ROCK1 in AgRP Neurons Regulates Energy Expenditure and Locomotor Activity in Male Mice

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Normal leptin signaling is essential for the maintenance of body weight homeostasis. Proopiomelanocortin- and agouti-related peptide (AgRP)-producing neurons play critical roles in regulating energy metabolism. Our recent work demonstrates that deletion of Rho-kinase 1 (ROCK1) in the AgRP neurons of mice increased body weight and adiposity. Here, we report that selective loss of ROCK1 in AgRP neurons caused a significant decrease in energy expenditure and locomotor activity of mice. These effects were independent of any change in food intake. Furthermore, AgRP neuron-specific ROCK1-deficient mice displayed central leptin resistance, as evidenced by impaired Signal Transducer and Activator of Transcription 3 activation in response to leptin administration. Leptin's ability to hyperpolarize and decrease firing rate of AgRP neurons was also abolished in the absence of ROCK1. Moreover, diet-induced and genetic forms of obesity resulted in reduced ROCK1 activity in murine arcuate nucleus. Of note, high-fat diet also impaired leptin-stimulated ROCK1 activity in arcuate nucleus, suggesting that a defect in hypothalamic ROCK1 activity may contribute to the pathogenesis of central leptin resistance in obesity. Together, these data demonstrate that ROCK1 activation in hypothalamic AgRP neurons is required for the homeostatic regulation of energy expenditure and adiposity. These results further support previous work identifying ROCK1 as a key regulator of energy balance and suggest that targeting ROCK1 in the hypothalamus may lead to development of antiobesity therapeutics. **(*Endocrinology* 154: 3660–3670, 2013)**

Obesity is the major risk factor for the development of type 2 diabetes (1, 2). Currently, obesity is reaching epidemic proportions in North America, affecting American society in increased morbidity and mortality as well as economic cost (3). Identifying the key genes and molecular pathways involved in obesity will provide improved understanding of how obesity and obesity-related diseases can be treated.

Leptin is a critical antiobesity hormone generated from adipocytes that is produced in proportion to fat mass. It is now well established that leptin circulates and binds its long isoform leptin receptor (LepRb) in the brain, where it controls food intake and energy expenditure (4, 5). Several brain regions of leptin receptor (LepR)-expressing neurons, including hypothalamic regions such as the lateral hypothalamic area, arcuate nucleus (ARC), ventromedial hypothalamus, and ventral premammillary nucleus, are critical to leptin's regulation of body weight and energy homeostasis (6). In the ARC, it is clear that LepR signaling in proopiomelanocortin (POMC)- and agouti-related peptide (AgRP)-containing neurons is required for normal body weight homeostasis (7, 8). Mice lacking LepR in POMC or AgRP neurons are obese on chow diet. Moreover, LepR deficiency in both POMC and AgRP neu-

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Abbreviations: AgRP, agouti-related peptide; ARC, arcuate nucleus; CLAMS, Comprehensive Lab Animal Monitoring System; GABA, γ-aminobutyric acid; GFP, green fluorescent protein; HFD, high-fat diet; LepR, leptin receptor; JAK2, Janus kinase 2; NPY, neuropeptide Y; PI3K, phosphatidylinositide 3-kinase; POMC, proopiomelanocortin; pSTAT3, phosphor-STAT3; PTP, protein tyrosine phosphatase; ROCK, Rho-kinase; SOCS3, suppressor of cytokine signaling 3.

rons has additive effects on body weight/adiposity and synergistic effects on insulin resistance (8). Given the important roles of leptin action on AgRP and POMC neurons in energy balance, there has been great interest in understanding the molecular mechanisms of leptin signaling in these neurons. However, the molecular mechanisms mediating leptin's regulation of hypothalamic neuronal activity are not well mapped.

Rho-kinase (ROCK) is a Ser/Thr protein kinase identified as a GTP-Rho-binding protein (9, 10). There are 2 isoforms of ROCK, ROCK1 (also known as ROKβ) (10, 11) and ROCK2 (also known as ROKα) (11, 12). ROCK isoforms have been implicated in a variety of cellular functions, including smooth muscle contraction, actin cytoskeleton organization, cell adhesion and motility, cytokinesis, and gene expression (13), all of which may be involved in the pathogenesis of metabolic-related diseases, including hypertension, arteriosclerosis, and diabetes (14, 15). Our previous study demonstrated that deficiency of ROCK1 in POMC neurons causes weight gain and prevents leptin-induced anorexia, hypothalamic Stat3 phosphorylation, and POMC neuronal activation in mice. Furthermore, deletion of ROCK1 in ARC of mice further increased body weight compared with deficiency of ROCK1 only in POMC neurons, indicating that ROCK1 action in other ARC neurons is important for leptin's action on energy balance (16). We also reported that loss of ROCK1 in AgRP neurons causes increased body weight and adiposity (16). In this study, we determined the role of ROCK1 in AgRP neurons in controlling food intake, energy expenditure, and locomotor activity. In addition, we examined the impact of ROCK1 activation in ARC in the development of obesity.

Materials and Methods

Experimental animals

C57BL6 mice (The Jackson Laboratory, Bar Harbor, Maine) were fed standard chow (16% kcal from fat and 56% from carbohydrates, Purina Lab diet 5008; Purina, Gray Summit, Missouri) or a high-fat/sugar diet (58% kcal from fat and 25% from sugars, Research Diets D12331; Research Diets, Inc, New Brunswick, New Jersey) for 3 months. Generation of AgRP-ires-Cre and loxP-flanked ROCK1 mice was described previously (16). Study animals were generated by mating AgRP-ires-Cre: ROCK1$^{loxP/+}$ mice with ROCK1$^{loxP/+}$ mice, from which AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ mice and their littermate control group, ROCK1$^{loxP/loxP}$ mice, were generated. To visualize the AgRP neurons, *NPY-hrGFP* BAC transgenic mice (17) were crossed with AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ mice to generate *NPY-hrGFP*; AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ mice. All animals were housed at 22°C–24°C with a 12-hour light, 12-hour dark cycle and ad libitum access to standard pelleted chow and water. All aspects of animal care and experimentation were conducted in accordance with the National Institutes Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication no. 85-23, revised 1996) and approved by the Institutional Animal Care and Use Committees of Beth Israel Deaconess Medical Center.

Body composition and food intake

Mice were weighed at weaning (3 wk) and weekly thereafter. Total fat and lean mass were measured using an Echo MRI system (Echo Medical Systems). Bone mineral density and bone mineral content were measured in the whole body except for the skull and tail with dual-energy x-ray absorptiometry (Lunar PIXImus, Madison, Wisconsin). For measurement of daily food intake, mice were individually housed for 1 week before measurement of food intake. Food intake was then measured over a 7-day period. Food intake data for 7 days were combined, averaged, and analyzed by unpaired Student's *t* test. For the analysis of food intake after fasting, after 1 week of acclimatized period, mice were then acclimatized to feeding from 3 pellets (maximum, ~9 g) every day for 3 days. Mice were then fasted overnight and measure food intake from 3 pellets (maximum, ~9 g) for 1, 8, and 24 hours. Food intake data were analyzed as above. To assess the exact amount of food intake, a white bedding paper under food bowl was used to collect food wastage during the course of food intake measurement. The uneaten food was collected and measured. This amount was excluded from the total amount of food intake.

Energy expenditure, respiratory exchange ratio, and locomotor activity

Energy expenditure was measured by assessing oxygen consumption with indirect calorimetry. Individually housed male mice maintained on a chow diet until 9 weeks of age were studied, using the Comprehensive Lab Animal Monitoring System (CLAMS) (Columbus Instruments). Mice were acclimated in the CLAMS chambers for 72 hours before data collection. Mice had free access to food and water for the duration of the studies. During the course of energy metabolism measurements (locomotor activity, O_2, and CO_2) using CLAMS, there are high variations (overlapping) of the measurements in individual time points between the groups. This cannot allow us to statistically analyze individual time points. To enhance statistical power of these measurements, we combined each value from individual time points and analyzed the data by unpaired Student's *t* test as it compares 2 groups. All data were normalized for lean mass of body weight.

Leptin-induced Stat3 phosphorylation

Mice were injected with leptin ip (3 mg/kg) and killed 30 minutes after the injection. Brain sections were evaluated for phosphor-STAT3 (pSTAT3) in AgRP/neuropeptide Y (NPY) neurons, as described (18). AgRP/NPY neurons were identified in sections by native fluorescence (green) of the green fluorescent protein (GFP) transgene. For pStat3 immunostaining, brain sections were incubated with an anti-pStat3 antibody (1:3000), followed by a biotinylated antirabbit antibody (1:1000), and then avidin-biotin complex labeling. pStat3 was visualized with Nickel-diaminobenzidine staining. In total, pSTAT3 was quantified in 346 random AgRP/NPY neurons from *NPY-*

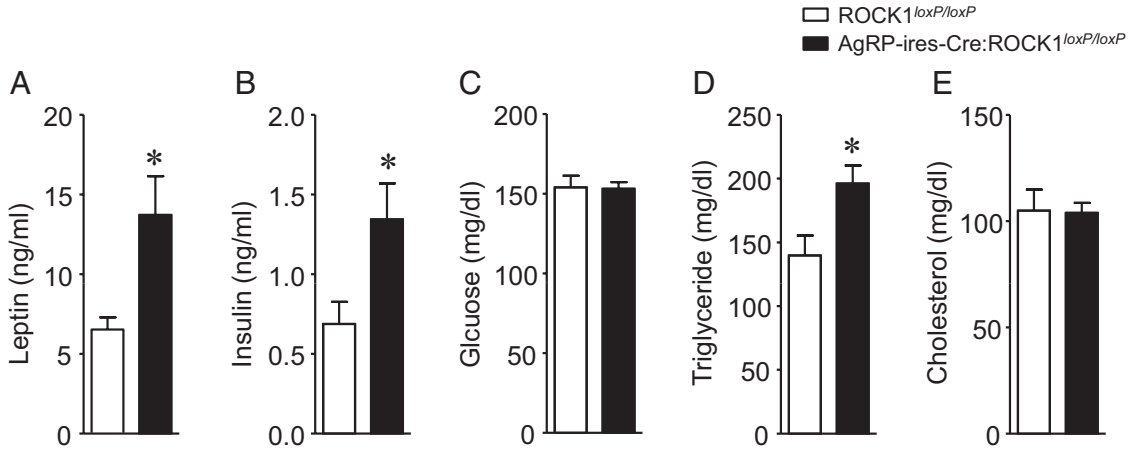

Figure 1. ROCK1 loss in AgRP neurons increases serum leptin, insulin, and triglycerides but not random-fed plasma glucose or serum cholesterol in mice. Serum (A) leptin, (B) insulin, (C) glucose, (D) triglycerides, and (E) cholesterol of AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ and control mice age of 18 weeks of age (n = 10) is shown. *, $P < .05$ vs control.

hrGFP; ROCK1$^{loxP/loxP}$ mice (n = 3) and in 352 AgRP/NPY neurons from *NPY-hrGFP*; AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ mice (n = 3).

ROCK1 activity assay

Hypothalamic tissue lysates (100-μg protein) were subjected to immunoprecipitation for 4 hours with 10 μL of a polyclonal ROCK1 antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, California), coupled to protein G-Sepharose beads (Pharmacia Biotechnology, Piscataway, New Jersey). Immune pellets were washed and resuspended in 50 μL of kinase mixture (20mM Tris [pH 7.5], 5mM $MgCl_2$, 100mM KCl, 0.1mM dithiothreitol, 100μM ATP, 1mM EDTA, 1μM microcystin-LR, 50μM long S6K substrate peptide [Millipore, Bedford, Massachusetts], and 1μCi [γ-32P] ATP) and incubated at 30°C for 30 minutes. Samples (40 μL) were spotted onto phosphocellulose p81 paper (Whatman, Princeton, New Jersey) and washed with 75mM orthophosphoric acid. Bound radioactivity was measured by scintillation counting (19).

Immunoblotting analysis

Hypothalamic lysates (25-μg protein) were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with polyclonal antibodies against ROCK1 (Santa Cruz Biotechnology, Inc) or actin (Santa Cruz Biotechnology, Inc). The bands were visualized with enhanced chemiluminescence and quantified by densitometry. The levels of ROCK1 protein were normalized by actin protein levels.

Electrophysiological studies

Brain slices from *NPY-hrGFP*; ROCK1$^{loxP/loxP}$ and *NPY-hrGFP*; AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ mice were prepared as described (20). Briefly, coronal sections (200μM) were cut with a Leica VT1000S Vibratome (Leica, Heerbrugg, Switzerland) and then incubated in oxygenated artificial cerebrospinal fluid at room temperature for at least 1 hour before recording. AgRP/NPY neurons were identified by GFP fluorescence, and whole-cell recordings were made using a MultiClamp 700B Amplifier (Axon Instruments, Union City, California) and pClamp 9.2 software (Axon Instruments). Recording electrodes had resistances of 2.5–4 MΩ when filled with the following solution containing 128mM K-gluconate, 10mM HEPES, 1mM EGTA, 10mM KCl, 1mM $MgCl_2$, 0.3mM $CaCl_2$, 5mM Mg-ATP, and 0.3mM Na-GTP (pH 7.35 with KOH). Leptin (100nM) or other agents were applied to bath solution through perfusion. Membrane potential and firing rate were analyzed with MiniAnalysis Program (Synaptosoft Inc) as described (20).

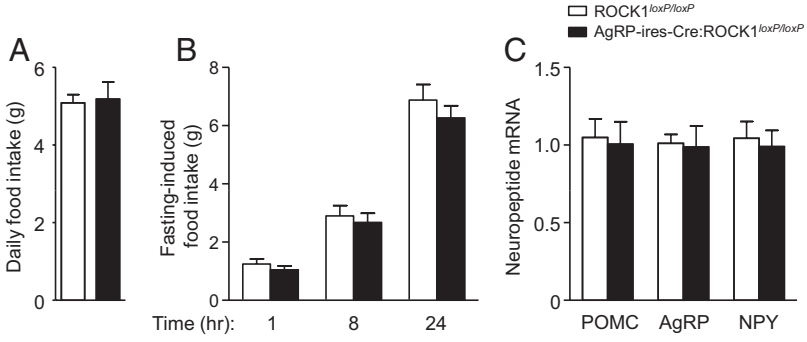

Figure 2. ROCK1 absence in AgRP neurons does not effect murine food intake or expression of feeding-related hypothalamic neuropeptides. (A) Daily food intake assessed on singly housed male at age of 8–9 weeks, (B) food intake after a 24-hour fast was measured at 1, 8, and 24 hours after food replacement, and (C) hypothalamic mRNA expression of neuronpeptides (POMC, AgRP, and NPY) assessed by real-time RT-PCR (at 10 wk of age) from AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ and control mice are shown (n = 8).

Blood parameters

Blood was collected from random-fed or overnight-fasted mice. Plasma glucose was measured using an OneTouch Ultra glucose meter (LifeScan, Inc, Milpitas, California). Serum triglyceride and cholesterol were measured by commercially available assays (Stanbio Laboratory). Serum insulin and leptin were measured by ELISA (Crystal Chem Inc). The range and sensitivity for these parameters are: glucose, 40–500 mg/dL; leptin, 1–25.6 ng/mL (sensitivity, 200 pg/mL using a 5-μL sample); insulin, 0.1–12.8 ng/mL

(sensitivity, 0.1 ng/mL using a 5-μL sample); total cholesterol, 1–750 mg/dL; and triglycerides, 1–1000 mg/dL.

Hypothalamic neuropeptide expression

Hypothalamic neuropeptide mRNAs were measured by quantitative RT-PCR, as described (7). Primer and probe sequences specific for POMC, AgRP, and NPY were previously described (7).

Statistical analyses

Data are presented as means ± SEM. Statistical analyses were performed using the StatView program (Abacus Concepts, Inc, Berkeley, California). Statistical significance among the groups was tested with unpaired or paired Student's *t* test and ANOVA when appropriate.

Results

ROCK1 deficiency in AgRP neurons increases serum leptin, insulin, and triglycerides but has no effects on glucose or cholesterol

We previously reported that body weight is increased approximately 12% in male AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ mice compared with ROCK1$^{loxP/loxP}$ controls at 18 weeks of age (17). Consistent with this increased body weight, we observed a more than 2-fold increase in serum leptin levels in these AgRP neuron-specific ROCK1 deletion mice compared with controls (ROCK1$^{loxP/loxP}$, 6.5 ± 0.8 ng/mL vs AgRP-ires-Cre: ROCK1$^{loxP/loxP}$, 13.7 ± 0.7 ng/mL) (Figure 1A), indicating development of whole-body leptin resistance in these mice. Similarly, serum insulin levels were also greatly elevated in AgRP neuron-specific ROCK1 deletion mice (ROCK1$^{loxP/loxP}$, 1.0 ± 0.3 ng/mL vs AgRP-ires-Cre: ROCK1$^{loxP/loxP}$, 1.7 ± 0.4 ng/mL) (Figure 1B), suggesting that these mice could have a high risk for developing insulin resistance. These effects are thought to be secondary due to increased adiposity. However, plasma glucose did not differ between genotypes (ROCK1$^{loxP/loxP}$, 153 ± 1 mg/dL vs AgRP-ires-Cre: ROCK1$^{loxP/loxP}$, 154 ± 7 ng/mL) (Figure 1C), indicating that ROCK1 loss in AgRP neurons did not affect glucose homeostasis. Similarly, serum cholesterol was unaffected by ROCK1 deletion in AgRP neurons (ROCK1$^{loxP/loxP}$, 105 ± 10 mg/dL vs AgRP-ires-Cre: ROCK1$^{loxP/loxP}$, 106 ± 5 mg/dL) (Figure 1E). Despite this, serum triglycerides levels were elevated in AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ mice compared with controls (ROCK1$^{loxP/loxP}$, 140 ± 16 mg/dL vs AgRP-ires-Cre: ROCK1$^{loxP/loxP}$, 191 ± 16 mg/dL) (Figure 1D). Together, these data show that selective deletion of ROCK1 in AgRP neurons causes whole-body leptin and insulin resistance.

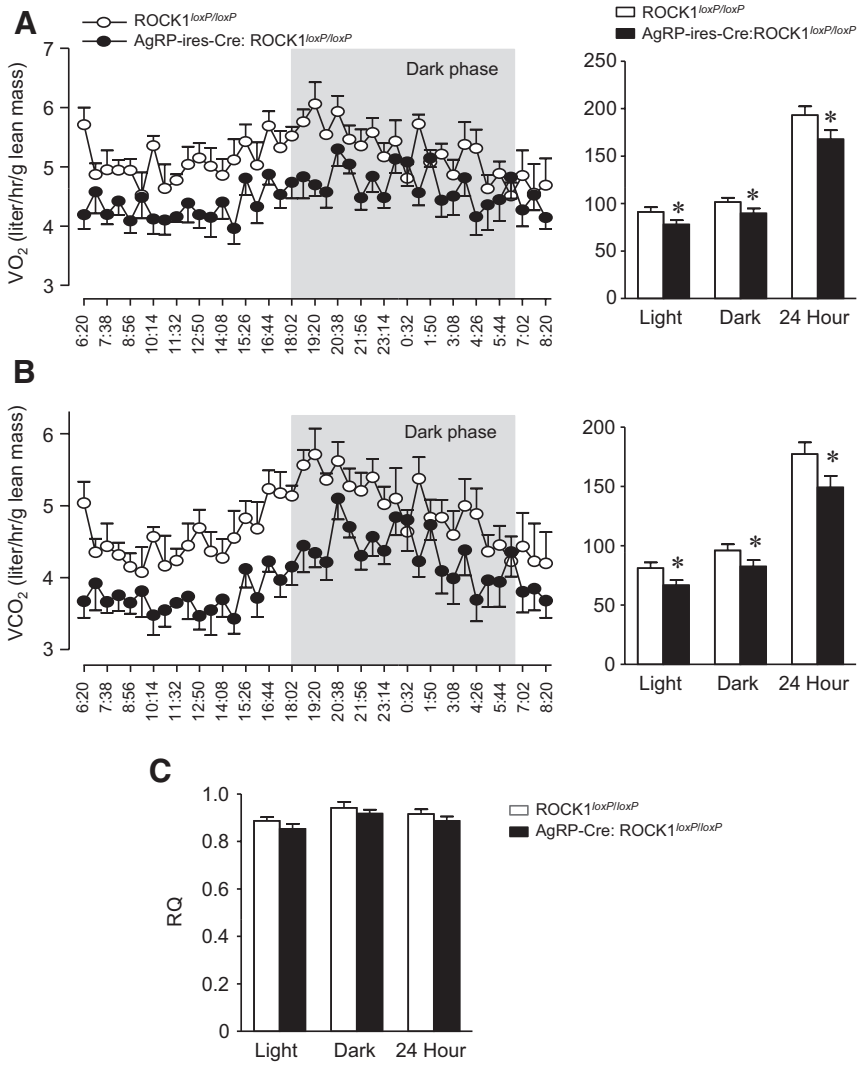

Figure 3. ROCK1 loss in AgRP neurons decreases energy expenditure of mice. (A) Hourly average oxygen consumption and corresponding light and dark phase oxygen consumption (12-h average). (B) Hourly average carbon dioxide consumption and corresponding light and dark phase carbon dioxide consumption (12-h average). (C) respiratory exchanges ratio of AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ and control mice at age of 10 weeks are shown. *, $P < .05$ vs control. VO_2, oxygen consumption; VCO_2, carbon dioxide production.

ROCK1 loss in AgRP neurons decreases energy expenditure and locomotion but does not affect food intake

To determine the mechanisms by which ROCK1 in AgRP neurons reg-

ulates energy balance, we measured food intake, energy expenditure, and locomotor activity of AgRP- ires-Cre: ROCK1$^{loxP/loxP}$ mice compared with ROCK1$^{loxP/loxP}$ controls. Daily food intake of AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ mice did not differ from controls at 8 weeks of age (ROCK1$^{loxP/loxP}$, 5.1 ± 0.2 g vs AgRP-ires-Cre: ROCK1$^{loxP/loxP}$, 5.2 ± 0.4 g) (Figure 2A) or during refeeding after a 24-hour fast (Figure 2B), suggesting that the increased adiposity of AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ mice does not result from increased food intake. Supporting this, hypothalamic POMC, AgRP, and NPY mRNA levels did not differ between genotypes (Figure 2C). Consistently, hypothalamic AgRP protein levels were similar in both groups (Supplemental Figure 1A, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). Leptin-stimulated AgRP mRNA levels tended to decrease but not statistically significant in both control and AgRP neuron-specific ROCK1-deficient mice (Supplemental Figure 1B).

In contrast, AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ mice have significantly lower oxygen consumption (VO_2) in during both the light and dark cycle compared with control mice (ROCK1$^{loxP/loxP}$, 4465 ± 91 mL/kg lean mass/h vs AgRP-ires-Cre: ROCK1 $^{loxP/loxP}$, 3854 ± 92 mL/kg lean mass/h; n = 7) (Figure 3A). Consistent with this, carbon dioxide production (VCO_2) was also significantly lower in AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ mice than control mice (ROCK1$^{loxP/loxP}$, 4005 ± 97 mL/kg lean mass/h vs AgRP-ires-Cre: ROCK1$^{loxP/loxP}$, 3431 ± 91 mL/kg lean mass/h; n = 7) (Figure 3B). The respiratory exchange ratio did not differ between genotypes (Figure 3C). We also observed a large difference in physical activity of AgRP neuron-specific ROCK1-deficient mice compared with controls. AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ mice showed much less movement than controls especially during the dark period (ROCK1$^{loxP/loxP}$, 2936 ± 297 counts vs AgRP-ires-Cre: ROCK1$^{loxP/loxP}$, 2178 ± 225 counts) (Figure 4, A and B). Collectively, our data demonstrate that AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ mice develop obesity mainly as a result of reduced energy expenditure and physical activity and not by increased food intake.

Deletion of ROCK1 impairs leptin-induced STAT3 phosphorylation in AgRP neurons

To determine whether ROCK1 deficiency increases adiposity via regulating leptin signaling in AgRP neurons, we first examined the response of AgRP/NPY neurons to leptin by assessing leptin-induced phosphorylation STAT3 in the hypothalamus. To determine the direct effects of ROCK1 on leptin activation of pSTAT3, *NPY-hrGFP*; AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ and *NPY-hrGFP;* ROCK1$^{loxP/loxP}$ mice were studied at 7 weeks of age, before differences in body weight become evident. In response to 3 mg/kg of leptin, approximately 60% of *NPY-hrGFP;* ROCK1$^{loxP/loxP}$ AgRP/NPY neurons exhibit pSTAT3 activation as detected by immunostaining, whereas only approximately 39% of AgRP/NPY neurons of *NPY-hrGFP*; AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ mice are positive for pSTAT3 (Figure 5, A and B). These data demonstrate that loss of ROCK1 in AgRP neurons decreases their leptin sensitivity.

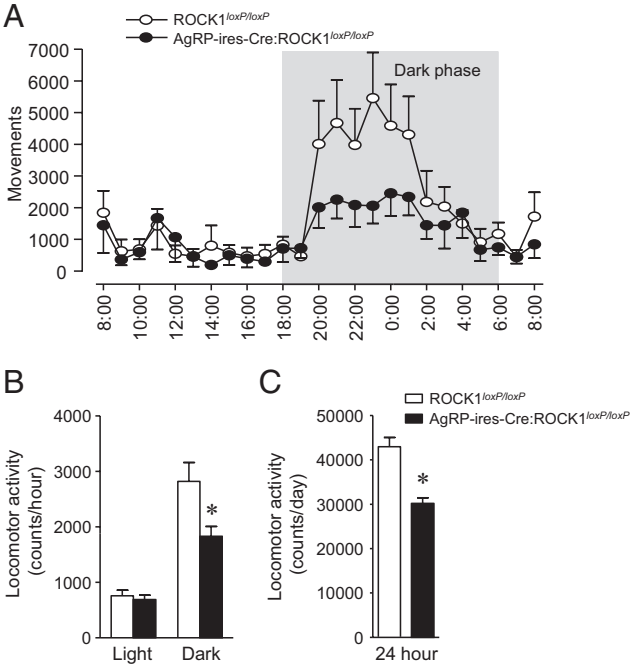

Figure 4. ROCK1 deficiency in AgRP neurons markedly lowers locomotor activity in mice. (A) Average hourly locomotor activity, (B) corresponding light and dark phase locomotor activity (12-h average), and (C) 24-hour locomotor activity (n = 8) of AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ and control mice are shown. *, $P < .05$ vs control.

ROCK1 is necessary for leptin-induced suppression of membrane potential and firing rate in AgRP/NPY neurons

AgRP neurons play critical roles in regulating energy expenditure and locomotion, and leptin inhibits AgRP neuronal activity to promote negative energy balance. To determine whether deletion of ROCK1 in AgRP neurons is necessary for leptin's inhibition of AgRP/NPY neurons, the membrane potential and firing rate of AgRP/NPY neurons from 4-week-old body weight-matched male *NPY-hrGFP*; AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ mice and *NPY-hrGFP;* ROCK1$^{loxP/loxP}$ littermates in response to leptin was measured. In control mice, leptin hypolarized AgRP neurons by −3.6 ± 0.6 mV and decreased firing rate by 2-fold (Figure 6, A, C, and D). In contrast, in *NPY-hrGFP*; AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ mice, leptin has no effects on membrane potential and firing rate of AgRP neurons

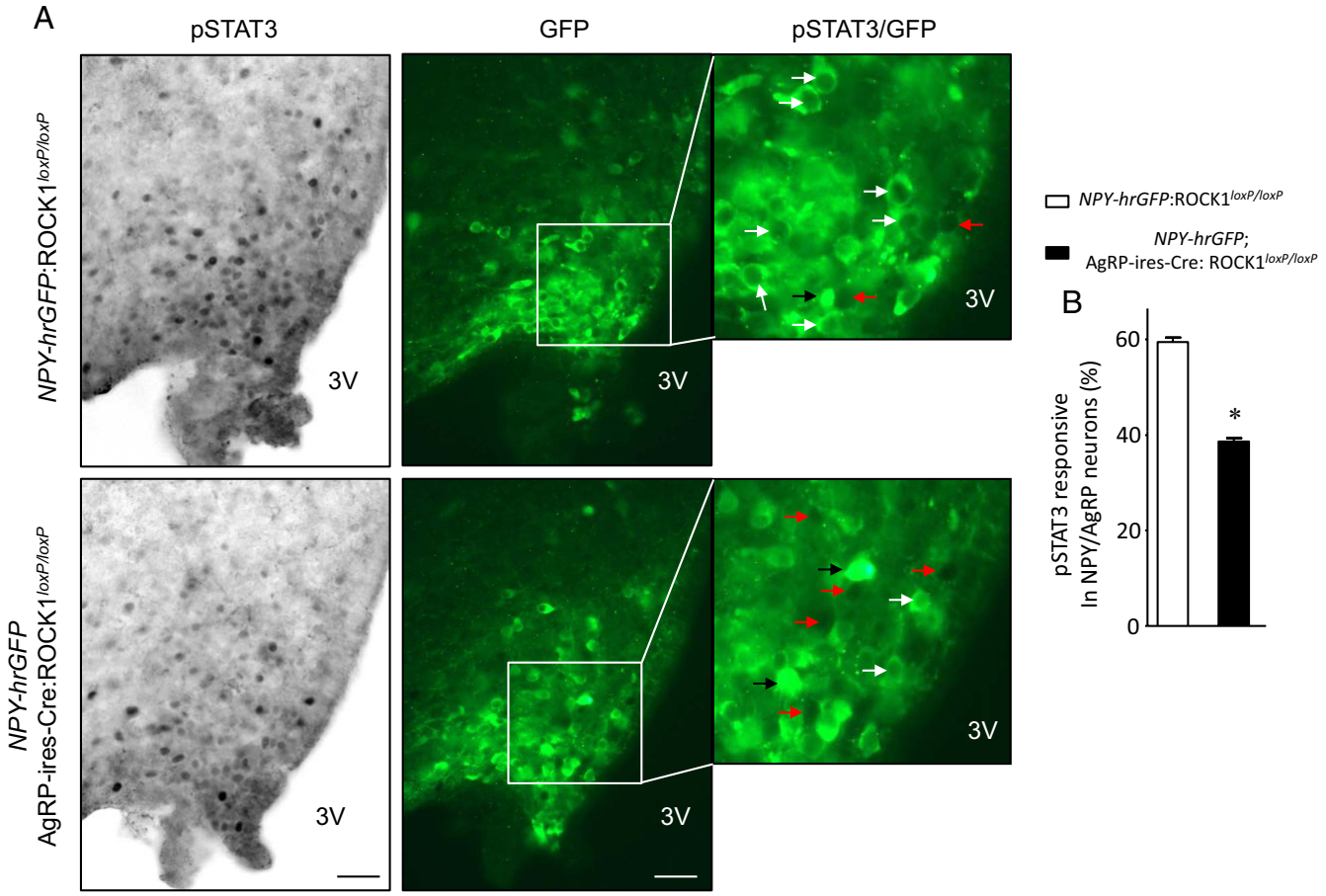

Figure 5. Lack of ROCK1 impairs leptin-induced pSTAT3 in AgRP neurons. (A) Immunochemical staining of hypothalamus from 7-week-old-fasted *NPY-hrGFP*; ROCK1$^{loxP/loxP}$ and *NPY-hrGFP*; AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ mice for AgRP/NPY GFP and phosphorylation of STAT3 is shown. (Left panel) Leptin-stimulated pSTAT3-reposive neurons are shown. (Middle panel) AgRP/NPY GFP neurons are shown. (Right panel) High-magnification images from pSTAT3/GFP images are shown. White arrows indicate pSTAT3 in AgRP/NPY neurons. Red arrows indicate pSTAT3 in non-AgRP/NPY neurons. Black arrows indicate non-pSTAT3 in AgRP/NPY neurons. (B) Quantification of the leptin-induced phosphorylation of STAT3 from AgRP/NPY neurons from the 2 genotypes is also shown. *, $P < .05$ vs control. Scale bar, 5.0 μm. 3V, third ventricle.

(Figure 6, B–D). Because prepubertal mice were used for this study, it is possible that gonadal steroid may influence the electrical activity of AgRP neurons. Nevertheless, These data suggest that ROCK1 is a key regulator of leptin inhibition of AgRP neuron activity in murine hypothalamus.

Deletion of ROCK1 in AgRP neurons increase body weight and adiposity on high-fat diet

On a chow diet, ROCK1 loss in AgRP neurons increased mouse weight by approximately 12% at 18 weeks of age (16). To determine whether ROCK1 deletion in AgRP neurons affects susceptibility to diet-induced obesity, AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ mice and their littermate controls were fed a high-fat/sugar diet from 4 weeks of age. At the age of 18 weeks, AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ mice were 46.1 ± 2.2 g, whereas control ROCK1$^{loxP/loxP}$ mice were 39.8 ± 2.9 g (Figure 7A), a 15% increase in body weight. Furthermore, as early as 9 weeks of age, AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ mice shown a 40% increase in fat mass compared with controls, despite having similar lean body mass and bone mineral mass and content (Figure 7, B, C, and F–H). Consistent with this, histological examination of white adipose and brown adipose tissue revealed that larger adipocyte cell size in both fat depots of AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ mice compared with controls (Figure 7D). Concurrently, adipose tissues of AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ mice were significantly increased compared with controls (Figure 7E). Together with our previous study, these data show that ROCK1 action in AgRP neurons promotes increased adiposity as well as increased sensitivity to diet-induced obesity.

Hypothalamic ROCK1 activity is decreased in diet-induced or genetic obesity in mice

Although ROCK1 deficiency in POMC or AgRP neurons impairs leptin action on energy balance, whether ROCK1 activity in obesity may ameliorate or contribute to its pathogenesis has not been explored. Of note, we

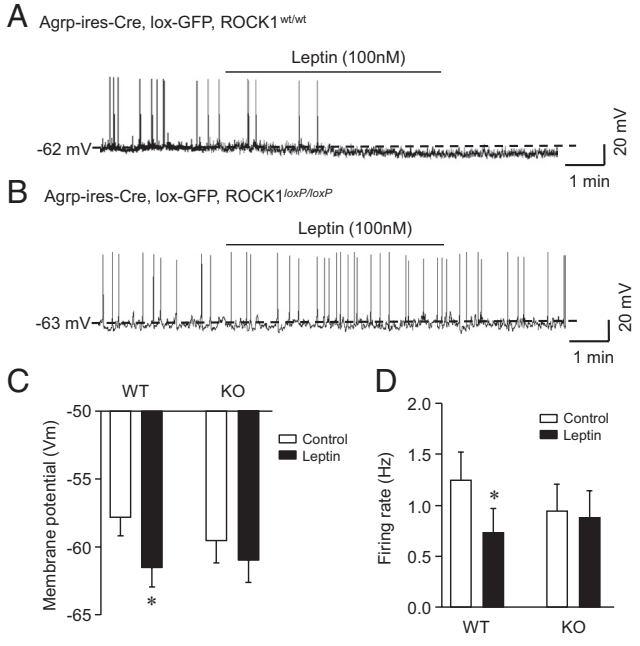

Figure 6. ROCK1 in AgRP/NPY neurons is necessary for leptin's effect to suppress neuron membrane potential and firing rate. (A) Leptin hypolarized AgRP/NPY neurons and decreased the frequency of action potentials in control male mice at 4 weeks of age. The bar indicates leptin exposure time. The dashed line indicates the baseline membrane potential. (B) Leptin did not alter membrane potential or firing rate of AgRP/NPY neurons from male *NPY-hrGFP*; AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ mice. (C) Leptin-induced membrane potential and (D) firing rate in WT (n = 14) and KO (n = 15) AgRP/NPY neurons. *, $P < .05$; **, $P < .01$ vs WT. WT, wild-type; KO, knockout.

previously showed that leptin increases ROCK1 activity in murine whole hypothalamus, an effect that was blocked by genetic deficiency of long form leptin receptor deficiency (*db/db*) in mice (16). These data suggested that leptin action and ROCK1 activity may reciprocally regulate each other in hypothalamic neurons. To test this hypothesis, we compared ROCK1 activity in ARC of *ob/ob* and *db/db* mice with lean. Consistent with an effect of leptin to stimulate ROCK1 activity, ARC ROCK1 activity was decreased *db/db* mice as well as *ob/ob* mice compared with lean controls (Figure 8I). The amount of ARC ROCK1 protein was decreased in *db/db* mice (control, 100 ± 4 AU vs *db/db*, 54 ± 7 AU; $P < .01$) but not *ob/ob* mice (control, 100 ± 4 AU vs *ob/ob*, 83 ± 12 AU; not significant) compared with control mice (Figure 8J). Impaired ARC ROCK1 activity in *db/db* mice could be due to decreased ROCK1 protein levels.

These data raised the possibility that in obesity, impaired leptin signaling may decrease ROCK1 activity in ARC neurons to promote leptin resistance. To test this, we examined ROCK1 activity in ARC of mice with diet-induced obesity compared with lean controls. C57BL6 mice fed a high-fat/high-sucrose diet from 6 weeks of age until 18 weeks of age exhibited increased body weight (chow, 31.1 ± 0.8 g vs high-fat diet (HFD), 47.1 ± 1.2 g), leptin (chow, 6.4 ± 2.7 ng/mL vs HFD, 21.3 ± 2.5 ng/mL), insulin (chow, 2.3 ± 0.2 ng/mL vs HFD, 4.4 ± 0.4 ng/mL), plasma glucose (chow, 168 ± 6 mg/dL vs HFD, 208 ± 8.9 mg/dL), serum triglyceride (chow, 84 ± 12 mg/dL vs HFD, 116 ± 17 mg/dL), and cholesterol (chow, 124 ± 6 mg/dL vs HFD, 156 ± 7 mg/dL), consistent with the development of diet-induced obesity and its related metabolic problems (Figure 8, A–F). At 3 weeks of HFD feeding, body weight was slightly increased but not statistically significant in HFD-fed mice compared with chow-fed mice (chow, 27.6 ± 0.6 g vs HFD, 30.0 ± 1.1 g; not significant). Basal ROCK1 activity in ARC was reduced in diet-induced obese mice compared with chow fed (chow, 100 ± 17% vs HFD, 43 ± 12%) (Figure 8G). Importantly, peripherally administered leptin significantly increased ROCK1 activity in the chow diet group, but this effect was blocked by high-fat diet group (chow leptin, 235 ± 18% vs HFD leptin, 96 ± 30%) (Figure 8G). The amount of ROCK1 protein in ARC was similar in both groups (Figure 8H). These findings indicate that impaired hypothalamic ROCK1 activity in mice fed high-fat diet may not due to increased adiposity or decreased its protein level. Similar observations were seen in mice fed HFD for 12 weeks (Supplemental Figure 2). Together, these data suggest that central leptin resistance lowers hypothalamic ROCK1 activity and ROCK1 impaired activation contributes to the pathogenesis of diet-induced obesity.

Discussion

Our previous work identified ROCK1 as a key mediator of leptin action in regulating food intake and adiposity in POMC neurons (16). Along with this, we reported that mice lacking ROCK1 in AgRP neurons displayed increased body weight and adiposity (16). In this study, we determined the physiological mechanisms by which ROCK1 in AgRP neurons regulates body weight homeostasis, with particular emphasis on energy metabolism. Here, we show that the increase in murine adiposity resulting from loss of ROCK1 in AgRP neurons is mainly due to decreased energy expenditure and, to a lesser extent, impaired locomotor activity. Furthermore, high-fat feeding impaired leptin-induced ROCK1 activity in murine ARC, suggesting that dysregulated hypothalamic ROCK1 activity may contribute to the etiopathogenesis of central leptin resistance and obesity. Combined with ROCK1's critical functions in POMC neurons to regulate feeding behavior (16), our data further support the idea that ROCK1 plays key roles in the central control of energy balance.

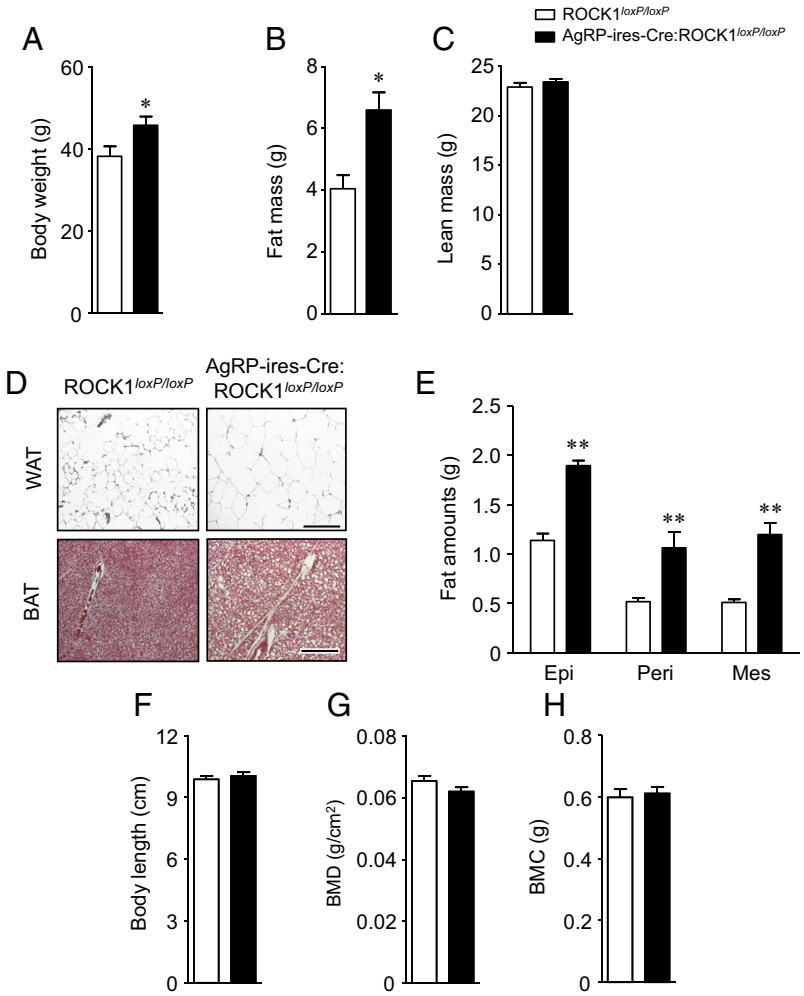

Figure 7. ROCK1 deletion in AgRP neurons increases murine body weight and adiposity on high-fat diet. (A) Body weight, (B) fat and (C) lean mass content, (D) white adipose tissue and brown adipose tissue morphology, (E) fat depots weight, (F) body length, (G) bone mineral density (BMD), and (H) bone mineral content of AgRP-ires-Cre: ROCK1$^{loxP/loxP}$ and control mice at 18 week of age (n = 10) is shown. **, $P < .01$ vs control; *, $P < .05$ vs control. BAT, brown adipose tissue; WAT, white adipose tissue; Epi, epididymal fat; Peri, perirenal fat; Mes, mesenteric fat; BMC, bone mineral content.

In contrast to the effects of ROCK1 deficiency in POMC neurons, ROCK1 loss selectively in AgRP neurons did not affect food intake of mice, suggesting that ROCK1 signaling in AgRP neurons is unlikely to regulate feeding behavior. Rather, ROCK1 absence in AgRP neurons impacted oxygen consumption and locomotion, revealing that ROCK1 action in AgRP neurons regulates energy balance through enhancing energy expenditure and physical activity. Consistent with this, recent studies show that leptin action in AgRP neurons is important for regulating metabolic rate and locomotor activity but not feeding behavior. For example, overexpression of the constitutively active form of STAT3 in AgRP neurons enhances energy expenditure and physical activity without affecting feeding behavior (21). Additionally, targeted deletion of either LepRb or STAT3 in AgRP-expressing neurons impaired oxygen consumption, but food intake was unchanged (8, 22). Furthermore, the magnitude of effect of ROCK1 deficiency in AgRP neurons on energy expenditure was similar to that of LepRb and STAT3, supporting the fact that ROCK1, like LepRb and STAT3, is a major mediator of leptin action in the context of energy balance.

AgRP neuronal activity is important for controlling energy balance. Activation of AgRP neurons is altered by nutritional condition, such as fasting and refeeding, and appetite-regulating hormones, including ghrelin and leptin (23). Pharmacological activation of AgRP neurons with designer receptors exclusively activated by designer drugs systems markedly decreases oxygen consumption, revealing a critical role of AgRP neuronal activation in the regulation of energy balance (23, 24). Leptin hyperpolarizes AgRP neurons and decreases fire rate, which contributes to promoting negative energy balance (25, 26). Like ROCK1 deficiency, phosphatidylinositide 3-kinase (PI3K) deletion in AgRP neurons also inhibits leptin-induced AgRP neuronal activity (27), raising the possibility that both ROCK1 and PI3K activate the same signaling cascade that can produce common metabolic outputs. In this regard, our previous studies clearly demonstrate that ROCK1 functions as a key upstream regulator of PI3K signaling in hypothalamic and other insulin-sensitive cell types (16, 28, 29).

Of note, it is possible that effect of ROCK1 deletion on energy expenditure but not food intake is the result of compensation of some sort in AgRP/NPY neurons. Clearly, acute activation of AgRP/NPY neurons in adult animals leads to dramatic increases in both feeding and energy expenditure (24, 30). However, many long-term genetic deletions of knockout models in AgRP/NPY neurons see effects on one and not the other; hence, the idea is that it may be compensation that adjusts food intake to normal.

It is clear that AgRP neurons send signals to neurons expressing melanocortin 3 receptor/melanocortin 4 receptor and/or oxytocin in the paraventricular hypothal-

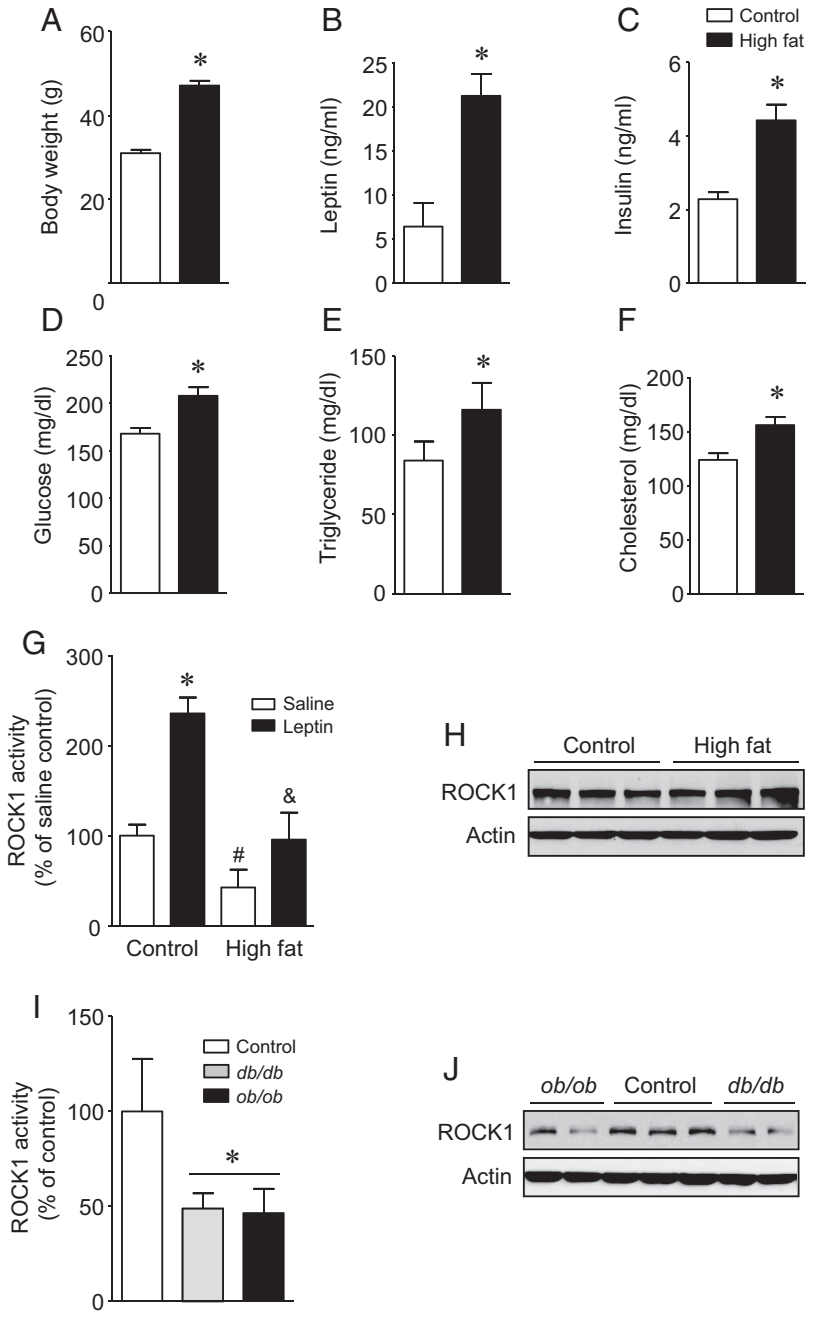

Figure 8. Diet-induced or genetic obesity decrease hypothalamic ROCK1 activity in mice. (A) Body weight, (B) serum leptin, (C) insulin and (D) plasma glucose, (E) serum, triglyceride, and (F) cholesterol from mice on chow or high-fat diet group at 18 weeks of age (n = 10) are shown. (G) Hypothalamic ROCK1 activity from mice on chow or high-fat diet group at 9 weeks of age (n = 6) is shown. Mice were injected with saline or leptin ip (3 mg/kg) and killed 30 minutes later. ROCK1 activity in the hypothalamus was measured by immune complex assay. (H) Hypothalamic ROCK1 protein from mice on chow or high-fat diet group is shown. Hypothalamic lysates were subjected to SDS-PAGE and immunoblot with an antibody for ROCK1. (I) Hypothalamic ROCK1 activity from *ob/ob*, *db/db*, and C57BL6 mice (n = 6) is indicated. Mice were injected with saline or leptin ip (3 mg/kg) and killed 30 minutes later. ROCK1 activity in the hypothalamus was measured by immune complex assay. (J) Hypothalamic ROCK1 protein from *ob/ob*, *db/db*, and C57BL6 mice is presented. Hypothalamic lysates were subjected to SDS-PAGE and immunoblot with an antibody for ROCK1. *, $P < .05$ vs saline control; #, $P < .05$ vs saline control; &, $P < .05$ vs leptin control; *, $P < .05$ vs control.

amus (30, 31). Both paraventricular hypothalamus neuron populations are involved in regulating food intake (30, 31). Evidence also demonstrates that AgRP neurons directly inhibit POMC neuronal activity by releasing the neurotransmitter γ-aminobutyric acid (GABA), leading to changes in energy expenditure and locomotor activity but not feeding behavior (32). Moreover, AgRP innervations onto POMC neurons are highly associated with leptin resistance induced by aging or chronic high-fat diet (33), suggesting that the release of GABA from AgRP neurons may play an important role in modulating energy metabolism in the context of leptin action. Our results demonstrate that hypothalamic ROCK1 activation is significantly reduced in mice with genetic or high-fat diet-induced obesity. Given that ROCK1 regulates neuronal leptin signal transduction (16), it is conceivable that ROCK1 activation in AgRP neurons may be involved in release of neurotransmitters like GABA in response to leptin. Indeed, disruption of GABA release in LepRb-expressing neurons displayed reduced energy expenditure, which led to increase body weight and sensitive to high-fat diet (34). Together, it is possible that ROCK1 deletion in AgRP neurons may affects the neuronal circuitry from AgRP neuron to POMC or yet unidentified neurons that play essential roles in controlling energy expenditure in conjunction with leptin action.

Leptin influences body weight not only by suppressing food intake but also by increasing energy expenditure, as reflected in metabolic rate and by oxygen consumption. Resistance to the effects of leptin on energy balance is a major contributor to development of obesity. Increased hypothalamic expression of negative regulators of proximal LepRb signaling components have been re-

ported in response to leptin, in contribution to the pathogenesis of leptin resistance in diet-induced obesity. These include suppressor of cytokine signaling 3 (SOCS3), a key inhibitor of Janus kinase 2 (JAK2) (35), protein tyrosine phosphatase (PTP)1B, a JAK2 tyrosine phosphatase (36), and the related T-cell PTP (37). Furthermore, loss of PTP1B enhanced leptin sensitivity and prevented obesity in mice on a high-fat diet (38). Similarly, haploinsufficiency of SOCS3 or neuron-specific deficiency of SOCS3 protected mice from diet-induced obesity and leptin resistance (39, 40). In this study, basal ROCK1 activity in hypothalamus was lower in mice fed a high-fat diet and in genetically obese mice. Furthermore high-fat feeding impaired leptin's ability to stimulate ROCK1 activity. Given that JAK2 is a substrate of ROCK1 (16), it is conceivable that increased PTP1B or SOCS3 expression by high-fat feeding inhibits JAK2 activation, leading to reduced ROCK1 activity. We cannot rule out the possibility that hyperglycemia and hyperinsulinemia could contribute to lower basal hypothalamic ROCK1 activity, because we found decreased ROCK1 activity in the hypothalamus of *db/db* or *ob/ob* mice that are hyperglycemic and hyperinsulinemic. Nevertheless, our data suggest that a defect in hypothalamic ROCK1 activity may contribute to the pathogenesis of central leptin resistance in obesity.

In conclusion, our work demonstrates that loss of ROCK1 from AgRP-expressing neurons impaired oxygen consumption and locomotor activity, leading to increased adiposity. Leptin action on neuronal activity and STAT3 phosphorylation of AgRP neurons is reduced in the absence of ROCK1. The molecular mechanism for this is involved in ROCK1-mediated JAK2 phosphorylation, which promotes downstream signaling pathways of leptin, including STAT3 and PI3K signaling, ultimately leading to control of energy balance (16). Moreover, defective hypothalamic ROCK1 activity is associated with the pathogenesis of diet-induced leptin resistance. These data suggest that ROCK1 activity is a key regulator of hypothalamic leptin signaling that is essential for normal body weight homeostasis, which when impaired, may contribute to the development of obesity.

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