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Rho-kinase Regulates Energy Balance by Targeting Hypothalamic Leptin Receptor Signaling

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Abstract

Leptin regulates energy balance. However, knowledge of the critical intracellular transducers of leptin signaling remains incomplete. Here we report that Rho-kinase 1 (ROCK1) regulates leptin action on body weight homeostasis by activating JAK2, an initial trigger of leptin receptor signaling. Leptin promotes the physical interaction of JAK2 and ROCK1, thereby increasing phosphorylation of JAK2 and downstream activation of Stat3 and FOXO1. Mice lacking ROCK1 in either POMC or AgRP neurons, mediators of leptin action, display obesity and impaired leptin sensitivity. In addition, deletion of ROCK1 in the arcuate nucleus markedly enhances food intake, resulting in severe obesity. Of note, ROCK1 is a specific mediator of leptin, but not insulin, regulation of POMC neuronal activity. Our data identify ROCK1 as a key regulator of leptin action on energy homeostasis.

Introduction

Obesity is often associated with an eating disorder, which contributes to dysregulation of body-weight homeostasis and energy balance[1]. The hypothalamus is a key site that plays an essential role in the physiologic regulation of food intake and body weight[2]. It is established that impaired leptin signaling in the hypothalamus causes hyperphagia, which then promotes

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AUTHOR CONTRIBUTIONS

Y.B.K., H.H., and B.B.L. designed the study. J.M.Z. and C.B. provided conceptual advice. H.H performed most of mice experiments and some of the *in vitro* studies. D.K. generated several lines of model mice for the study. K.H.B and B.L performed most of the *in vitro* studies including PLA and FCCS experiments. C.Y was responsible for electrophysiology studies. S.K. carried out AAV injection experiments. D.H.L bred and maintained ROCK1 floxed mice. S.W.L. generated adenovirus encoding ROCK1. B.C.O. performed immunoblotting analyses. M.S.K. performed food intake studies with adenovirus. C.B. carried out IHC experiments. All authors analyzed and interpreted experimental data. J.M.Z., C.B., B.B.L., and Y.B.K. co-wrote the manuscript

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adiposity and weight gain[3]. Over the past decade, there have been intensive efforts to identify mediators of leptin action in the hypothalamus that control feeding behavior, energy expenditure, and glucose metabolism[4].

In the central nervous system, a long-form leptin receptor (LepRb) is expressed in the hypothalamic arcuate nucleus (ARC), which is involved in the regulation of body weight and energy metabolism[5,6]. Two distinct populations of neurons, pro-opiomelanocortin (POMC)- and agouti-related protein (AgRP)/neuropeptide Y (NPY)-producing neurons, in the hypothalamic ARC, are directly regulated by leptin[7,8]. The anorexigenic POMC neurons are activated by leptin, whereas the orexigenic AgRP/NPY-producing neurons are inhibited by it[5,9]. Consistent with this, hypothalamic POMC mRNA levels are reduced in leptin-deficient *ob/ob* mice, and they are elevated by leptin supplementation[10–12]. Studies have shown that mice lacking LepRb in POMC[13] or AgRP neurons[14] are obese on normal chow diet and that LepRb deficiency in both POMC and AgRP neurons has additive effects on body weight. These data clearly suggest that leptin signaling in POMC- and AgRP-expressing neurons in the hypothalamic ARC is required for the regulation of normal body-weight homeostasis.

Rho-kinase (ROCK) is a Ser/Thr protein kinase identified as a GTP-Rho-binding protein[15]. ROCK isoforms (ROCK1 and ROCK2) in endothelial cells, heart, and skeletal muscle are involved in the pathogenesis of metabolic-related diseases such as hypertension and diabetes[16,17]. We and others have reported that ROCK isoforms in peripheral insulin-sensitive cells and tissues are important regulators of insulin receptor-PI3K signaling and glucose metabolism[17–21]. Along with this, accumulating evidence shows that key insulin signaling mediators in hypothalamic neurons, including PI3K, PTEN, and mTOR, regulate adiposity and energy metabolism in mice[22–25]. In the hypothalamus, leptin binding to LepRb activates JAK2 tyrosine kinase, which is constitutively associated with the receptor, leading to enhanced downstream signaling, including Stat3 phosphorylation and PI3K activation[3].

Since hypothalamic insulin and leptin signaling pathways overlap[2], we investigated the physiological role of ROCK1 in hypothalamic control of food intake and body weight, with particular emphasis on the metabolic action of leptin in hypothalamic arcuate neurons. Here we demonstrate that hypothalamic ROCK1 activation is necessary for the homeostatic regulation of feeding behavior and adiposity by targeting JAK2 in the neuronal leptin receptor signaling pathway, suggesting that ROCK1 is a central regulator of leptin action.

Results

ROCK1 induces JAK2 phosphorylation by direct interaction

The role of ROCK1 in leptin signaling is not known. To determine whether ROCK1 regulates leptin signaling, we measured leptin-induced JAK2 phosphorylation in hypothalamic GT1-7 cells, which express the endogenous LepRb. ROCK1 Inhibition prevented JAK2 phosphorylation by leptin (Fig. 1a). Leptin-stimulated JAK2 phosphorylation was reduced by either DN-ROCK1 overexpression or ROCK inhibitor treatment, both of which block ROCK1 catalytic activity, indicating ROCK1 activity is necessary for leptin-dependent JAK2 phosphorylation (Fig. 1b–c). Furthermore, the physical interaction of ROCK1 and JAK2, and ROCK1-associated JAK2 phosphorylation, were greatly increased in GT1-7 cells and murine hypothalamus *in vivo* in response to leptin (Fig. 1d–f). Importantly, this interaction was also observed within GT1-7 cells by fluorescence cross-correlation spectroscopy (FCCS) analyses and by proximity ligation assays (PLA) (Fig. 1g–j). However, ROCK1 binding to LepRb was not detected during leptin stimulation

(Supplementary Fig. 1a). Collectively, these data suggest that ROCK1 activation is required for leptin-mediated JAK2 activation, the initial key trigger of leptin signaling.

To further investigate the effects of ROCK1 on downstream LepRb signaling pathways, we measured Stat3 phosphorylation and SOCS3 expression in cultured cell lines. ROCK1 inhibition decreased leptin-induced Stat3 phosphorylation in GT1-7 cells and CHO cells expressing LepRb, whereas ROCK1 overexpression enhanced Stat3 activation by leptin (Fig. 2a and Supplementary Fig. 1b). Leptin markedly increased the gene expression of SOCS3 by ~4-fold in GT1-7 cells but this effect was completely blocked by suppression of ROCK1 (Fig. 2b). These data suggest that ROCK1 functions as an enhancer of proximal LepRb-JAK2 signaling, thereby stimulating downstream LepRb pathways.

Leptin-mediated FOXO1 nuclear export is dependent on ROCK1

To determine whether ROCK1 acts as an upstream effector of PI3K signaling, we assessed leptin-induced FOXO1 nuclear export, an indicator of activation of the PI3K/Akt signaling pathway by leptin[26]. In the basal state, the majority of FOXO1 is localized in the nucleus. Upon leptin stimulation, FOXO1 nuclear export was increased but this effect was blocked when ROCK1 expression was inhibited (Fig. 2c–d). Similarly, leptin-mediated FOXO1 nuclear export was blocked by JAK2 or Rho-kinase inhibitors (Supplementary Fig. 1c). These data, combined with the results of cell-fractionation analyses (Supplementary Fig.1d–f), suggest that ROCK1 acts as a key upstream regulator of the PI3K signaling pathway.

Leptin increases ROCK1 activity in hypothalamus

ROCK1 protein is expressed in most neurons in the brain, including hypothalamic arcuate neurons such as POMC and AgRP/NPY neurons and is highly co-localized (>95%) with the LepRb or pStat3 in arcuate neurons (Supplemental Fig. 2a–e). To determine whether leptin increases the enzyme activity of hypothalamic ROCK1 *in vivo*, we measured ROCK1 activity by immuncomplex assay. An intracerebroventricular (icv) injection of leptin in C57BL/6 mice increased ROCK1 activity in the hypothalamus. Intraperitoneal (ip) leptin also stimulated hypothalamic ROCK1 activity (Fig. 3a). In addition, a low dose of icv leptin increased ROCK1 activity in the hypothalamus of *ob/ob* mice (Fig. 3b). However, in *db/db* mice, hypothalamic ROCK1 did not respond to leptin, evidencing that ROCK1 activity stimulated by leptin is mediated via LepRb. Acute nutritional changes (24 hr fasting and 2 hr refeeding) did not affect hypothalamic ROCK1 activity (not shown). Collectively, these data imply that ROCK1 is involved in leptin signaling in the hypothalamus.

Deletion of ROCK1 in POMC neurons causes obesity

To explore the importance of ROCK1 on POMC neurons in control of energy metabolism, we characterized the metabolic phenotype of POMC neuron-specific ROCK1-deficient mice. As important negative controls, we noted that POMC-Cre mice and $ROCK1^{flox/flox}$ mice have similar body weight compared to wild-type (not shown). However, *POMC-Cre,* $ROCK1^{flox/flox}$ mice had significantly increased body weight compared with $ROCK1^{flox/flox}$ mice (Fig. 4a). When examined at the age of 20-weeks, body weights of *POMC-Cre,* $ROCK1^{flox/flox}$ mice were found to be ~10% higher (Male (g): 37.2 ± 1.2 vs 40.9 ± 1.3*, $*p < 0.05$, $t = -2.06$, Female (g): 28.4 ± 1.0 vs 30.7 ± 0.8*, $*p < 0.05$, $t = -2.10$) (Fig. 4a). The increased body weight is likely due to an increase in fat mass (Fig. 4b–c). In addition, *POMC-Cre,* $ROCK1^{flox/flox}$ mice had the same body length as controls (Supplementary Fig. 3f). Furthermore, femur length, bone mineral density, and bone mineral content were normal (Supplementary Fig. 3g–i). POMC neuron morphologies and projections were similar in $ROCK1^{flox/flox}$ and *POMC-Cre,* $ROCK1^{flox/flox}$ mice (Supplementary Fig. 4a–c). Together, our findings demonstrate that deficiency of ROCK1 in the POMC neurons causes an

impairment of energy metabolism that can lead to an increase in body fat, establishing a new role for ROCK1 in body-weight homeostasis.

Deficiency of ROCK1 in POMC neurons increases food intake—We further characterized the metabolic phenotype of POMC neuron-specific ROCK1-deficient mice. Daily food intake in mice lacking ROCK1 in POMC neurons was significantly increased compared with control mice (Supplementary Fig. 3a). It thus appears that increased adiposity can be explained by hyperphagia in these mice, suggesting that ROCK1 plays a pivotal role in regulating food intake.

At ~20 weeks of age, POMC neuron-specific ROCK1-deficient mice displayed hyperleptinemia and hyperinsulinemia, indicating a higher risk for leptin and insulin resistance (Supplementary Fig. 3b–c). Indeed, *POMC-Cre, ROCK1*$^{flox/flox}$ mice had subtle insulin resistance as evidenced by a modest defect in the ability of insulin to decrease blood glucose (Supplementary Fig. 5a). Neither glucose nor serum corticosterone was altered in mice lacking ROCK1 in POMC neurons (Supplementary Fig. 3d–f). Mice lacking ROCK1 in POMC neurons had reduced hypothalamic NYP mRNA levels, while POMC and AgRP mRNA also tended to be reduced in these mice (Supplementary Fig. 3j).

ROCK1 deletion in POMC neurons suppresses locomotion—Leptin signaling in hypothalamic neurons is involved in regulating locomotor activity[27–29]. To determine whether blockade of ROCK1 signaling in POMC neuron affects locomotor activity, we measured ambulatory movements of *POMC-Cre, ROCK1*$^{flox/flox}$ mice. Locomotor activity for 24 hours was decreased in *POMC-Cre, ROCK1*$^{flox/flox}$ mice compared with *ROCK1*$^{flox/flox}$ mice (Fig. 4d). The majority of the decrease occurred during the dark period (Fig. 4e) and these results were confirmed by assessing locomotor activity for 2 weeks, using an infrared beam break system (Supplementary Fig. 5c). However, energy expenditure was unchanged by ROCK1 deficiency in POMC neurons (Fig. 4f). These data suggest that ROCK1 plays a key role in regulating POMC neuron-mediated locomotor activity.

ROCK1 deletion impairs leptin action in POMC neurons

To explore whether deficiency of ROCK1 in POMC neurons alters cellular and whole-body leptin sensitivity, we measured leptin-stimulated Stat3 phosphorylation in weight-matched *ROCK1*$^{flox/flox}$ and *POMC-Cre, ROCK1*$^{flox/flox}$ 6 week-old mice. For Stat3 phosphorylation, leptin-responsive POMC neurons identified by double IHC analysis were counted. Of the 242 POMC neurons examined in *ROCK1*$^{flox/flox}$ mice, leptin induced Stat3 phosphorylation in ~ 55% of the neurons. In contrast, in *POMC-Cre, ROCK1*$^{flox/flox}$ mice, leptin induced Stat3 phosphorylation in only ~ 25% of the 251 POMC neurons, (Fig. 5a–b). Quantification analysis indicates that leptin-dependent Stat3 phosphorylation in individual POMC neurons was decreased by 50% in *POMC-Cre, ROCK1*$^{flox/flox}$ mice compared with *ROCK1*$^{flox/flox}$ mice (Fig. 5b). However, Stat3 phosphorylation by leptin in non-POMC neurons of hypothalamic arcuate was comparable in both groups (Fig. 5c). Similar results of Stat3 phosphorylation were seen in 20 week-old mice (Fig. 5b–c and Supplementary Fig. 6a). Immunoblotting of Stat3 phosphorylation also showed a similar reduction in arcuate tissue from *POMC-Cre, ROCK1*$^{flox/flox}$ mice compared with *ROCK1*$^{flox/flox}$ mice (Supplementary Fig. 6b). Collectively, these findings suggest that central leptin resistance in POMC neurons is due to direct loss of ROCK1, and not to obesity per se.

Food intake in *ROCK1*$^{flox/flox}$ mice was markedly decreased at 8 – 24 hours after the injection of ip leptin. However, the ability of leptin to decrease food intake was impaired in *POMC-Cre, ROCK1*$^{flox/flox}$ mice (Fig. 5d). Moreover, fasting-induced food intake was elevated in these mice (Fig. 5e). Together, our data suggest that ROCK1 in POMC neurons

is required for maximal LepRb neuronal signaling and the anorexigenic effect of leptin, and further indicate that the increased adiposity and caloric intake of POMC neuron-specific ROCK1-deficient mice are caused by deficient LepRb signaling in POMC neurons.

ROCK1 is required for leptin-induced POMC neuronal activity

Neuronal leptin action likely involves modulation of the electrical activity of hypothalamic neurons[23,30]. Specifically, leptin normally depolarizes and increases the firing rate of POMC neurons[30]. To determine the role of ROCK1 signaling in leptin action on POMC neurons, we performed electrophysiologic recordings on GFP-labeled POMC neurons in hypothalamic slices of *POMC-Cre, lox-GFP, ROCK1$^{wt/wt}$* (control) and *POMC-Cre, lox-GFP, ROCK1$^{flox/flox}$* (KO) 4 week-old mice. Whole-cell patch recordings revealed that the basal activities of these neurons were similar between genotypes (Fig. 6 a–b). Leptin induced depolarization and increased firing rate in 10 of 18 POMC neurons in control mice (Fig. 6a). In contrast, leptin had no effect on membrane potential and firing rate in the 11 POMC neurons from *POMC- Cre, lox-GFP, ROCK1$^{flox/flox}$* mice we examined (Fig. 6b). After observing no effect of leptin on POMC neurons lacking ROCK1, a recording buffer containing higher concentration of K^+ (40 μM NaCl was replaced by 40 μM KCl) was used at the end of each recording to ensure that these POMC neurons of *POMC-Cre, lox-GFP, ROCK1$^{flox/flox}$* mice were indeed capable of being activated (not shown). In control mice, leptin depolarized POMC neurons by 4.0 ± 0.2 mV and increased the firing rate by 2.1-fold, whereas in *POMC-Cre, lox-GFP, ROCK1$^{flox/flox}$* mice, leptin failed to depolarize POMC neurons and increase the firing rate (Fig. 6c–d). Collectively, these data demonstrate that ROCK1 is required for leptin-dependent regulation of neuronal activity of POMC neurons.

In contrast, insulin hyperpolarized POMC neurons and decreased their firing rate in control mice (Fig. 6e). These effects were also found in POMC neuron-specific ROCK1-deficient mice (Fig. 6f), indicating that insulin-mediated changes in neuronal activity of POMC neurons occur independently of ROCK1. These findings are consistent with previous studies showing that a subset of POMC neurons is insulin-responsive[22–24]. Taken together, our data further suggest that ROCK1 regulation of POMC neuronal activity is leptin-specific.

Loss of ROCK1 in AgRP neurons leads to obesity

To determine the physiological role of ROCK1 in AgRP neurons on body weight homeostasis, we assessed the adiposity of AgRP neuron-specific ROCK1-deficient mice. Consistent with the results of POMC neuron-specific ROCK1-deficient mice, body weight was increased ~12% in AgRP neurons-specific ROCK1-deficient mice compared with control mice at the age of 18-weeks (Male (g): 34.3 ± 1.5 vs 39.8 ± 1.2*, *$p < 0.01$, $t = -2.83$) (Fig. 7a). These effects were due to an increase in fat mass but not lean mass (Fig. 7b). Concurrently, adipose tissues of AgRP neurons-specific ROCK1-deficient mice were significantly increased compared to control (Fig. 7c). Together, our findings demonstrate that ROCK1 plays an important role in the regulation of body weight and adiposity in AgRP expressing neurons.

Hypothalamic ROCK1 promotes food intake and adiposity

To further test whether deficiency of ROCK1 in the broader hypothalamic ARC area of neurons leads to severe obesity, we injected adeno-associated virus encoding *Cre* recombinase (AAV-Cre) bilaterally into hypothalamic arcuate of *ROCK1$^{flox/flox}$ mice* and adenovirus encoding ROCK1 into hypothalamic arcuate of C57BL/6 mice. Control mice were injected with AAV encoding GFP (*AAV-GFP*) or adenovirus encoding GFP (*Ad-GFP*). We targeted a larger area, the mediobasal hypothalamus encompassing the ARC, VMH, and DMH (Fig. 8a). Immunoblotting analysis indicated that ROCK1 expression

levels in the hypothalamus were reduced in *AAV-Cre* injected *ROCK1*$^{flox/flox}$ mice and were increased in adenovirus ROCK1-injected mice (Fig. 8a).

Twenty weeks after the AAV injection, body weight was greatly increased in *AAV-Cre* injected *ROCK1*$^{flox/flox}$ mice compared to that of *AAV-GFP*-injected *ROCK1*$^{flox/flox}$ mice (body weight (g): 32.7 ± 1.6, *AAV-GFP* vs 42.3 ± 2.6*, *AAV-Cre*, *$p < 0.05$, t = 2.95) (Fig. 8b). These effects are most likely due to increased food intake (Fig. 8e). In parallel, total fat mass also was increased but lean mass was similar in *AAV-Cre* injected *ROCK1*$^{flox/flox}$ mice (Fig. 8c). Conversely, adenovirus-mediated overexpression of hypothalamic ROCK1 notably decreased food intake and body weight for 7 days after the adenovirus injection (Fig. 8d–e). Collectively, these data suggest that ROCK1 activation in the ARC neurons is necessary for normal food intake and body-weight homeostasis.

Discussion

The current study explored the role of ROCK1 in the regulation of food intake and energy metabolism, focusing on the possibility that hypothalamic ROCK1 activation is required for normal body-weight regulation. Although ROCK1 has not previously been suspected to play a role in control of energy balance, our data demonstrate the necessity of hypothalamic ROCK1 in the regulation of food intake and normal body-weight homeostasis, by phosphorylation and activation of leptin receptor-associated JAK2. Thus, we identify ROCK1 as a key regulator of adiposity, and a potentially novel therapeutic target for the prevention and treatment of obesity.

Our experimental evidence demonstrates that leptin-dependent JAK2 tyrosine phosphorylation is markedly reduced by inactivation of ROCK1, suggesting that ROCK1 acts at a proximal step in the LepRb signaling pathway., We also show that upon leptin stimulation, ROCK1 physically interacts with JAK2, but not LepRb, the initial crucial component in intracellular LepRb signal transduction. Tyrosine phosphorylation of JAK2 proteins in ROCK1-JAK2 complexes is greatly elevated in samples from leptin-treated cells or hypothalamus tissues indicating enhanced JAK2 enzymatic activity. It is therefore predicted that all LepRb-pathways downstream of JAK2 are influenced by ROCK1, including Stat3 and FOXO1 regulation, both of which are impaired in cells lacking ROCK1 activity during leptin stimulation. In this regard, it should be noted that leptin-mediated Stat3 tyrosine phosphorylation in ROCK1 knockdown GT1-7 cells and in POMC neurons lacking ROCK1 is impaired by ~50% when JAK2 phosphorylation is nearly abolished. The discrepancy may be due to the fact that leptin-induced Stat3 phosphorylation can occur in the absence of JAK2, presumably via Src and Fyn signaling[31]. Thus, it is likely that the partial inhibition of leptin-stimulated Stat3 phosphorylation by ROCK1 loss is due to JAK2-independent leptin signaling.

A crucial metabolic signal is the fat-derived anorexigenic hormone leptin, which directly activates hypothalamic POMC neurons, an important site for the regulation of energy homeostasis[13,30]. We thus investigated the role of ROCK1 in POMC-expressing neurons. Interestingly, the metabolic phenotypes of POMC neuron-specific ROCK1-deficient mice are nearly identical to those of POMC neuron-specific LepRb-deficient mice[13]. For example, the degree of obesity (~10%) seen in *POMC-Cre, ROCK1*$^{flox/flox}$ mice is similar to that seen in *POMC-Cre, LepRb*$^{flox/flox}$ mice, and also consistent with the recent findings that restoration of LepRb in only POMC neurons in LepRb-deficient mice causes a ~10% decrease in body weight relative to male *Lepr*$^{db/db}$ control mice of same age[29]. Thus, ROCK1 signaling in POMC neurons is a key downstream regulator of LepRb signaling that is necessary for the homeostatic control of body weight. Furthermore, the degree of obesity

caused by ROCK1 deficiency is consistent with the possibility that ROCK1 is a key cellular mediator of LepRb signaling in POMC neurons.

An important function of leptin is to modulate the function of specific neuronal subtypes by altering neuronal electrical activity in hypothalamic neurons[23,30]. Electrophysiological studies have shown that leptin depolarizes and increases the firing rate of POMC neurons[31] and inhibits the tone of AgRP neurons[9,30]. Specifically, leptin's effect on POMC neuronal activity is thought to be mediated via the PI3K signaling pathway[22]. In addition to this current dogma, our data suggest the requirement of ROCK1 in leptin-induced neuronal activity of POMC neurons, as revealed by the ablation of leptin's effects on membrane potential and firing rate in the absence of ROCK1. These results are similar to findings in mice lacking PI3K in POMC neurons, raising the possibility that both ROCK1 and PI3K activate the same signaling node to regulate neuronal activity. This notion is further supported by our findings showing that the PI3K-FOXO1 pathway is regulated via a JAK2/ROCK1-dependent signaling mechanism. Thus, we propose that ROCK1 is a key upstream regulator of PI3K and that ROCK1-PI3K activation is necessary for the control of leptin-dependent electrical activity in the hypothalamic POMC neurons, establishing a new ROCK1-PI3K signaling axis in neuronal leptin action.

It is yet unclear to what extent POMC neuronal activity is required for the anti-obesity actions of leptin. Hill et al reports that PI3K signaling in POMC neurons is not a key contributor of long-term body-weight regulation despite its critical role in acute control of POMC neuronal activity[22]. Our study demonstrates that modulation of ROCK1 in POMC neurons has a significant impact on both long-term body-weight homeostasis and on neuronal activity. If indeed it is the case that increased firing of POMC neurons by leptin does not influence long-term energy balance as surprisingly reported by Hill et al, it has to be concluded that the increased adiposity of *POMC-Cre, ROCK1$^{flox/flox}$* mice is caused by dysregulation of signaling pathways other than the PI3K pathway. For example, it is possible that the observed impaired leptin-activated Stat3 signaling in *POMC-Cre, ROCK1$^{flox/flox}$* mice is responsible for the obesity of these animals, since global deletion of the Stat3 activation site on LepRb causes massive obesity[32]. On the other hand, selective deletion of Stat3 in POMC neurons only causes mild obesity in female mice[33], whereas we show obesity in both males and females. This might suggest that the deletion of ROCK1 affects several intracellular pathways in addition to Stat3. This possibility is consistent with our data showing that ROCK1 acts at the level of JAK2, the key upstream mediator of most if not all LepRb signaling pathways.

Given that inhibition of ROCK1 expression or activity impairs leptin signaling, it is hypothesized that ROCK1 deficiency would block leptin signaling in AgRP neurons, thereby leading to the impaired metabolic action of leptin. As a result, mice lacking ROCK1 in AgRP-expressing neurons would be expected to be obese, as our data show. Taken together with our data showing ROCK1 deficiency in POMC neurons impairs leptin but not insulin stimulated neuronal activity, these data suggest that ROCK1 regulation of AgRP (and POMC) neuron functions in body weight homeostasis is leptin-specific, rather than a general perturbation of AgRP neuronal physiology.

Considering that other neurons and sites in the hypothalamus are also involved in leptin's regulation of normal body weight[6], it is hypothesized that ROCK1 deletion in wider regions of the hypothalamus can further accelerate the development of adiposity compared to deletion of ROCK1 in arcuate POMC or AgRP/NPY neurons. Consistent with this, our data show that a dramatic increase (~30%) in body weight is observed when ROCK1 is deleted in the hypothalamic regions that include ARC (possibly containing POMC, AgRP/NPY, and other neurons), VMH and DMH. The relative magnitude of increased body weight is ~3-fold

compared to that of POMC neuron-specific ROCK1-deficient mice at the age of ~20 weeks, highlighting the importance of ROCK1 in other regions of the hypothalamus for energy balance. Supporting this, it has been shown that LepRb deficiency in both POMC and AgRP neurons (within ARC region) or in both POMC and SF-1 neurons (ARC and non-ARC region) has additive effects on body weight[14,34]. Whether deficiency of ROCK1 on these neurons has additive or synergistic effects on body-weight gain is yet to be determined.

It should be noted that the metabolic phenotypes resulting from hypothalamic ROCK1 knockout or knockdown may also involve changes in other intracellular signaling pathways such as insulin or BDNF, both of which stimulate PI3K signaling[20,35]. Indeed, our previous work demonstrates that insulin's ability to activate PI3K is impaired by ROCK1 deficiency in skeletal muscle[20]. Our data also do not establish the exact role of ROCK1 activity in normal physiological or pathophysiological leptin action. While ROCK1 activity was increased in the hypothalamus upon supraphysiologic leptin stimulation, it was not changed by acute physiological fasting (24 hr) or refeeding (2 hr). These data imply that though hypothalamic ROCK1 is required for intact leptin signaling, dynamic alterations in ROCK1 level or activity may play a limited role in acute changes in energy balance under physiologic or pathophysiologic circumstances. Further investigations are therefore needed to clarify this important question.

In conclusion, this study demonstrates that ROCK1 is an important positive regulator of leptin receptor signaling in hypothalamic neurons. Upon leptin stimulation, ROCK1 is activated and rapidly phosphorylates JAK2 on serine residues, which in turn promotes downstream signaling pathways of leptin, including Stat3 and PI3K signaling, ultimately leading to control of energy balance (Supplementary Fig. 8c). We thus propose that ROCK1 will need to be posted as a new component of the current leptin signaling paradigm in the hypothalamus. This model provides a new mechanism that advances our understanding of central leptin action and body weight regulation.

Methods

Cell culture, transfection and transduction

For knockdown studies, immortalized hypothalamic GT1-7 cells[36] or CHO cells expressing the long-form leptin receptor[37] were transiently transfected with siRNA for murine ROCK1 or luciferase, and stimulated with leptin (100 nM) or vehicle (control) for 15 min. The sequence used for ROCK1 siRNA is 5' UCCAAGUCACAAGCAGACAAGGAUU-3'. For overexpression studies, GT1-7 cells were transfected with DN-ROCK1 cDNA and CHO cells were transduced by adenovirus for wild type ROCK1 or β-galactosidase (β-Gal) as described[19]. For inhibitor studies, GT1-7 cells were pretreated with AG490 (30 μM, JAK2 inhibitor) or Y-27632 (10 μM, ROCK inhibitor) for 1 – 3 hr. For SOCS3 mRNA measurement, GT1-7 cells were treated with leptin (100 nM) or vehicle for 1 hr. SOCS3 mRNA was measured by TaqMan quantitative RT-PCR and normalized to 18S ribosomal RNA content. The primers for SOCS3 are FP 5'-GTCACCCACAGCAAGTTTCC-3' and RP 5'-TCCAGTAGAATCCGCTCTCC-3'.

Proximity ligation assay (PLA)

GT1-7 cells were treated with leptin (100 nM) or vehicle for 15 min and fixed with methanol. Cells were incubated with primary antibodies against ROCK1 (1:100, goat, Santa Cruz Biotechnology, Santa Cruz, CA) or JAK2 (1:200, rabbit, Cell Signaling, Danvers, MA) overnight. PLA was performed using the Duolink *in situ* PLA kit (Olink Bioscience, Uppsala, Sweden) with PLA PLUS or MINUS probes for rabbit or mouse anti-serum. The nuclei of cells were stained using Hoechst 33342 dye (Olink Bioscience). The number of *in*

situ PLA signals per cell was counted by semi-automated image analysis using BlobFinder software program (Olink Bioscience).

Fluorescence cross-correlation spectroscopy (FCCS)

The molecular interaction between ROCK1 and JAK2 was assessed using a FCCS[38]. In brief, GT1-7 cells were transfected with very low amounts (5 ng each) of JAK2-RFP and ROCK1-EGFP using lipopectamine (Invitrogen, Carlsbad, CA) in order to minimize perturbation to cells. Cells were serum-starved and treated with leptin (100 nM) or vehicle for 1 min. FCCS was performed in live neuronal cells using inverted confocal laser scanning microscopy (LSM710-ConfoCor3, Carl Zeiss, Thornwood, NY). EGFP was excited with the 488 nm laser line and RFP with the 543 nm laser line. The emission signals were split by a dichroic mirror (570 nm beam splitter) and detected at 505–530 nm in the green channel for EGFP, and at over 610 nm in the red channel for tagRFP. Positive controls (EGFP–tagRFP fusion protein-expressing cells) and negative controls (EGFP or tagRFP alone expressing cells) in each independent experiment were included. No cross-correlation signal was found in EGFP or tagRFP alone expressing cells, indicating that there was no background cross-correlation. Measurements in the number of cells that gave a count rate of fluorescent intensity below 100 kilo centipoise (kcp) and a count per molecule greater than 2 kcps were performed. Measurement for a sample was conducted 10 times for 15 – 20 seconds. Data were analyzed using ConfoCor3 software (Carl Zeiss) and shown for an average value.

Immunofluorescent analysis

GT1-7 cells were fixed in methanol, and rinsed with PBS. The fixed cells were incubated with a rabbit anti-FOXO1 antibody (1:50, Abcam) at 4 °C overnight. Cells were washed and incubated for 1 hour with an Alexa Fluor 488 anti-rabbit IgG (1:500, Invitrogen). For counterstaining of nuclei, cells were incubated with DAPI (4',6-diamidino-2-phenylindole; 1 mg/ml, Sigma-Aldrich, St. Louis, MO). Images of green fluorescence were assessed by laser confocal fluorescence microscopy (LSM-710, Carl Zeiss) and quantitated by High content screening (HCS, Thermo).

Immunoblotting and immunoprecipitation

For immunoblotting, cell and tissue lysates (10 – 30 μg protein) were separated by SDS-PAGE and transferred to nitrocellulose membranes. For immunoprecipitation, cell or tissue lysates (100–200 μg) were incubated with 2 μg of a polyclonal ROCK1 antibody (Santa Cruz Biotechnology) coupled to protein G-sepharose (Sigma) overnight at 4 °C. Immunoprecipitates were washed and separated by SDS-PAGE, and transferred to nitrocellulose membranes. The membranes were incubated with either polyclonal antibodies against phospho-Tyr^{1007}JAK2 (Cell Signaling), phospho-Tyr^{705} Stat3 (Cell signaling), JAK2 (Cell Signaling), phosphoserine (Millipore), Stat3 (Cell Signaling), ROCK1 (Santa Cruz Biotechnology), ROCK2 (Santa Cruz), FOXO1 (Abcam), GAPDH (Santa Cruz Biotechnology), RNA polymerase II (RPII) (Abcam), or actin (Santa Cruz Biotechnology). Proteins were visualized with enhanced chemiluminescence and quantified by densitometry.

Animals care

Animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The mice were fed standard chow (Teklad F6 Rodent Diet 8664, Harlan Teklad; Madison, WI) and housed under controlled temperature at 22 – 24°C and 14 h-light/ 10 h-dark cycle. Animals were individually housed for studies of food intake, locomotor activity, energy expenditure, and after implantation of cannulas for stereotactic injections. Otherwise, mice were group housed.

ROCK1 activity

Hypothalamic lysates (100 μg protein) were subjected to immunoprecipitation for 4 hours with 10 μl of a polyclonal ROCK1 antibody (Santa Cruz Biotechnology), coupled to protein G-Sepharose beads (Pharmacia Biotechnology). Immune pellets were washed and resuspended in a 50 μl of kinase mixture (20 mM Tris, pH 7.5, 5 mM $MgCl_2$, 100 mM KCl, 0.1 mM DTT, 100 μM ATP, 1 mM EDTA, 1 μM microcystin-LR, 50 μM long S6K substrate peptide (Millipore) and 1 μCi [γ-^{32}P] ATP] and incubated at 30° C for 30 minutes[19]. Forty microliters of samples were spotted onto phosphocellulose p81 paper (Whatman, Clifton, NJ), and washed with 75 mM orthophosphoric acid. Radioactivity of the paper was determined by scintillation counting.

Generation of ROCK1 floxed mice, and POMC neuron- and AgRP neuron-specific ROCK1-deficient mice (Supplementary Figure 7–8)

Mice bearing a *LoxP*-flanked *ROCK1* allele (*ROCK1$^{flox/flox}$* mice) were generated by inGenious Targeting Laboratory (Stony Brook, NY). Briefly, a BAC clone (C57BL/6, RPC123 clone) containing a 9.32kb fragment of *ROCK1* genomic DNA was used to generate a targeting vector. Four independent *ROCK1*$^{flox/+}$ ES clones were identified, which were injected into C57BL/6 blastocysts to generate chimeric mice. The chimeric mice were bred with wild type C57BL/6 mice for germline transmission. Heterozygous animals were then crossed with mice expressing *flpe*-recombinase in the germline (Flipper mice, from The Jackson Laboratory) to delete the *FRT*-flanked Neo cassette. Offspring of these mice were heterozygous for the desired *ROCK1$^{flox/+}$* allele. *POMC-Cre, ROCK1$^{flox/flox}$* mice were generated by mating *ROCK1$^{flox/flox}$* mice with *POMC-Cre*[13] or *AgRP-ires-Cre* transgenic mice[39]. To detect AgRP neurons, *AgRP-ires-Cre, NPY-hrGFP, ROCK1$^{flox/flox}$* mice were generated by crossing *AgRP-ires-Cre, ROCK1$^{flox/flox}$* mice with *NPY-hrGFP BAC* transgenic mice[40]. The *NPY-hrGFP BAC* transgenic mice demonstrated that NPY neurons are completely colocalized with AgRP neurons[40]. Immunohistochemistry analysis indicated that ROCK1 expression in POMC or AgRP/NPY neurons was intact in *ROCK1$^{flox/flox}$* mice but was absent in POMC neuron- or AgRP/NPY neuron-specific ROCK1 deficient mice (Supplementary Fig. 8a and 8c), confirming the loss of ROCK1 in these neurons. ROCK1 expression in central and peripheral tissues was not different between genotypes (Supplementary Fig. 8b and 8d). POMC neuron-specific and AgRP/NPY neuron-specific ROCK1-deficient mice were maintained with a mixed genetic background of 129, C57BL/6, and FVB strains.

Immunohistochemistry (IHC)

For double fluorescence detection of ROCK1 and POMC, coronal brain sections from 16-week-old *ROCK1$^{flox/flox}$* and *POMC-Cre, ROCK1$^{flox/flox}$ male* mice were generated and immunohistochemistry was performed as described[41]. Brain sections were incubated with anti-ROCK1 (1:100) and anti-POMC (1:3000) antibodies, and further incubated with fluorescent-labeled (green for ROCK1 and red for POMC) secondary antibodies. For nonfluorescent pStat3, brain sections were incubated with an anti-pStat3 antibody (1:3000) and biotinylated anti-Rabbit antibodies (1:1000), followed by avidin-biotin complex labeling, and developed with Nickel-diaminobenzidine (DAB). For the detection of ROCK1 in AgRP expressing neurons, IHC for ROCK1 was performed on coronal brain sections from 12-week-old male *NPY-hrGFP, ROCK1$^{flox/flox}$* and *AgRP-ires-Cre, NPY-hrGFP, ROCK1$^{flox/flox}$*mice. Brain sections were incubated with anti-ROCK1 antibody (1:100) and further incubated with fluorescent-labeled (red for ROCK1) secondary antibodies. AgRP/NPY neurons were identified by *NPY-hrGFP* fluorescence.

Leptin-Induced Stat3 phosphorylation

Mice were injected with leptin intraperitoneally (3 mg/kg) or vehicle and sacrificed 30 min after the injection. Coronal brain sections were subjected to double IHC for pSTAT3 and POMC as described[41]. In total, pSTAT3 was quantified in 242 (6 weeks old) or 210 (20 weeks old) random POMC neurons from *ROCK1*$^{flox/flox}$ male mice (n = 3) and in 251 (6 weeks old) or 246 (20 weeks old) POMC neurons from *POMC-Cre, ROCK1*$^{flox/flox}$ male mice (n = 3).

Electrophysiological Studies

Brain sections from 4-week-old male *POMC-Cre, lox-GFP, ROCK1*$^{wt/wt}$ and *POMC-Cre, lox-GFP, ROCK1*$^{flox/flox}$ were prepared as described[34]. Briefly, coronal sections (200 μM) were cut with a Leica VT1000S Vibratome and then incubated in oxygenated an artificial CSF solution at room temperature for at least 1 hr before recording. POMC neurons were identified by GFP fluorescence, and whole-cell recordings were made using a MultiClamp 700B Amplifier (Molecular Devices, LLC Sunnyvale, CA) and pClamp 9.2 software (Molecular Devices). Recording electrodes had resistances of 2.5–4 MΩ when filled with the following solution containing 128 mM K-gluconate, 10 mM HEPES, 1 mM EGTA, 10 mM KCl, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, 5 mM Mg-ATP, and 0.3 mM Na-GTP (pH 7.35 with KOH). Leptin, insulin or vehicle was applied to bath solution through perfusion. Membrane potential and firing rate were analyzed with MiniAnalysis Program (Synaptosoft, Inc., Decatur, GA) as described [34].

Blood parameters and food intake

Mice were weighed at weaning (3 weeks) and weekly thereafter. Blood was collected from random fed or overnight fasted male mice. Blood glucose was measured using an OneTouch Ultra glucometer (LifeScan Inc., Milpitas, CA). Serum insulin and leptin were measured by enzyme-linked immunosorbent assay (Crystal Chem Inc., Chicago, IL). Serum corticosterone was measured with a commercially available kit (ICN Biomedical Inc., Costa Mesa, CA). Leptin- or fasting-induced food intake was measured in male mice at 12 – 14 week of age.

Body composition, locomotor activity and energy expenditure

Fat and lean body mass were assessed using EchoMRI (Echo Medical Systems, Houston, TX). Fat pads were harvested and weighed. Locomotor activity or energy expenditure was measured in 18-week-old male mice using a comprehensive lab animal monitoring system (CLAMS, Columbia Instruments; Columbus, OH). Locomotor activity was also monitored for 2 weeks using an infrared beam break systems (OptoM3 apparatus, Columbia Instruments; Columbus, OH).

Hypothalamic neuropeptide Expression

Neuropeptide mRNA was determined by quantitative PCR as described[13]. Primer and probe sequences for POMC, AgRP and NPY were also as described[13].

Stereotaxic adenovirus-associated vector (AAV) or adenovirus injection

AAV-eGFP or *AAV-Cre* was bilaterally injected into the mediobasal hypothalamus (2.2×10^{13} pfu) of 4-week-old male *ROCK1*$^{flox/flox}$ mice as described[42]. Adenovirus encoding ROCK1 (10^{11} pfu) or GFP was also bilaterally injected into the mediobasal hypothalamus of 10-week-old C57BL/6 male mice as described[43]. Results from animals shown to have received correct injections were included in analyses, as described[42,43].

Statistical Analysis

Data are presented as means ± standard error of the mean (s.e.m). Statistical analyses were performed using Stat View software (Abacus Concepts, Inc., Berkeley, CA). Unpaired Student's *t* tests were used throughout this study to compare two distinct groups. For analysis of neuronal activities, paired Student's *t* tests were used to compare activity before and after leptin treatment. When more than two groups were compared, one-way analysis of variance (ANOVA) followed by post-hoc tests (Fisher's PLSD) was used. For all tests, $P<0.05$ was considered to be statistically significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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REFERENCES

1. Bjorbaek C. Central leptin receptor action and resistance in obesity. J Investig Med. 2009; 57:789–794.
2. 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]
3. Myers MG, Cowley MA, Munzberg H. Mechanisms of leptin action and leptin resistance. Annu Rev Physiol. 2008; 70:537–556. [PubMed: 17937601]
4. 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]
5. Elmquist JK, Elias CF, Saper CB. From lesions to leptin: hypothalamic control of food intake and body weight. Neuron. 1999; 22:221–232. [PubMed: 10069329]
6. Myers MG Jr, Munzberg H, Leinninger GM, Leshan RL. The geometry of leptin action in the brain: more complicated than a simple ARC. Cell Metab. 2009; 9:117–123. [PubMed: 19187770]
7. Cheung CC, Clifton DK, Steiner RA. Proopiomelanocortin neurons are direct targets for leptin in the hypothalamus. Endocrinology. 1997; 138:4489–4492. [PubMed: 9322969]
8. Wilson BD, et al. Physiological and anatomical circuitry between Agouti-related protein and leptin signaling. Endocrinology. 1999; 140:2387–2397. [PubMed: 10218993]
9. van den Top M, Lee K, Whyment AD, Blanks AM, Spanswick D. Orexigen-sensitive NPY/AgRP pacemaker neurons in the hypothalamic arcuate nucleus. Nat Neurosci. 2004; 7:493–494. [PubMed: 15097991]
10. Mizuno TM, et al. Hypothalamic pro-opiomelanocortin mRNA is reduced by fasting and [corrected] in ob/ob and db/db mice, but is stimulated by leptin. Diabetes. 1998; 47:294–297. [PubMed: 9519731]
11. Schwartz MW, et al. Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the rostral arcuate nucleus. Diabetes. 1997; 46:2119–2223. [PubMed: 9392508]
12. Thornton JE, Cheung CC, Clifton DK, Steiner RA. Regulation of hypothalamic proopiomelanocortin mRNA by leptin in ob/ob mice. Endocrinology. 1997; 138:5063–5066. [PubMed: 9348241]
13. Balthasar N, et al. Leptin receptor signaling in POMC neurons is required for normal body weight homeostasis. Neuron. 2004; 42:983–991. [PubMed: 15207242]

14. van de Wall E, et al. Collective and individual functions of leptin receptor modulated neurons controlling metabolism and ingestion. Endocrinology. 2008; 149:1773–1785. [PubMed: 18162515]
15. Matsui T, et al. Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. Embo J. 1996; 15:2208–2216. [PubMed: 8641286]
16. Hu E, Lee D. Rho kinase as potential therapeutic target for cardiovascular diseases: opportunities and challenges. Expert Opin Ther Targets. 2005; 9:715–736. [PubMed: 16083339]
17. Chun KH, et al. In vivo activation of ROCK1 by insulin is impaired in skeletal muscle of humans with type 2 diabetes. Am J Physiol Endocrinol Metab. 2011; 300:E536–E542. [PubMed: 21189360]
18. Begum N, Sandu OA, Ito M, Lohmann SM, Smolenski A. Active Rho kinase (ROK-alpha) associates with insulin receptor substrate-1 and inhibits insulin signaling in vascular smooth muscle cells. J Biol Chem. 2002; 277:6214–6222. [PubMed: 11739394]
19. Furukawa N, et al. Role of Rho-kinase in regulation of insulin action and glucose homeostasis. Cell Metab. 2005; 2:119–129. [PubMed: 16098829]
20. Lee DH, et al. Targeted disruption of ROCK1 causes insulin resistance in vivo. J Biol Chem. 2009; 284:11776–11780. [PubMed: 19276091]
21. Chun KH, et al. Regulation of glucose transport by ROCK1 differs from that of ROCK2 and is controlled by actin polymerization. Endocrinology. 2012; 153:1649–1662. [PubMed: 22355071]
22. Hill JW, et al. Acute effects of leptin require PI3K signaling in hypothalamic proopiomelanocortin neurons in mice. J Clin Invest. 2008; 118:1796–1805. [PubMed: 18382766]
23. Al-Qassab H, et al. Dominant role of the p110beta isoform of PI3K over p110alpha in energy homeostasis regulation by POMC and AgRP neurons. Cell Metab. 2009; 10:343–354. [PubMed: 19883613]
24. Plum L, et al. Enhanced PIP3 signaling in POMC neurons causes KATP channel activation and leads to diet-sensitive obesity. J Clin Invest. 2006; 116:1886–1901. [PubMed: 16794735]
25. Cota D, et al. Hypothalamic mTOR signaling regulates food intake. Science. 2006; 312:927–930. [PubMed: 16690869]
26. Fukuda M, et al. Monitoring FoxO1 localization in chemically identified neurons. J Neurosci. 2008; 28:13640–13648. [PubMed: 19074037]
27. Coppari R, 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]
28. Mesaros A, et al. Activation of Stat3 signaling in AgRP neurons promotes locomotor activity. Cell Metab. 2008; 7:236–248. [PubMed: 18316029]
29. Huo L, et al. Leptin-dependent control of glucose balance and locomotor activity by POMC neurons. Cell Metab. 2009; 9:537–547. [PubMed: 19490908]
30. Cowley MA, et al. Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. Nature. 2001; 411:480–484. [PubMed: 11373681]
31. Ren D, Li M, Duan C, Rui L. Identification of SH2-B as a key regulator of leptin sensitivity, energy balance, and body weight in mice. Cell Metab. 2005; 2:95–104. [PubMed: 16098827]
32. Bates SH, et al. STAT3 signalling is required for leptin regulation of energy balance but not reproduction. Nature. 2003; 421:856–859. [PubMed: 12594516]
33. Xu AW, Ste-Marie L, Kaelin CB, Barsh GS. Inactivation of signal transducer and activator of transcription 3 in proopiomelanocortin (Pomc) neurons causes decreased pomc expression, mild obesity, and defects in compensatory refeeding. Endocrinology. 2007; 148:72–80. [PubMed: 17023536]
34. Dhillon H, et al. Leptin directly activates SF1 neurons in the VMH, and this action by leptin is required for normal body-weight homeostasis. Neuron. 2006; 49:191–203. [PubMed: 16423694]
35. Yoshii A, Constantine-Paton M. Postsynaptic BDNF-TrkB signaling in synapse maturation, plasticity, and disease. Dev Neurobiol. 2010; 70:304–322. [PubMed: 20186705]
36. Mellon PL, et al. Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis. Neuron. 1990; 5:1–10. [PubMed: 2196069]

37. Bjorbaek C, Uotani S, da Silva B, Flier JS. Divergent signaling capacities of the long and short isoforms of the leptin receptor. J Biol Chem. 1997; 272:32686–32695. [PubMed: 9405487]

38. Bacia K, Kim SA, Schwille P. Fluorescence cross-correlation spectroscopy in living cells. Nat Methods. 2006; 3:83–89. [PubMed: 16432516]

39. Tong Q, Ye CP, Jones JE, Elmquist JK, Lowell BB. Synaptic release of GABA by AgRP neurons is required for normal regulation of energy balance. Nat Neurosci. 2008; 11:998–1000. [PubMed: 19160495]

40. van den Pol AN, et al. Neuromedin B and gastrin-releasing peptide excite arcuate nucleus neuropeptide Y neurons in a novel transgenic mouse expressing strong Renilla green fluorescent protein in NPY neurons. J Neurosci. 2009; 29:4622–4639. [PubMed: 19357287]

41. Huo L, Grill HJ, Bjorbaek C. Divergent regulation of proopiomelanocortin neurons by leptin in the nucleus of the solitary tract and in the arcuate hypothalamic nucleus. Diabetes. 2006; 55:567–573. [PubMed: 16505217]

42. Balthasar N, et al. Divergence of melanocortin pathways in the control of food intake and energy expenditure. Cell. 2005; 123:493–505. [PubMed: 16269339]

43. Kim MS, et al. Role of hypothalamic Foxo1 in the regulation of food intake and energy homeostasis. Nat Neurosci. 2006; 9:901–906. [PubMed: 16783365]

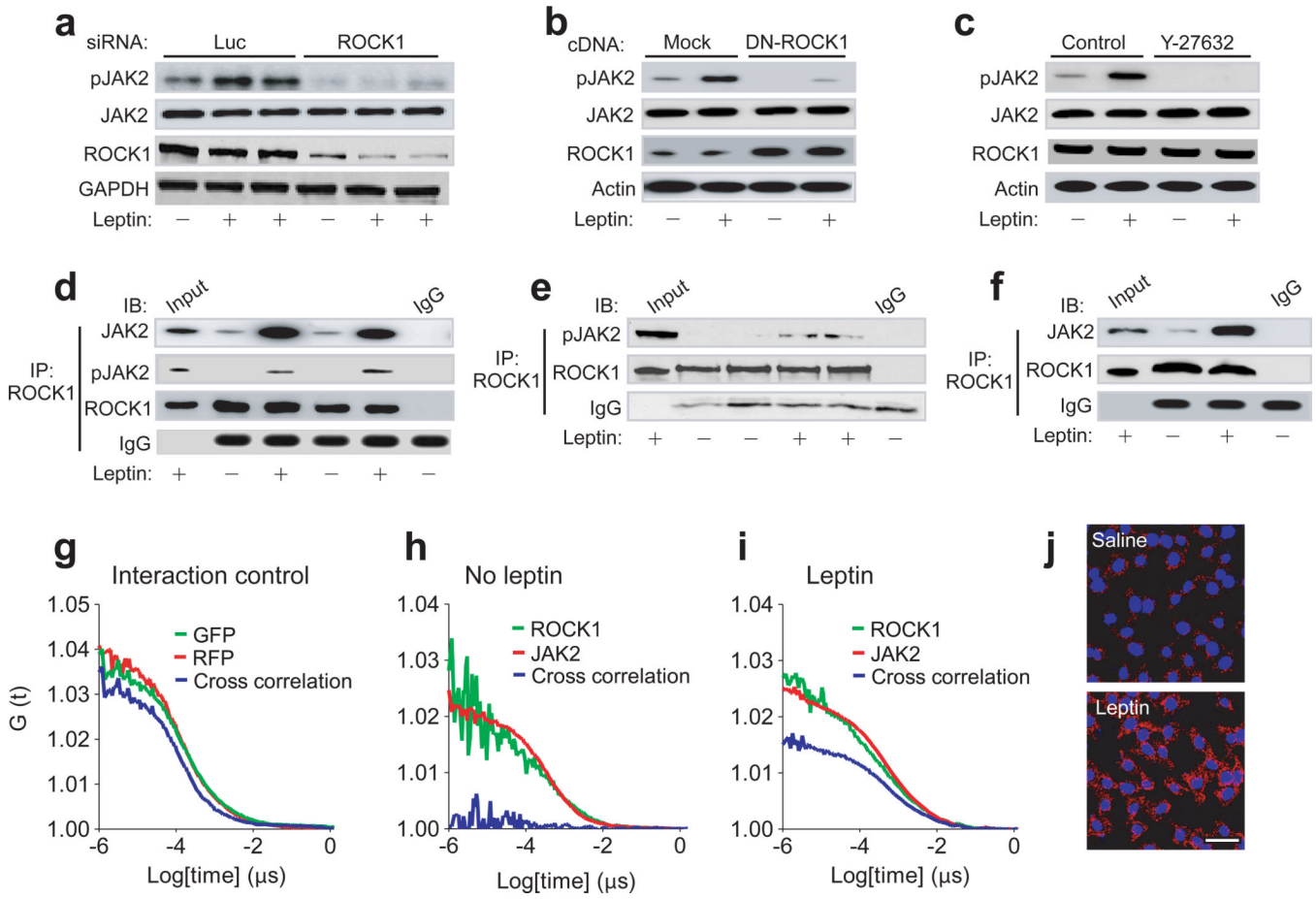

Figure 1. ROCK1 interacts with and phosphorylates JAK2 in hypothalamic GT1-7 cells and hypothalamus

(**a**) Leptin increases JAK2 phosphorylation. Cells were transfected with luciferase (Luc) or ROCK1 siRNA, and treated ± leptin.

(**b**) DN-ROCK1 inhibits leptin-stimulated JAK2 phosphorylation. Cells were transfected ± DN-ROCK1 cDNA and later treated ± leptin.

(**c**) ROCK inhibition blocks leptin-stimulated JAK2 phosphorylation. Cells were pretreated ± ROCK inhibitor Y-27632 and stimulated ± leptin.

(**d**) Leptin increases the physical interaction between ROCK1 and JAK2. Cells were treated ± leptin and lysates (input) subjected to immunoprecipitation with a ROCK1 or non-specific IgG antibody.

(**e–f**) Leptin increases ROCK1 and JAK2 interaction and JAK2 phosphorylation in hypothalamus *in vivo*. C57BL/6 mice were injected ± leptin. Hypothalamic lysates (input) were immunoprecipitated with a ROCK1 or non-specific (IgG) antibody.

For **a–e**, JAK2, pJAK2 Tyr^{1007}, ROCK1, GAPDH, actin, and IgG light and heavy chains were measured by immunoblotting.

(**g–i**) Interaction dynamics between ROCK1 and JAK2 by FCCS. Cells were treated ± leptin. X axis: lag time during measurements, Y axis: autocorrelation and cross-correlation function [G(t)]. Cross-correlation indicates the physical interaction strength between ROCK1 and JAK2. Interaction control (**g**) shows high cross correlation between red fluorescent protein (RFP) and green fluorescent protein (GFP). The physical interaction between ROCK1 and JAK2 in the absence (**h**) or presence (**i**) of leptin is shown. Data are means for 10 measurements.

(**j**) PLA shows that leptin stimulates physical interaction between ROCK1 and JAK2. Cells were treated ± leptin. Each red spot represents a ROCK1-JAK2 interaction. Nuclei were stained with Hoechst 33342. Bar = 2.5 μm.
Full-length blots are in Supplementary Fig. 9.

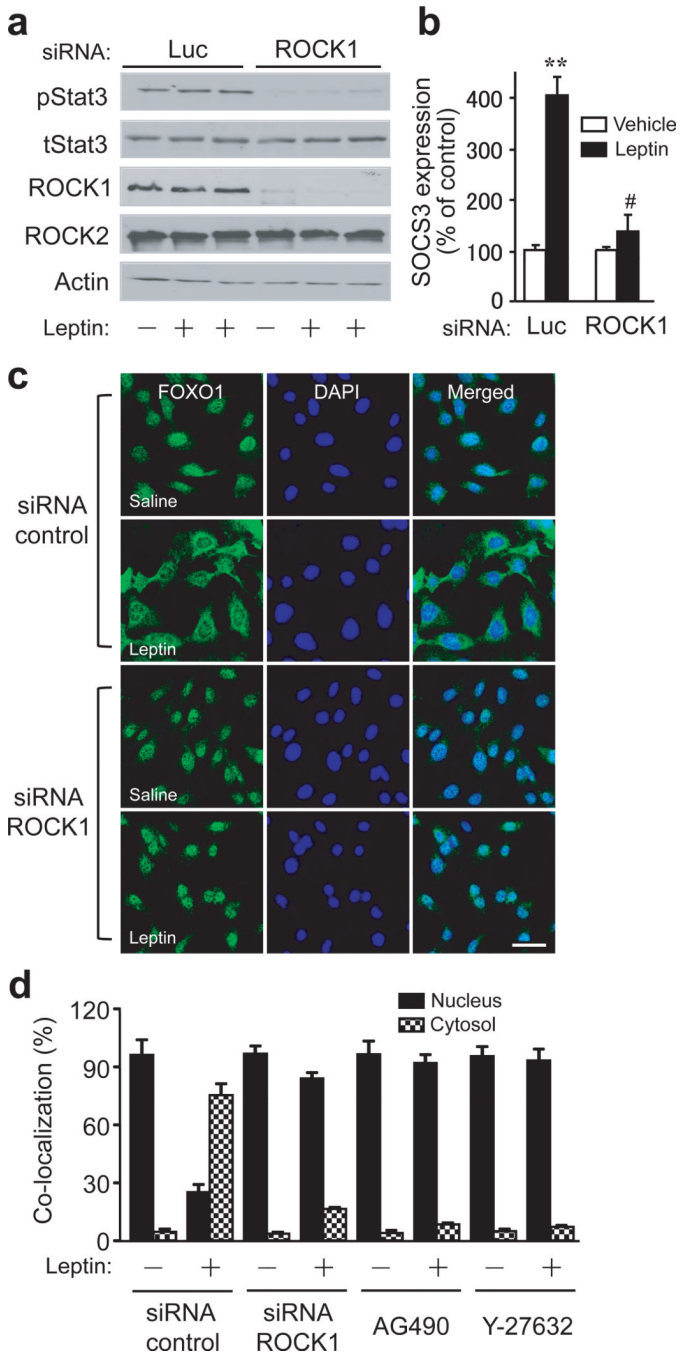

Figure 2. ROCK1 stimulates Stat3 phosphorylation and FOXO1 nuclear export

(**a**) ROCK1 inhibition blocks leptin-stimulated Stat3 phosphorylation. GT1-7 cells were transfected with luciferase (Luc) siRNA or ROCK1 siRNA, and then treated with leptin (100 nM) or vehicle for 15 min. pStat3 Tyr^{705}, Stat3, ROCK1, ROCK2, and actin were measured by immunoblotting. Full-length blots are presented in Supplementary Fig. 9. (**b**) SOCS3 mRNA level is decreased by ROCK1 inhibition. GT1-7 cells were transfected with Luc or ROCK1 siRNA, and treated with leptin (100 nM) or vehicle for 1 hr. SOCS3 mRNA levels were measured by quantitative PCR. Data are representative of two

independent experiments and are presented as means ± s.e.m. ANOVA analysis with Fisher's PLSD. $F = 28.3$, $^{**}p < 0.01$ vs. luc vehicle, $^{\#}p < 0.01$ vs. luc leptin.

(**c**) ROCK1 inhibition blocks leptin-dependent FOXO1 nuclear export. GT1-7 cells were transfected with Luc or ROCK1 siRNA, and treated with vehicle or leptin (100 nM) for 15 min. Cells were fixed and then subjected to immunocytochemistry for FOXO1. Nuclei were stained with DAPI (blue). Bar = 2.5 μm.

(**d**) Quantitation of FOXO1 in the nucleus and cytosol. High content screening analysis of FOXO1 in ROCK1 or Luc siRNA transfected cells, AG490 (JAK2 chemical inhibitor)-treated cells, or Y-27362 (ROCK chemical inhibitor)-treated cells was performed. Data are presented as mean ± s.e.m.

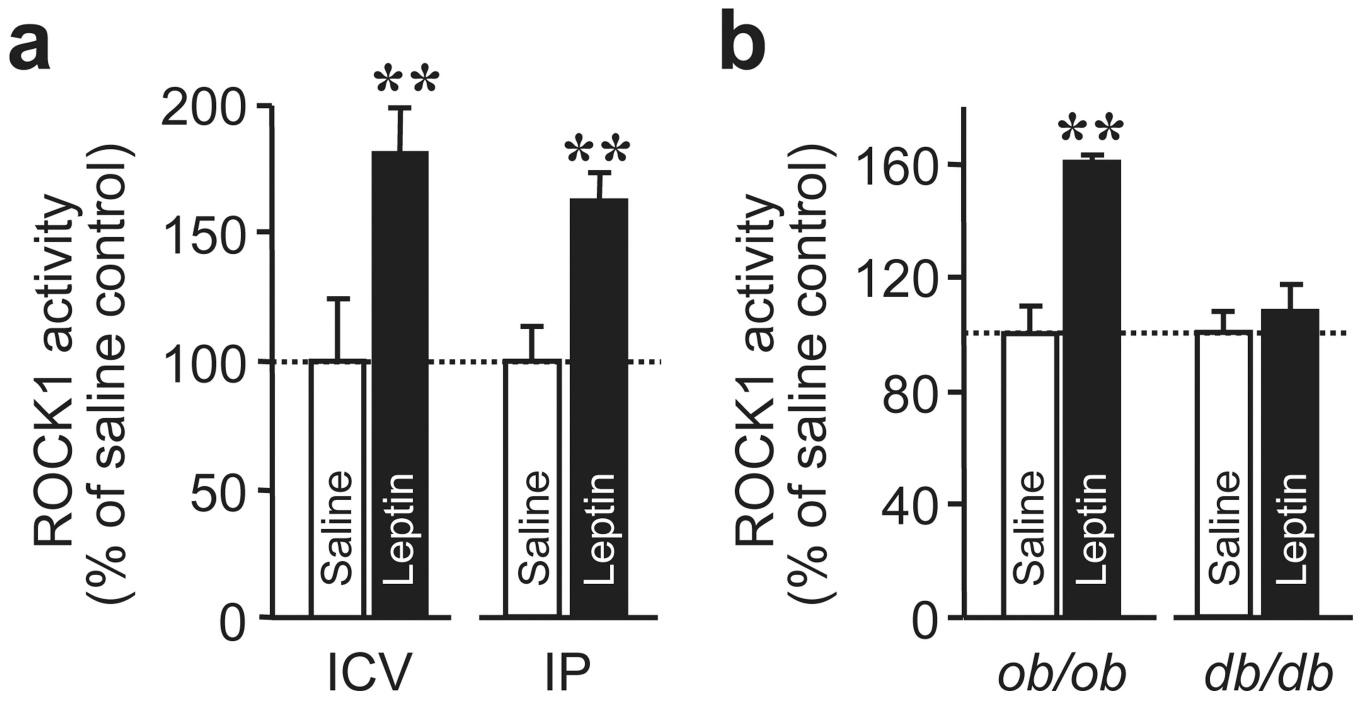

Figure 3. Leptin increases ROCK1 activity in the hypothalamus

(a) ROCK1 activity in hypothalamus of C57BL/6 mice. Mice were injected with saline or leptin either icv (1 μg, $n = 6–7$) or ip (3 mg/kg, $n = 6$), and sacrificed 30 min or 60 min later, respectively. ROCK1 activity was measured by immuncomplex assay. Data are presented as means ± s.e.m. *$p < 0.01$ vs. saline-injected control by unpaired Student's t test, $t = 3.60$ (for icv), $t = 3.03$ (for ip).

(b) Hypothalamic ROCK1 activity in *ob/ob* and *db/db* mice. *ob/ob* (leptin-deficient, $n = 3$) and *db/db* (LepRb-deficient, $n = 5$) mice were injected with saline or leptin either icv (0.2 μg/kg) or ip (3 mg/kg), and sacrificed 30 min later. ROCK1 activity was measured by immuncomplex assay. Data are presented as mean ± s.e.m. **$p < 0.01$ vs. saline-injected control by unpaired Student's t test, $t = 11.1$.

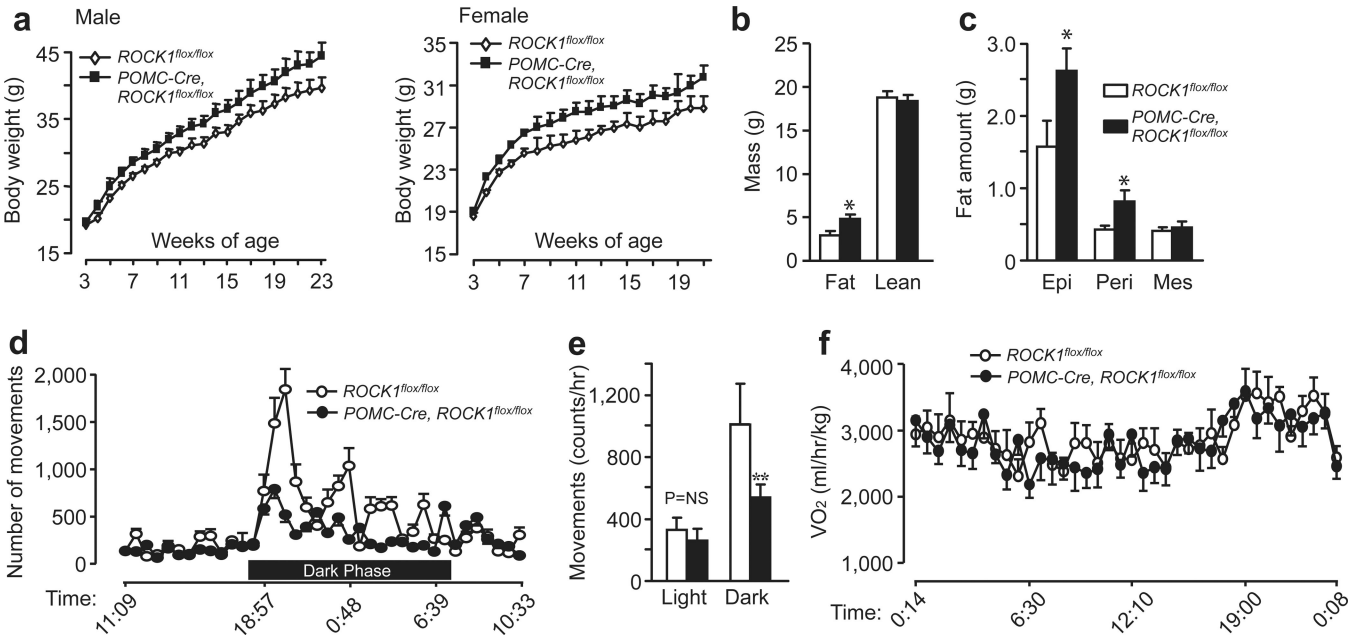

Figure 4. ROCK1 deficiency in POMC neurons leads to obesity

(**a**) Body-weights of male and female *ROCK1*$^{flox/flox}$ (control, $n = 11$) and *POMC-Cre, ROCK1*$^{flox/flox}$ (KO, $n = 12$) mice over time are shown. Data are presented as means ± s.e.m.

(**b**) Fat and lean body mass by MRI of 20-week-old male mice are indicated (control: $n = 6$, KO: $n = 7$). Data are presented as means ± s.e.m. *$p < 0.05$ vs. control mice by unpaired Student's t test, $t = -2.66$.

(**c**) Fat pads weights of 24-week-old male mice are shown ($n = 6$). Epi: epididymal fat, Peri: perirenal fat, Mes: mesenteric fat. Data are presented as means ± s.e.m. *$p < 0.05$, vs. control mice by unpaired Student's t test, $t = -2.27$ (for Epi) and $t = -2.81$ (for Peri),

(**d–e**) Diurnal and nocturnal locomotor activity of 18-week-old male mice ($n = 8$). All mice were weight-matched. Locomotor activity was measured using a comprehensive lab animal monitoring system (CLAMS) over one day (**d**) and averaged for light (10h) and dark periods (14h). (**e**). Data are presented means ± s.e.m. *$p < 0.01$ vs. control mice by unpaired Student's t test, $t = 2.74$.

(**f**) Energy expenditure of 16-week-old male mice (control: $n = 11$, KO: $n = 12$). All mice were weight-matched. Oxygen consumption was measured using a CLAMS. Data are presented as means ± s.e.m.

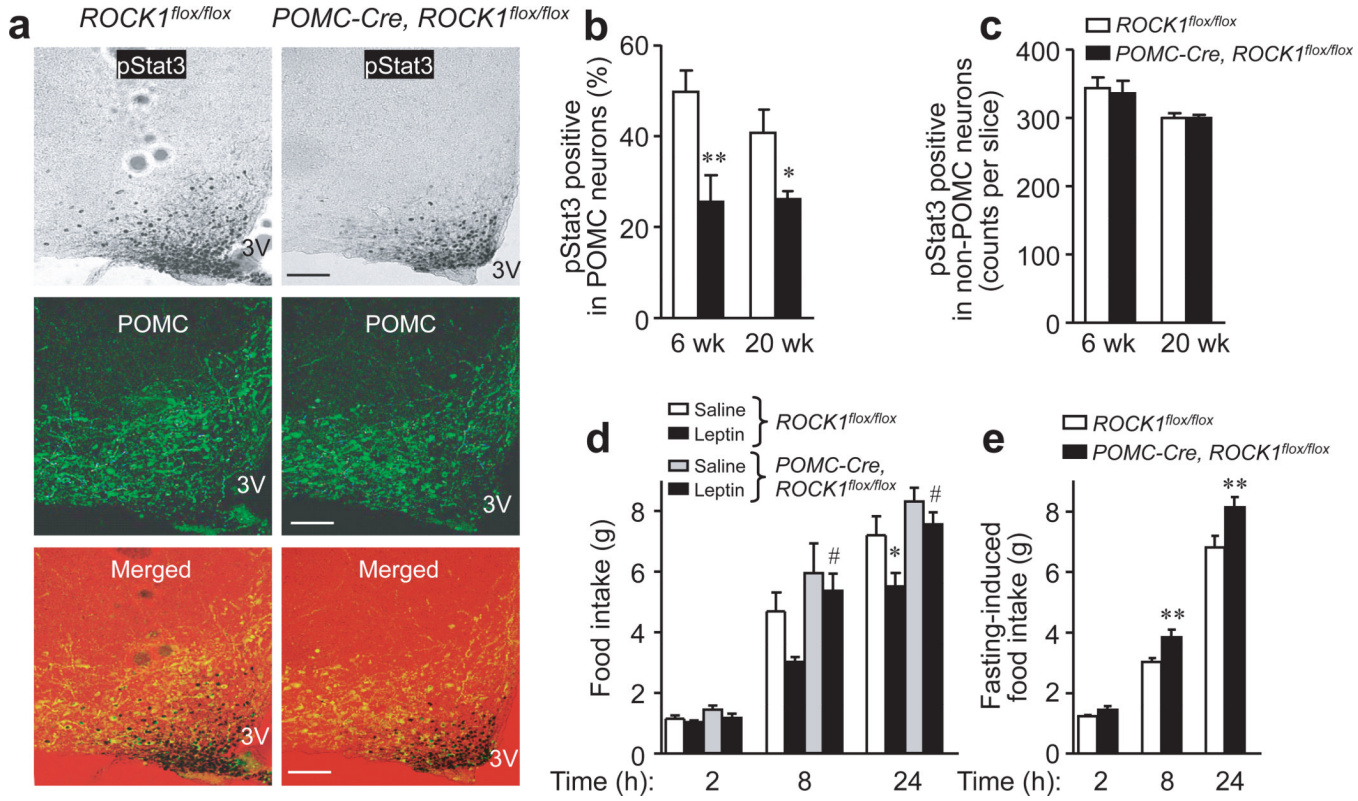

Figure 5. ROCK1 deficiency in POMC neurons impairs leptin-stimulated Stat3 phosphorylation and food intake

(**a**) Immunohistochemical staining of Stat3 phosphorylation and POMC expression in coronal brain section from 6-week-old male *ROCK1*$^{flox/flox}$ (control) and *POMC-Cre, ROCK1*$^{flox/flox}$ mice is shown. Mice were injected with saline or leptin ip (3mg/kg), and sacrificed 30 min later. Genotypes were weight-matched. 3V: third ventricle. Bar = 50 μm.

(**b**) Quantitation of pStat3-positive POMC neurons in coronal brain sections shown in 6-week-old and 20-week-old male mice. pStat3 positive neurons were counted from 6 brain sections from 6-week-old and 3 brain sections from 20-week-old male mice. Data are presented as means ± s.e.m. *$p < 0.05$ and **$p < 0.01$ vs. age-matched control mice by unpaired Student's t test, $t = 3.21$ (6 wk), $t = 2.70$ (20 wk).

(**c**) Quantitation of pStat3 positive non-POMC neurons. pStat3 positive neurons were counted from 6 brain sections from 6-week-old and 3 brain sections from 20-week-old male mice. Data are presented means ± s.e.m.

(**d**) Food intake of 12-week-old male mice after ip injection of saline or leptin (3 mg/kg) ($n = 7$). Data are presented as means ± s.e.m. *$p < 0.05$ vs. saline-injected control mice and $^{\#}p < 0.01$ vs. leptin-injected control mice by ANOVA with Fisher's PLSD. $F = 4.18$ (8 hr), $F = 5.27$ (24 hr).

(**e**) Food intake of 14-week-old male mice after overnight fasting (control: $n = 9$, KO: $n = 10$). Data are presented as means ± s.e.m. **$p < 0.01$ vs. control mice by unpaired Student's t test, $t = -3.34$ (8 hr), $t = -2.93$ (24 hr).

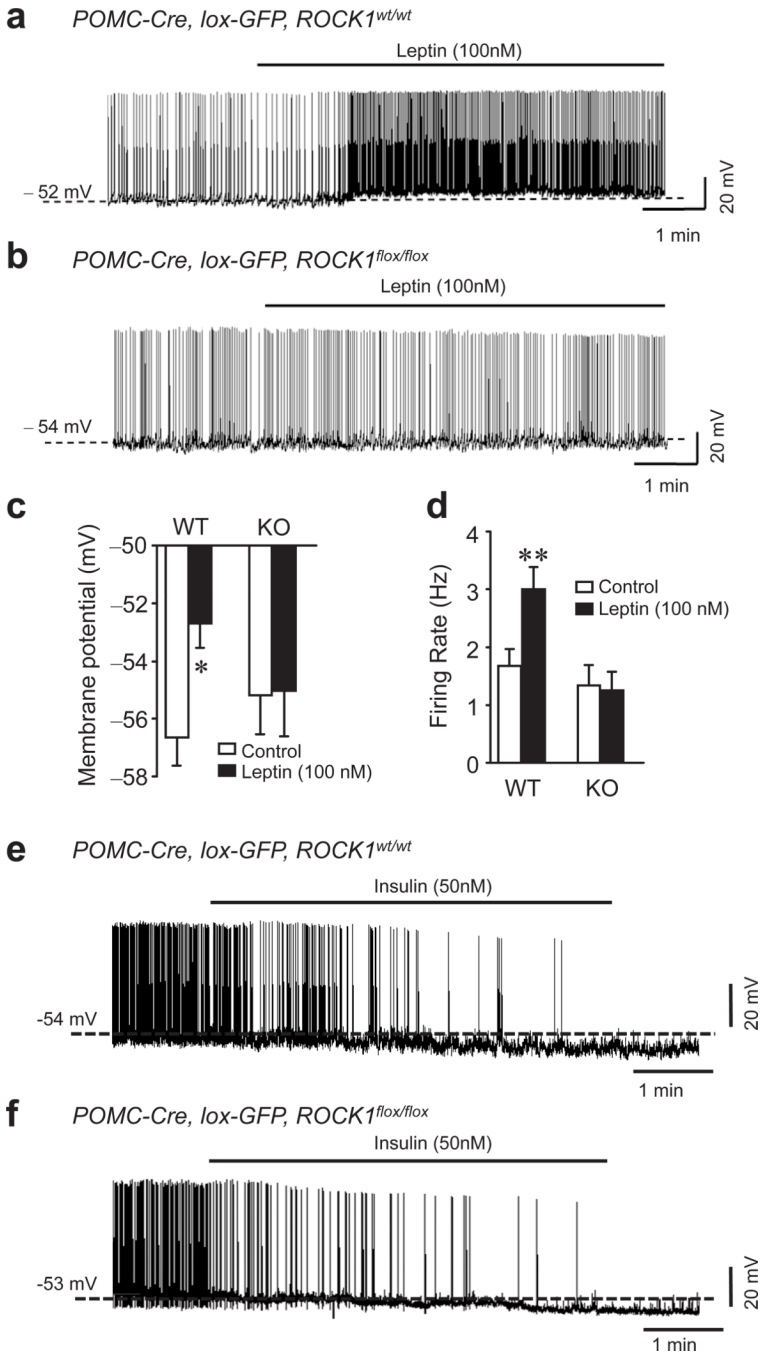

Figure 6. Leptin-induced POMC neuronal activity is dependent on ROCK1

(**a**) Leptin depolarizes and increases the frequency of action potentials in POMC neurons in hypothalamic brain slices from 4-week-old *POMC-Cre, lox-GFP, ROCK1*$^{wt/wt}$ male mice. (**b**) Leptin does not depolarize or increase the frequency of action potentials in hypothalamic POMC neurons from 4-week-old *POMC-Cre, lox-GFP, ROCK1*$^{flox/flox}$ male.

(**c–d**) The membrane potential (**c**) and firing rate (**d**) of POMC neurons from mice shown in **a** and **b** were quantified. A total of 18 recordings were made on GFP-positive neurons from *POMC-Cre, lox-GFP, ROCK1*$^{wt/wt}$ mice (WT). The mean membrane potential or firing rate of the 10 neurons responding to leptin is shown. The mean membrane or firing rate of the 11

total recordings from GFP-positive neurons from *POMC-Cre, lox GFP, ROCK1$^{flox/flox}$ mice* (KO), none of which responded to leptin, is shown. Data are presented as means ± s.e.m. $^{**}p < 0.01$ vs. before leptin treatment (control) using paired Student's *t* test, $t = -17.18$ (membrane potential), $t = -6.32$ 9 (firing rate).

(**e–f**) Insulin hyperpolarizes and decreases the frequency of action potentials in hypothalamic POMC neurons from 4-week-old *POMC-Cre, lox-GFP, ROCK1$^{wt/wt}$* (**e**) and *POMC-Cre, lox-GFP, ROCK1$^{flox/flox}$* (**f**) male mice. Five recordings were made on GFP-positive neurons from *POMC-Cre, lox-GFP, ROCK1$^{wt/wt}$* mice, of which 2 responded to insulin. Seven recordings on GFP-positive neurons from *POMC-Cre, lox GFP, ROCK1$^{flox/flox}$* mice were made, of which 3 responded to leptin. a,b,e,f are whole-cell recording traces from responsive neurons are shown. Bars indicate hormone exposure time. Dashed lines indicate baseline membrane potential.

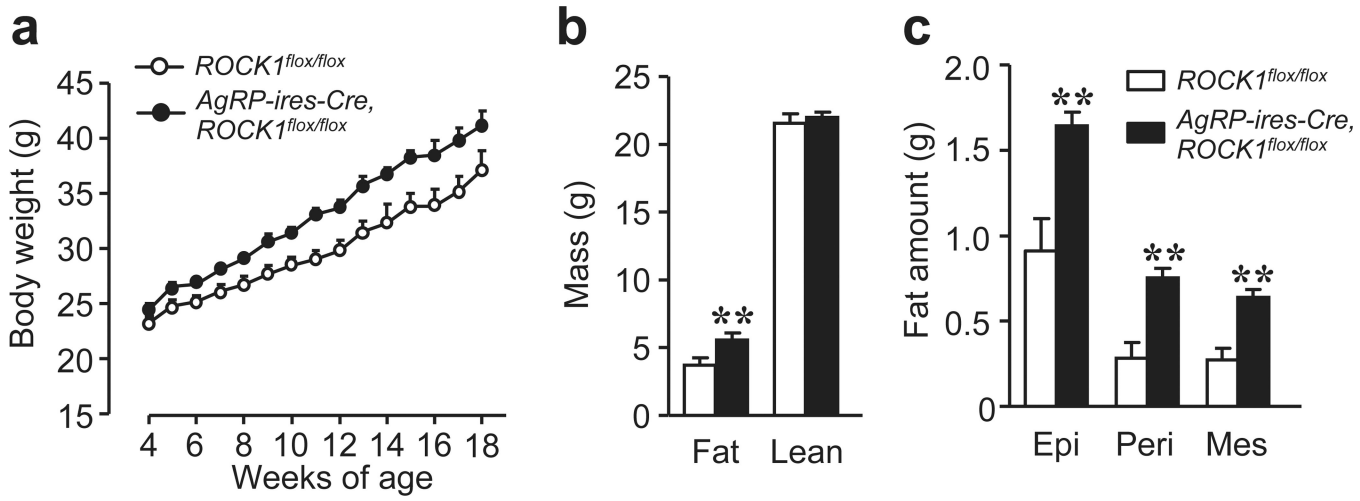

Figure 7. Loss of ROCK1 in AgRP neurons leads to obesity

(**a**) Body-weights of male *ROCK1*$^{flox/flox}$ (control, $n = 10$) and *AgRP-ires-Cre, ROCK1*$^{flox/flox}$ (KO, $n = 13$) mice over time are shown. Data are presented as means ± s.e.m.

(**b**) Fat and lean body mass by MRI of 12-week-old male mice are shown (control: $n = 9$, KO: $n = 13$). Data are presented as means ± s.e.m. *$p < 0.05$ vs. control mice by unpaired Student's t test, $t = -2.39$.

(**c**) Fat pads weights from 20-wk-old male mice are shown ($n = 8$). Epi: epididymal fat, Peri: perirenal fat, Mes: mesenteric fat. Data are presented as means ± s.e.m. *$p < 0.01$ vs. control mice by unpaired Student's t test. $t = 5.56$ (for Epi), $t = 4.37$ (for Peri), $t = 4.51$ (for Mes).

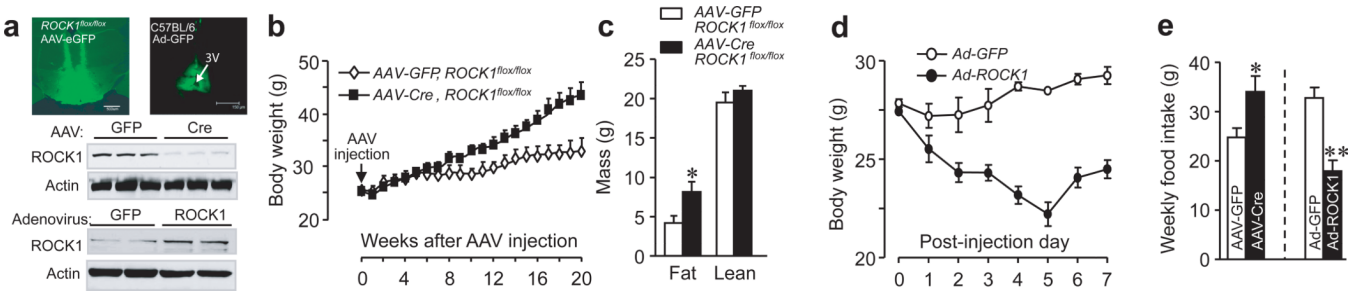

Figure 8. Hypothalamic ROCK1 signaling regulates food intake and adiposity

(**a**) Targeted *ROCK1* deletion or overexpression in hypothalamus of mice. *AAV-GFP* or *AAV-Cre* was injected bilaterally into the mediobasal hypothalamus of male *ROCK1*$^{flox/flox}$ mice. Adenovirus expressing GFP or ROCK1 was injected bilaterally into the mediobasal hypothalamus of male C56BL/6 mice. Immunohistochemical analysis of GFP expression was performed in coronal brain sections (top). ROCK1 and actin in hypothalamic tissue punches from coronal brain slices were measured by immunoblotting (middle and bottom). AAV: Bar = 500 μm. Adenovirus: Bar = 150 μm.

(**b**) Body-weights of *ROCK1*$^{flox/flox}$ mice after intrahypothalamic *AAV-GFP* ($n = 4$) or *AAV-Cre* injection ($n = 5$). Data are presented as means ± s.e.m

(**c**) Fat mass and lean mass by MRI of 20-week-old male mice from **b** are indicated (*AAV-GFP:* $n = 4$, *AAV-Cre:* $n = 5$). Data are presented as means ± s.e.m. *$p < 0.05$ vs *AAV-GFP*-injected *ROCK1*$^{flox/flox}$ mice by unpaired Student's *t* test. $t = 2.31$.

(**d**) Body-weights of C57BL/6 mice after intrahypothalamic injection of adenovirus encoding GFP ($n = 5$) or WT-ROCK1 ($n = 6$) are shown. Data are presented as means ± s.e.m.

(**e**) Weekly food intake of mice from **b** and **d**. Data are presented means ± s.e.m. *$p < 0.05$ vs *Ad-GFP*-injected *ROCK1*$^{flox/flox}$ mice by unpaired Student's *t* test, $t = 2.34$. **$p < 0.01$ vs adenovirus GFP-injected mice by unpaired Student's *t* test, $t = 4.82$.