2 tyrosine phosphatase controls energy balance and metabolism. Proc Natl Acad Sci U S A. 2004; 101:16064–16069. [PubMed: 15520383]

Zhang R, Dhillon H, Yin H, Yoshimura A, Lowell BB, Maratos-Flier E, Flier JS. Selective inactivation of Socs3 in SF1 neurons improves glucose homeostasis without affecting body weight. Endocrinology. 2008a; 149:5654–5661. [PubMed: 18669597]

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. 2008b; 135:61–73. [PubMed: 18854155]
The small GTPase Rap1 in the brain is activated in high-fat diet-induced obesity

Loss of neuronal Rap1 protects against diet-induced obesity and glucose imbalance

Rap1 controls neural leptin sensitivity

Brain Rap1 interacts with ER stress pathways in leptin resistance and obesity
**Figure 1. Validation of Rap1ΔCNS mice**

(A) PCR genotyping analyses were performed from several tissues of Rap1ΔCNS and control (Rap1 floxed) mice. Cre-deleted alleles are detected only in hypothalamus, neocortex, and testis.

(B) Hypothalamic Rap1 mRNA levels were measured using qPCR analyses (n=3 per group).

(C) Rap1 Western blot and densitometric quantification of hypothalamic Rap1. β-Actin was used as a loading control (n=4 per group).
(D) Representative images of brain slices from Rap1ΔCNS mice and control mice stained for Rap1. *(Left)* immunohistochemistry images. *(Right)* Quantification of immunohistochemistry. *P<0.05, **P<0.01, ***P<0.001* compared to control mice based on t-tests in (B, C and D). Arcuate nucleus (ARC), ventromedial hypothalamus (VMH), dorsomedial hypothalamus (DMH). Scale bar, 200 µm. See also Figure S1 and S2.
Figure 2. Loss of neuronal Rap1 protects against diet-induced obesity

(A–L) Weekly body weight of HFD-fed males (A) and females (B) (n=10–17 per group). Body composition in HFD-fed male (E) and female (F) mice at 20 weeks of age. Serum leptin levels in HFD-fed male (I) and female (J) mice at 24 weeks of age. The HFD was initiated at 4 weeks of age. Weekly body weight of normal chow-fed males (C) and females (D) (n=9–12 per group). Body composition in normal chow-fed male (G) and female (H) mice at 20 weeks of age. Serum leptin levels in normal chow-fed male (K) and female (L) mice at 24 weeks of age.
(M–R) Metabolic profile of 2-week HFD-fed male Rap1ΔCNS mice (n=8 per group) on food intake (M), O2 consumption (N), CO2 production (O), heat production (P), ambulatory activity (Q), and respiratory exchange ratio (R) during 24 hour, dark, or light cycles. Note that mice at 7 weeks of age had comparable body weight (control: 23.41 ± 0.7g vs. Rap1ΔCNS: 23.07 ± 0.7g, P>0.05, t-tests), fat mass (control: 4.41 ± 0.7g vs. Rap1ΔCNS: 3.16 ± 0.2g, P>0.05, t-tests) and lean mass (control: 16.86 ± 0.4g vs. Rap1ΔCNS: 17.27 ± 0.5g, P>0.05, t-tests) at the time of the CLAMS study.

(S) Hypothalamic mRNA expression of the feeding-related neuropeptide genes. Hypothalami were collected from HFD-fed male mice at 28 weeks of age (n=6 per group). qPCR analyses were performed to measure mRNAs. *P<0.05, **P<0.01, ***P<0.001 for Two-way ANOVA followed by Sidak's multiple comparisons tests in (A and B) or t-tests in (E, F, G, H, J, M, R and S). See also Figure S3, S4 and S5.
Figure 3. Improved glucose homeostasis in Rap1ΔCNS mice

(A–D) Glucose homeostasis parameters of Rap1ΔCNS or control mice fed a high-fat diet for 24 weeks (n=7–14 per group). Glucose (A), serum insulin levels (B), GTT (C) and ITT (D).

(E–H) Glucose profile of age- and body weight-matched lean cohorts (control: 21.23 ± 0.910 g vs. Rap1ΔCNS: 21.78 ± 0.78 g, P > 0.05 based on t-tests) at 7 weeks of age (n=7–12 per group). Glucose (E), serum insulin levels (F), GTT (G) and ITT (H).

(I) Cellular insulin sensitivity (n=4 per group). Western blot (Left) and quantification (Right) of Akt (Thr308) and GSK-3β (Ser9) phosphorylation in liver, fat and muscle 10 minutes after...
a bolus injection of insulin (1 U/kg, i.p.) or saline into Rap1ΔCNS or control mice fed a HFD for 24 weeks.

(J) qPCR analysis of hepatic mRNA expression of genes encoding G6pc and Pepck of 24-week HFD-fed Rap1ΔCNS mice (n=6 per group).

*P<0.05, **P<0.01, ***P<0.001 for t-tests in (A, B, E and J), Two-way ANOVA followed by Sidak's multiple comparisons tests in (C, D, G and H), or One-way ANOVA followed by Tukey's multiple comparison test in (I). See also Figure S6.
Figure 4. Leptin sensitivity is increased in Rap1ΔCNS mice

(A and B) Male mice were maintained on a high-fat diet for 8 weeks and were injected with leptin (3 mg/kg, twice per day, i.p.) or vehicle during the indicated period. Body weight (A) and food intake (B) were measured every day. Age- and body weight-matched cohorts were used (n=8 per group).

(C) Leptin (3 mg/kg, i.p.) was administered to the indicated mice (n=3 per group). (Left) Representative immunohistochemistry images for pSTAT3. Scale bar, 100 µm. (Right) Quantification of immunohistochemistry.
(D) Hypothalamic expression of genes involved in leptin resistance in Rap1ACNS and control mice. Hypothalami were collected from age-matched normal chow or 4 weeks HFD-fed male mice at 12 weeks of age (after high-fat diet feeding, control: 36.77 ± 1.0 g vs. Rap1ACNS: 34.59 ± 0.6 g, P>0.05 based on t-tests, after normal chow feeding, control: 32.06 ± 1.7 g vs. Rap1ACNS: 29.78 ± 1.3 g, P>0.05 t-tests) (n=4–5 per group). *P<0.05, **P<0.01, ***P<0.001 for Two-way ANOVA followed by Bonferroni’s multiple comparisons tests in (A and B) or One-way ANOVA followed by Tukey’s multiple comparison test in (C and D). See also Figure S3 and S7.
Figure 5. ESI-05 reverses leptin resistance in HFD-induced obese mice
(A and B) ESI-05 enhances leptin-induced body weight reduction (A) and food intake suppression (B). Leptin (2 µg) or vehicle was i.c.v. infused with or without ESI-05 (0.2 nmol) to HFD-fed obese C57BL/6 mice (HFD for 5 months, n=8–10 per group) or lean normal chow-fed C57BL/6 mice (n=5 per group) (twice per day for 3 days).
(C) Western blot images (Top) and quantification (Bottom) of hypothalamic STAT3 (Tyr\textsuperscript{705}) and S6K (Thr\textsuperscript{389}) phosphorylation 1 hour after a bolus injection of leptin (2 µg, i.c.v.) or

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saline into HFD-fed mice that received ESI-05 (0.2 nmol, i.c.v.) or vehicle 3 hours before leptin injection (n=5 per group).

(D) Representative immunohistochemistry images of hypothalamic pSTAT3. HFD-fed obese mice received ESI-05 (2 nmol, i.c.v.) or vehicle followed 3 hours later by i.c.v. injection of leptin (2 µg) for 1 hour. Scale bar, 100 µm.

(E and F) Effect of ESI-05 on leptin sensitivity in Rap1^{ACNS} mice. Body weight change (E) and food intake (F). HFD-fed obese control or Rap1^{ACNS} mice (HFD for 5 weeks, n=5–7 per group) received i.c.v. injections of leptin (2 µg) with or without ESI-05 (0.2 nmol) twice a day over 3 days.

(G and H) Effect of ESI-05 on body weight and food intake in HFD-induced obese mice. Body weight change (G) and food intake (H). HFD-fed obese C57BL/6J mice (HFD for 16 weeks, n=10 per group) received i.c.v. injections of ESI-05 (0.2 nmol for Day 1–14, 1 nmol for Day 15–18), once a day.

*P<0.05, **P<0.01, ***P<0.001 for Two-way ANOVA followed by Tukey’s multiple comparison test in (A, B, E and F) or Sidak’s multiple comparison test in (C, G and H).
Figure 6. Rap1 mediates leptin resistance conferred by chemically-induced ER stress

(A) Effect of ESI-05 on multiple forms of leptin resistance in organotypic brain slices. The slices were incubated with either forskolin (Fsk, 20 µM), thapsigargin (Tg, 30 µM), tunicamycin (TU, 30 µM), dithiothreitol (DTT, 1 mM) or a high dose of leptin (Hyperleptinemia, 120 nM) in the presence or absence of ESI-05 (50 µM) for 6 hours, and then stimulated with leptin (120 nM, 60 min). Leptin-induced pSTAT3 are shown. Scale bar, 100 µm.

(B) Quantification of hypothalamic pSTAT3 (n=3–21 per group) in organotypic brain slices.
(C) Activation of brain Rap1 by chemically induced ER stress. Lean C57BL/6 mice were administered with Tunicamycin (10 µg, i.c.v.) for the indicated period (n=5–6 per group). Proteins were extracted from the treated brains and Rap1 activity was measured.

(D) ESI-05 blocks ER stress-induced leptin resistance in vivo. Tunicamycin (10 µg, i.c.v.) was injected with or without ESI-05 (0.2 nmol, i.c.v.) into the brain of lean C57BL/6 mice. Three hours later, leptin (5 µg, i.c.v.) was administered to the mice (n=4–5 per group). The hypothalami were collected at 60 min after leptin injection and subjected to Western blot analysis using pSTAT3 antibodies.

(E) Relative mRNA expression of Socs-3, Ptp1b, Tcptp and Shp2 in brains of the mice centrally receiving Tunicamicin (10 µg) with or without ESI-05 (0.2 nmol) for 4 hours (n=12–13 per group).

*P <0.05, **P<0.01, ***P<0.001 for One-way ANOVA followed by Tukey's multiple comparison test in (B, C, D and E).
Figure 7. Blockade of Rap1 signaling in the CNS protects mice from HFD induction of hypothalamic ER stress and IL-6

(A) Western blot images (Left) and quantification (Right) of the amount of active form of Rap in the brain of lean mice or HFD-induced obese mice that received ESI-05 (0.2 nmol, i.c.v., twice a day for 3 days) or vehicle (n=10 per group).

(B) Relative mRNA expression of Socs-3, Ptp1b, Tcptp, Shp2, Il-6, Xbp1s, Chop, Edem, Atf4 and Grp94, in the hypothalamus of ESI-05-treated HFD-induced obese mice or lean control mice. Mice were maintained on a HFD or a normal chow for 33 weeks and received ESI-05 (0.2 nmol, i.c.v., twice a day) or vehicle for 3 days (n=9 per group).
(C) Western blot images (Left) and quantification (Right) of SOCS-3, BIP, CHOP, eIF2α, PERK, β-Actin, and phosphorylated forms of eIF2α and PERK in the hypothalamus of HFD-induced obese mice that were given ESI-05 (0.2 nmol, i.c.v., twice a day for 10 days) or vehicle (n=7–10 per group).

(D) Relative mRNA expression of Il-6, Xbp1s, Bip, Chop, Edem, Atf4 and Grp94 in the hypothalamus of Rap1ΔCNS, Rap1ΔCNS or control animals were challenged with a HFD for 4 weeks. Age- and body weight-matched cohorts were used (n=4–5 per group).

(E) Relative mRNA expression of Xbp1s, Bip, Chop, Edem, Atf4 and Grp94 in brains of the mice. The hypothalami were collected from HFD-fed obese or normal chow fed mice (HFD for 62 weeks, n=4–7 per group) that received vehicle or TUDCA (2.5 µg, i.c.v.) for 3 days. Hypothalamic mRNA levels were determined by qPCR analyses.

(F and G) A chemical chaperone decreased both Rap1 activity (F) and mRNA expression of Socs-3 (G) in the brain of HFD-induced obese mice. The brain samples were collected from HFD-fed obese or normal chow fed mice (HFD for 62 weeks, n=4–7 per group) that received TUDCA (2.5 µg, i.c.v.) for 3 days, and subjected to Rap1 assay and qPCR analyses.

*P <0.05, **P<0.01, ***P<0.001 for One-way ANOVA followed by Tukey's multiple comparison test in (A, B, D, E, F, and G) or t-tests in (C).