Leptin contributes to the control of resting metabolic rate (RMR) and blood pressure (BP) through its actions in the arcuate nucleus (ARC). The renin-angiotensin system (RAS) and angiotensin AT1 receptors within the brain are also involved in the control of RMR and BP, but whether this regulation overlaps with leptin’s actions is unclear. Here, we have demonstrated the selective requirement of the AT1a receptor in leptin-mediated control of RMR. We observed that AT1a receptors colocalized with leptin receptors (LEPRs) in the ARC. Cellular coexpression of AT1a and LEPR was almost exclusive to the ARC and occurred primarily within neurons expressing agouti-related peptide (AgRP). Mice lacking the AT1a receptor specifically in LEPR-expressing cells failed to show an increase in RMR in response to a high-fat diet and deoxycorticosterone acetate–salt (DOCA-salt) treatments, but BP control remained intact. Accordingly, loss of RMR control was recapitulated in mice lacking AT1a in AgRP-expressing cells. We conclude that angiotensin activates divergent mechanisms to control BP and RMR and that the brain RAS functions as a major integrator for RMR control through its actions at leptin-sensitive AgRP cells of the ARC.

Introduction
The renin-angiotensin system (RAS) contributes to blood pressure (BP) and fluid balance control. Selected tissues contain an autocrine/paracrine RAS, in which local synthesis of angiotensin II (ANG II) and its receptors regulate regional function (1). All components of the RAS have been documented in specific brain nuclei, and the brain RAS has been implicated in BP control. Both ANG type 1 (AT1) and type 2 (AT2) receptors are expressed in the brain, and it is believed that the majority of the central effects of ANG II on cardiovascular function are mediated through the AT1 receptor in humans and its AT1a homolog in rodents (2, 3). Interestingly, the AT1 receptor is robustly expressed in the arcuate nucleus (ARC) (4–6), a site implicated in metabolic control but less appreciated in cardiovascular control. Although microinjection of exogenous ANG II into the ARC increases mean arterial pressure (7), it is not known whether ANG II signaling in the ARC is necessary for BP regulation. The role of ANG II in the ARC therefore remains unclear.

In contrast, there is a well-defined role for leptin in the ARC in the control of both BP and energy balance. Leptin action at proopiomelanocortin (POMC) neurons of the ARC is critical for BP regulation (8), and both POMC and agouti-related peptide (AgRP) neurons mediate the metabolic effects of leptin in the ARC. While the brain RAS has been implicated in the control of selected aspects of energy balance including food intake and energy expenditure, the specific brain regions involved and the neurocircuitry that mediates these effects are unknown (9, 10).

Here, we examined the concept that AT1a receptors in the ARC are involved in energy homeostasis. Specifically, we document a critical role for AT1a expressed in cells that also express the leptin receptor (LEPR) in resting metabolic rate (RMR) control. In addition, we document a specific directionality of the leptin-AT1a interaction and establish a primary role for AT1a within the subset of LEPR-expressing cells that also express AgRP. These findings document a local autocrine/paracrine leptin-RAS interaction within the ARC, clarify the divergent control of BP versus RMR by the ARC, and highlight a major integrative role for the ARC RAS in RMR control.

Results
Brain ANG II stimulates RMR via AT1 receptors. To establish a primary role for AT1 receptors in the RMR-stimulating effects of central ANG II, RMR was determined by respirometry in animals after i.c.v. infusion of either artificial cerebral spinal fluid (aCSF) or ANG II. Mice receiving i.c.v. ANG II exhibited a significant increase in RMR compared with aCSF-treated animals after 10 days of treatment (Figure 1, A and B), with no significant alterations in the respiratory exchange ratio (RER) (Figure 1C). Mice were also subsequently treated with losartan in their drinking water for 7 days (0.8 mg/ml). While mice that received i.c.v. aCSF did not show any change in RMR with losartan, mice treated with i.c.v. ANG II showed a significant reduction in RMR (Figure 1D). Taken together, these data support the idea that central ANG II signaling stimulates RMR via the ANG II AT1 receptor; however, it remains unclear which AT1 receptors in the brain are important for this control.

LEPR and AT1a colocalize within the ARC. We examined expression patterns of both LEPR and AT1a by using the NZ44-transgenic mouse, which expresses GFP inserted into the AT1a locus in a recombinated BAC transgene (11), and mice that conditionally
express a red fluorescent reporter after Cre-mediated recombination in LEPR-expressing cells (Lepr-Cre ROSA-stoplox flox tdTomato) (12–14). We identified LEPR- and AT1a coexpressing cells by the colocalization of red and green fluorescence in the brain. Cells expressing both receptors were abundant in the ARC of the hypothalamus, with minimal colocalization in the lateral hypothalamus, dorsomedial hypothalamus, and nucleus tractus solitarii (Figure 2A). Cells expressing either, but not both, receptors were detected in other regions. These findings support the notion of a primary colocalization of LEPR and AT1a within the ARC.

AT1a in leptin-sensitive cells is necessary for energy homeostasis. To determine whether ANG II signaling specifically in leptin-sensitive cells is involved in the control of energy balance, we created a transgenic mouse model that lacks the AT1a receptor in any cell expressing the long (b) signaling form of LEPR (AT1aLepKo Lepr-Cre mice, referred to hereafter as AT1aLepKo mice). Cre-recombinase activity was confirmed by PCR amplification of the AT1a gene using primers that generate different-sized products, depending on Cre-mediated recombination. Cre-recombinase-mediated recombination of the AT1a gene was detected in the supraoptic nucleus (SON) and the ARC, but not in the subfornical organ (SFO), paraventricular nucleus (PVN), or cortex, probably because of the low abundance of Lepr-Cre-expressing cells in these regions (Figure 2B). Critically, with the exception of lung tissue in which AT1a expression was already very low at baseline, we observed no changes in AT1a mRNA expression in peripheral tissues (Figure 2C). Thus, the physiological phenotypes observed in AT1aLepKo mice were likely due to the loss of ANG II receptor signaling on LEPR-containing cells within the ventromedial ARC. Importantly, the loss of AT1a receptors on LEPR-containing cells does not block leptin signaling, as phosphorylation of STAT3 (p-STAT3) was preserved in the ARC of AT1aLepKo mice in response to leptin (Figure 2D).

Next, we assessed whether AT1aLepKo mice have altered energy homeostasis. Under ad libitum Chow-fed conditions (Teklad Diet 7013; 18% kcal from fat), AT1aLepKo mice exhibited normal body mass and fat mass (Figure 3, A and B). When maintained on a chow diet, these mice had normal food intake, digestive efficiency, physical activity, RMR, and brown adipose tissue (BAT) uncoupling protein 1 (Ucp1) mRNA levels (Figure 3, C–G). While both control and AT1aLepKo mice responded to 5 weeks of 45% high-fat diet (HFD) (OpenSource D12451) treatment, with elevations in body weight and fat mass, AT1aLepKo mice gained significantly more weight and fat mass than did their control littermates (Figure 3, A and B). There were no significant differences in food intake (Figure 3C) or digestive efficiency (Figure 3D) between AT1aLepKo and littermate control mice during HFD treatment, indicating a normal total daily energy input. Whereas control littermates responded to HFD treatment with a significant increase in RMR after just 2 weeks of treatment (Figure 3F), AT1aLepKo mice failed to show RMR stimulation, and the induction of BAT Ucp1 expression was severely compromised (Figure 3G). Of critical importance, the lack of RMR stimulation in response to an HFD occurred prior to significant alterations in body weight and composition, consistent with the concept that the reduced increase in RMR is causal for the increased body adiposity observed in AT1aLepKo mice.

Sympathetic nerve activity (SNA) responses to leptin are attenuated in mice with global AT1a disruption and in rats administered i.c.v. losartan (15), suggesting that central ANG II signaling through AT1a receptors is required for leptin action. Consistent with this hypothesis, the BAT SNA response to leptin was significantly attenuated in AT1aLepKo mice (Figure 2D). The reduction in leptin-stimulated BAT SNA in AT1aLepKo mice probably explains the loss of RMR and UCP1 responses to HFD in these animals and the subsequent elevation in fat mass, as it has been previously shown that enhanced SNA is necessary to counteract weight gain following high caloric intake (13). Thus, we conclude that AT1a receptors expressed in leptin-sensitive cells are required to mediate the RMR response to HFD and leptin via activation of thermogenic SNA.

AT1a receptors in LEPR-expressing cells selectively mediate RMR but not BP. The DOCA-salt model (deoxycorticosterone acetate plus a high dietary sodium load) of hypertension is a well-established model of low-renin hypertension mechanistically involving brain ANG II and AT1a receptors (16–18). It is notable that DOCA-salt also robustly stimulates RMR, an effect that also requires brain AT1a receptors (19). However, it is not known whether RMR and BP responses are mediated by AT1a receptors on the same population of cells in the brain. Given the HFD results obtained above, we asked whether the RMR responses to DOCA-salt required AT1a receptors expressed in LEPR-expressing cells and whether these cells also mediate the BP response. Control litter-
A unidirectional interaction between AT1A and LEPR in RMR control. To elucidate the directionality of the central interaction between leptin and ANG II in RMR control, we next examined the effects of stimulating the brain RAS in LEPR-deficient animals (db/db mice). At baseline, db/db mice had significant elevations in body and fat mass as previously reported (Figure 4, D and E). Fol-omes responded to DOCA-salt treatment with a significant elevation in RMR, but this increase was markedly blunted in AT1A−/−/LepR−/− mice (Figure 4A; genotype-DOCA interaction P = 0.02). This suggests that the RMR response to both leptin (HFD) and ANG II (DOCA-salt) is impaired in AT1A−/−/LepR−/− mice. In contrast, there was a normal increase in BP (Figure 4B) and reduction in heart rate (Figure 4C) in AT1A−/−/LepR−/− mice in response to DOCA-salt. Taken together, these results are consistent with a selective role for AT1A receptors in leptin-sensitive cells for metabolic, but not cardiovascular, control physiology.

A unidirectional interaction between AT1A and LEPR in RMR control. To elucidate the directionality of the central interaction between leptin and ANG II in RMR control, we next examined the effects of stimulating the brain RAS in LEPR-deficient animals (db/db mice). At baseline, db/db mice had significant elevations in body and fat mass as previously reported (Figure 4, D and E). Fol-
The metabolic effects of leptin require functional AT1A receptors on LEPR-containing cells, while the RMR effects of brain ANG II do not require functional leptin signaling. We conclude that there is a specific directionality to the central interaction between leptin and ANG II in RMR control. Moreover, BP responses to DOCA-salt RMR stimulation, both control and db/db mice responded with significant elevations in RMR (Figure 4F). Consistent with an increase in RMR, DOCA-salt-treated db/db mice exhibited a significant reduction in fat mass (Figure 4E). This, in combination with the findings above, leads to the conclusion that the metabolic effects of leptin require functional AT1A receptors on LEPR-containing cells, while the RMR effects of brain ANG II do not require functional leptin signaling. We conclude that there is a specific directionality to the central interaction between leptin and ANG II in RMR control. Moreover, BP responses to DOCA-salt treatment in control and db/db mice showed significant increases in RMR (Figure 4F). Consistent with this, fat mass in db/db mice was significantly reduced after DOCA-salt treatment (Figure 4E), revealing a directionality in the interaction between leptin and ANG II in RMR control. Additionally, BP responses to DOCA-salt treatment in control and db/db mice showed significant increases in RMR (Figure 4F). Consistent with this, fat mass in db/db mice was significantly reduced after DOCA-salt treatment (Figure 4E), revealing a directionality in the interaction between leptin and ANG II in RMR control.

Figure 3. AT1A-Lepr-KO mice exhibit impaired responses to an HFD and leptin. (A-D) Body mass (A) (n = 28 chow-fed control mice; n = 35 HFD-fed control mice; n = 26 chow-fed AT1A-Lepr-KO mice; n = 31 HFD-fed AT1A-Lepr-KO mice); fat mass (B) (n = 28 chow-fed control mice; n = 35 HFD-fed control mice; n = 26 chow-fed AT1A-Lepr-KO mice; n = 31 HFD-fed AT1A-Lepr-KO mice); home cage food intake (C) (n = 12 chow-fed control mice; n = 29 HFD-fed control mice; n = 15 chow-fed AT1A-Lepr-KO mice; n = 14 HFD-fed AT1A-Lepr-KO mice); and digestive efficiency (D) (n = 5/group) of control and AT1A-Lepr-KO mice on a chow diet or after 5 weeks of 45% HFD treatment. (E) Physical activity of control and AT1A-Lepr-KO mice on a chow diet (n = 10 control mice; n = 9 AT1A-Lepr-KO mice). (F and G) ANCOVA-adjusted RMR (F) (n = 13 chow-fed control mice; n = 22 HFD-fed control mice; n = 15 chow-fed AT1A-Lepr-KO mice; n = 12 HFD-fed AT1A-Lepr-KO mice) and BAT Ucp1 expression (G) (n = 4 chow-fed control mice; n = 4 HFD-fed control mice; n = 5 chow-fed AT1A-Lepr-KO mice; n = 8 HFD-fed AT1A-Lepr-KO mice) in control and AT1A-Lepr-KO mice on a chow diet or after 2 weeks of HFD treatment. (H) Changes in BAT SNA following i.v. administration of leptin (60 μg) in control and AT1A-Lepr-KO mice (n = 6 control mice; n = 5 AT1A-Lepr-KO mice). RVI, rectified/integrated voltage. Data represent the mean ± SEM. *P < 0.05, by Tukey’s multiple comparisons procedure.
cleavage product of the Pomc gene and a marker for POMC neurons. To determine whether AgRP neurons express the AT1A receptor, mice expressing tdTomato under control of the Agrp promoter (Agrp-Cre ROSA-stopfloxed-ttdTomato mice) were bred with NZ44 mice. While POMC neurons do not appear to express the AT1A receptor, we observed a significant localization of the AT1A receptor on AgRP neurons (Figure 5C). These data are consistent with RNA-sequencing data demonstrating expression of AT1A receptors on AgRP, but not POMC, neurons isolated by laser-capture microdissection (21). To complement this reporter-based method, we also used FISH (RNAscope) to examine the colocalization of endogenous AT1a, Pomc, and Agrp mRNA transcripts in the ARC of WT C57BL/6J mice. As expected, we detected AT1a transcripts in cells also expressing AgRP, but not in cells expressing POMC (Figure 5D and Supplemental Data; supplemental material available online with this article; https://doi.org/10.1172/JCI88641DS1).

α-Melanocyte-stimulating hormone action on second-order neurons is suppressed in AT1A LepR-KO mice. We next hypothesized that the loss of AT1a in AgRP neurons of AT1A LepR-KO mice would result in disinhibited inhibitory tone to second-order neurons and thereby attenuated RMR responses to exogenous α-melanocyte-stimulating hormone (αMSH). To test this concept, we administered salt are preserved in db/db mice (20), further supporting a model of divergent BP and RMR control by the brain RAS, involving AT1a receptors in distinct neural pathways.

Reduced AgRP and normal POMC responses to HFD in the ARC in AT1A LepR-KO mice. To determine whether AT1a is localized to a specific subset of LEPR-expressing cells of the ARC, we first examined the expression of Pomc and Agrp genes within this brain region in AT1A LepR-KO and littermate control mice under chow- and HFD-fed conditions. Five weeks of HFD feeding caused a significant increase in POMC expression in the hypothalamus of both AT1A LepR-KO and control littermate mice (Figure 5A). In contrast, HFD feeding caused a significant suppression of hypothalamic Agrp mRNA expression in control mice, but no significant suppression was observed in AT1A LepR-KO mice (Figure 5B). These data support the concept that loss of AT1a in LEPR-expressing cells has the specific effect of modulating the function of AgRP, but possibly not POMC, neurons of the hypothalamus.

AT1a disruption in AgRP-expressing cells specifically interrupts RMR control. We next investigated the colocalization of AT1a receptors with POMC and AgRP neurons. To determine whether POMC neurons express the AT1a receptor, we immunostained brain tissue from NZ44 mice for adrenocorticotropin (ACTH), a
Nle$^{4}$, D-Phe$^{7}$-$\alpha$MSH, an analog of $\alpha$MSH that has been demonstrated to stimulate RMR after peripheral administration (22), to both control and AT1A LepR-KO mice and examined acute RMR responses. As expected, while both control littermates and AT1A LepR-KO mice responded to acute $\alpha$MSH treatment with significant elevations in RMR, this response was blunted in AT1A LepR-KO mice as compared with that in control animals (Figure 5E). Importantly, a volume-matched injection of 0.9% saline had no effect on RMR (data not shown). These results indicate that loss of AT1A receptors in leptin-sensitive cells interferes with the action of $\alpha$MSH on second-order neurons and thereby leptin-mediated control of SNA and RMR.

We speculate that AT1A in LEPR-expressing cells is required to disinhibit SNA and RMR activation by $\alpha$MSH signaling.

Specific disruption of AT1A in AgRP neurons of AT1A AgRP-KO mice. Given the colocalization of AT1A and AgRP within the ARC, we explored the functional role of AT1A within AgRP neurons in RMR control in mice lacking AT1A receptors specifically in AgRP-expressing cells (AT1A AgRP-KO mice). Mice expressing Cre-recombinase via the Agrp promoter (Agrp-Cre) (23) were bred with mice harboring the conditional AT1A allele. Like AT1A LepR-KO mice, AT1A AgRP-KO mice exhibited a normal RMR when maintained on a standard chow diet (Figure 6A) but had suppressed BAT SNA in response to i.c.v. injection of leptin (Figure 6B) and suppressed RMR responses to acute $\alpha$MSH injection (Figure 6C). Notably, these effects were similar to those observed in AT1A LepR-KO mice.

Together, we conclude that AT1A receptors, localized to the subset of AgRP- and LEPR-expressing cells within the ARC, are critically and specifically involved in the control of RMR.

Disinhibition of GABA in AT1A AgRP-KO mice. Finally, we investigated possible mediators of AT1A signaling, focusing on mechanisms that have been documented in AgRP neurons such as GABA signaling. Previously, it was established that disruption of the LEPR in vesicular GABA transporter–expressing (VGAT-expressing) cells induces obesity (24), similar to that seen in AT1A LepR-KO mice. Moreover, genetic disruption of VGAT within AgRP neurons elicited phenotypes opposite those of AT1A LepR-KO mice.
mice, as VGATAgRP-KO animals were resistant to HFD-induced weight gain selectively due to an elevated RMR (23). As it has been demonstrated that expression of glutamate decarboxylase enzyme 1 (GAD1) (also known as GAD67) positively correlates with GABA release (25), we examined the expression of GABA synthetic enzymes within the ARC. mRNA levels for both of the glutamate decarboxylase enzymes \( \text{Gad1} \) and \( \text{Gad2} \) (also known as GAD65) (Figure 6, D and E) and \( \text{Vgat} \) (Figure 6F) were significantly increased in AT1A AgRP-KO mice compared with mRNA levels in littermate controls. Further, AT1A LepR-KO mice showed similar increases in \( \text{Gad1} \) and \( \text{Gad2} \) levels in the ARC (\( \text{Gad1} \), 1.5-fold increase, \( P < 0.05 \); \( \text{Gad2} \), 1.6-fold increase, \( P < 0.05 \)). Together, these findings support a role for AT1A within ARC AgRP neurons in the control of GABA signaling.

**Discussion**

Our current study underscores a major role of the RAS within the brain in the control of energy homeostasis. This is mediated in large part through actions of ANG II upon AT1A localized to a subset of LEPR-expressing neurons within the ARC that also express AgRP. These AT1A receptors are required for ANG II and leptin to fully stimulate SNA and RMR, but not BP. These AT1A receptors appear to tonically suppress GABA expression, and thus angiotensinergic stimulation of the ARC ultimately results in the stimulation of SNA and the RMR through the disinhibition of POMC and aMSH stimulation of second-order neurons (Figure 7).

It is well established that the CNS develops resistance to leptin during obesity. More recently, it has been suggested that this resistance is selective, whereby the cardiovascular effects of leptin remain intact, while the metabolic effects of leptin are abolished (26). Current research on the mechanisms underlying selective leptin resistance (SLR) focuses on deficits in leptin signaling. However, our data support a possible role for central ANG II signaling in SLR, as we have shown that ANG II AT1A receptors specifically in leptin-sensitive cells are dispensable for BP control, but necessary for RMR control and thus energy balance. The general concept that AT1 receptors mediate leptin sensitivity is also supported in a recent study by Müller-Fielitz et al. (27). In contrast to the effects observed in our studies, it was demonstrated that peripheral treatment with the AT1 antagonist telmisartan prevented leptin resistance in rats fed a high-calorie/cafeteria diet (27). Further, TGR(ASrAOGEN) rats, which exhibit glial-specific knockdown of angiotensinogen expression, are small and resistant to weight gain with high-calorie diets (28). It is unclear how species differences, peripheral actions of telmisartan, and developmental or receptor sensitivity changes in the TGR(ASrAOGEN) rats (29) may complicate the interpretation of these studies in concert with the current study. In addition, we and others have documented interacting but opposing effects of the brain versus the peripheral RAS in the control of the RMR (9, 19, 30–33). Thus, further elucidation of site-, receptor-, and second-messenger–specific actions of ANG II within the brain and
periphery in the control of leptin sensitivity and cardiovascular and metabolic function is warranted.

An anatomical dissociation of cardiovascular versus metabolic control mechanisms by leptin has been previously implied by other studies. Supporting the idea of possible cross-talk between leptin and ANG II in other brain regions, Smith et al. demonstrated that the SFO of young Sprague-Dawley rats responds to leptin with induction of p-STAT3 and that a large fraction of neurons from this region exhibit altered excitability in response to leptin (34). Young et al. demonstrated that i.c.v. delivery to Leprfl/fl mice of an adenovirus encoding Cre-recombinase, which provides surprisingly specific targeting of the SFO, abolished renal SNA responses to peripheral or central leptin administration, while similar disruption of the SFO and ANG II AT1A receptor abolished BAT SNA responses to leptin (35, 36). As the SFO is a well-characterized target for cardiovascular control by ANG II, it is intriguing that LEPR appears to be present in this region and that its disruption selectively modulates cardiovascular but not metabolic control, while disruption of AT1A in this region attenuates metabolic responses to leptin. In the current study, we detected tdTomato reporter expression in the SFO of Lepr-Cre; ROSA-stoplox-stopflox-tdTomato mice, but no overlap with the GFP reporter (Figure 2A), which may indicate that the LEPR and AT1A receptors are present in distinct neurons of this brain region in mice. Further, this lack of colocalization in the SFO may explain the lack of BP effect in AT1A, Lepr-KO mice (Figure 4B).

It is likely that leptin signaling contributes to the transcriptional regulation of RAS signaling components. Indeed, i.c.v. administration of leptin leads to elevated AT1A receptor expression in the ARC, and ob/ob mice have decreased expression and activity of angiotensin-converting enzyme (ACE) (15, 37, 38). Further, administration of leptin to ob/ob mice enhances plasma RAS expression (37, 38), and angiotensinogen is expressed in AgRP neurons of the ARC (21). Leptin-activated second-messenger pathways, such as p-STAT3, have also been shown to promote the transcription of angiotensinogen in other cell types (39–44). In addition, i.c.v. administration of the ACE inhibitor captopril attenuates SNA responses to central leptin (15). Thus, it is reasonable to hypothesize that leptin signaling may activate an autocrine RAS signaling mechanism within AgRP neurons of the ARC, ultimately to control the RMR.

In conclusion, our data demonstrate a critical requirement of the ANG II AT1A receptor, specifically localized to leptin-sensitive AgRP neurons, in the control of RMR. This functional interaction appears to be specific to the control of RMR and may therefore contribute to the development of SLR. The results presented here illustrating a selective effect of AT1A in AgRP neurons upon RMR, but not ingestive behavior, also raise the intriguing possibility that a specific subset of AgRP neurons expressing AT1A contribute to RMR control. In contrast, AT1A-signaling may not be functional within the AgRP neurons that contribute to ingestive behavior. Further exploration of each of these novel concepts is warranted.

**Methods**

**Animal subjects.** The AT1A, Lepr-KO colony was maintained on a C57BL/6J background by iterative breeding of a male mouse expressing Cre recombinase under control of the Lepr promoter (12) with a female mouse with loxP sites flanking the AT1a allele (JAX-016211; The Jackson Laboratory) (45). The AT1A, AgRP-KO colony was maintained on a C57BL/6J background by iterative breeding of a male mouse expressing Cre recombinase under control of the Agpr promoter (JAX-012899; The Jackson Laboratory) with a female mouse with loxP sites flanking the AT1a allele. Experiments were conducted in 10- to 15-week-old male and female mice. Littermate controls were used throughout, and no differences for any endpoint were discovered when comparing among littermate controls of various genotypes (see the Supplemental Data for additional details regarding the littermate controls). Mice were housed at 25°C on a standard 12-hour light/12-hour dark cycle with ad libitum access to water and standard chow (18% kcal from fat; Teklad diet 7013; Envigo) or an HFD (45% kcal from fat; OpenSource diet D12451; Research Diets Inc.). Inbred male C57BL/6J and db/db mice (JAX-000642) maintained on the C57BL/6J background were obtained from The Jackson Laboratory. NZ44 mice backcrossed onto the C57BL/6J background (originally derived by the GENSAT Project at The Rockefeller University, New York, New York, USA) were obtained from Teresa Milner’s laboratory at Weill Cornell Medical College (New York, New York, USA) (11).

**Intracerebroventricular delivery of ANG II.** Mice were instrumented with an i.c.v. cannula (ALZET Brain Infusion Kit III) connected to an osmotic minipump (ALZET Model 1004) for chronic i.c.v. administration of aCSF (Tocris; 3525) or ANG II (5 ng/h; Sigma-Aldrich; A9525). Under anesthesia with i.p. ketamine/xylazine, the mice were placed in a stereotaxic frame, the minipump was inserted under the skin, and the cannula was implanted using the following coordinates: 1.1 mm lateral, 0.5 mm caudal to bregma, and 3.0 mm ventral from the surface of the skull. The cannula was secured to the skull using Vetbond (3M) and dental cement.

**DOCA-salt model.** A 50-mg pellet of DOCA (Sigma-Aldrich) was implanted into the s.c. cavity under isoflurane anesthesia. Animals were subsequently singly housed and allowed ad libitum access to standard chow and both tap water and 0.15 M NaCl water for 3 weeks.

**Brain punches.** Mice were euthanized and brains were harvested, frozen in 2-methylbutane and then in embedding medium (Tissue-Tek O.C.T. Compound; Sakura Finetek), and stored at −80°C. Coronal sections (50-μm) of the brain were cut using a cryostat, and micropunches of the cortex and SFO were obtained using a 0.75-mm needle (Stoelting). Bilateral punches of the PVN, ARC, and SON were taken using 0.50-mm and 0.75-mm needles, respectively. Genomic DNA was isolated using the AllPrep DNA/RNA Micro Kit (QIAGEN; catalog 80284), and Cre-mediated recombination was confirmed by PCR amplification of the STOP-LacZ cassette using the primers 5′-ATGAACAGACAACTTGTCCATCTG-3′ and 5′-ATTGGTGTGCAGTTGAGCTGACG-3′.
examined by PCR using Taq Platinum (Invitrogen, Thermo Fisher Scientific). ATla gene primers were designed to produce a 2,761-bp band for the intact ATla gene or a 309-bp band for the recombined fragment, because of the excision of exon 3 as previously described (46). Total RNA was isolated from brain punches by TRizol (Thermo Fisher Scientific) extraction, and cDNA was made using SuperScript III Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific). Real-time quantitative PCR for mouse Pomp, Agyp, Gad1, Gad2, Vgat, and actin was performed using TaqMan Gene Expression Assays (Life Technologies, Thermo Fisher Scientific; Mm00435874_m1, Mm00475829_g1, Mm04207432_g1, Mm00484623_m1, and Mm00494138_m1, respectively), and gene expression levels were compared using the Livak method (47).

**SNr recording.** BAT sympathetic nerves were recorded under α-chloralose anesthesia as previously described (15, 30). Briefly, animals were anesthetized and instrumented with a colonic temperature probe. Cannulae were implanted into the common carotid artery (to record BP) and jugular vein (for i.v. injections). A sympathetic nerve subserving BAT was isolated and suspended on 36-gauge platinum-iridium electrodes and secured with silicone gel. Electrodes were interfaced to a high-impedance probe (HIP-511; Grass Instruments), and the neural signal from BAT was amplified 100,000 times (1 × 10^4), filtered at low- and high-frequency cutoffs of 100 and 1,000 Hz, respectively, and routed to a resetting voltage integrator (B600c; University of Iowa Bioengineering). Data were recorded using an ADInstruments PowerLab with the associated Chart software.

**Home cage experiments.** Mice were singly housed in home cages, and standard bedding was replaced by an absorbent pad to facilitate the measurement of food intake and collection of feces at both 10 and 15 weeks of age. Mice were acclimated to the cages for 72 hours before data collection. Body mass and food intake measurements and feces collection were done at the same time daily for 4 days. Energy per gram feces was determined by bomb calorimetry as previously described (48, 49). This energy was applied to feces and food intake to determine energy intake and output, and thus caloric absorption.

**RMR.** The RMR was determined using respirometry as described previously (19, 30, 46, 50). Briefly, mice were placed into thermally controlled, air-tight chambers maintained at thermoneutrality (30°C), and oxygen consumption and carbon dioxide content of effluent air (flowing at 300 ml/min, corrected to standard temperature and pressure) were continually recorded (AEI analyzers, logged using an ADInstruments PowerLab with the associated Chart software). Analyzers were calibrated daily using soda lime and calibration gas (Praxair). To determine the effect of oMSH treatment on the RMR, baseline RMR measurements were taken in the morning and again in the afternoon following i.p. injection of UMSH.

**BP by radiotelemetry.** BP and heart rate were assessed using radiotelemetric BP probes (DSI; model TA11PA-C10), with cannulae implanted into the common carotid artery, as previously described (19). Briefly, probes were implanted under ketamine/xylazine anesthesia, and animals were allowed to recover for at least 1 week before 3 consecutive 24-hour periods were recorded (baseline). Data were recorded for 30 seconds every 5 minutes during the recording period and averaged across days within subject before statistical comparisons were made across groups.

**Body composition.** Body composition was determined using nuclear magnetic resonance (NMR) (Bruker; LF90) as previously described (49–52). Awake animals were lightly restrained in a polycarbonate tube during the 1-minute recording and then immediately placed back into home cages.

**IHC.** Brain tissue used for IHC was perfused with 4% PFA and incubated in 30% sucrose. Coronal sections (40-μm) were cut using a frozen sledge microtome (American Optical Scientific Instruments), and primary antibodies were added to free-floating sections at 4°C overnight. After washing with 1× PBS, secondary antibodies were added at room temperature for 1 hour. p-STAT3 staining was performed as previously described (53). Briefly, mice were fasted overnight and administered 1 μg/g leptin i.p. the following morning. Thirty minutes later, mice were perfused, and brains were sectioned as described above. Free-floating sections were incubated with the primary antibody for 72 hours, followed by incubation with the secondary antibody for 1 hour. All sections were mounted onto microscope slides and viewed using a Nikon Labophot 2 microscope. Quantification was performed using ImageJ (NIH). The following primary antibodies were used: chicken GFP (Aves Lab Inc., GFP-1010); ACTH (Harbor-UCLA Research and Education Institute); and p-STAT3 (Cell Signaling Technology; 9131S). The following secondary antibodies were used: Alexa Fluor 488 goat anti-chicken (Life Technologies, Thermo Fisher Scientific; A11039); Alexa Fluor 488 goat anti-rabbit (Thermo Fisher Scientific; A10008); and Alexa Fluor 568 goat anti-rabbit (Thermo Fisher Scientific; A11011).

**FISH.** Mice were euthanized and brain tissue was collected and frozen in 2-methylbutane and then in embedding medium (Tissue-Tek O.C.T. Compound; Sakura Finetek) and stored at −80°C. Next, 10-μm coronal sections were cut using a cryostat and underwent the RNAscope Multiplex FISH protocol for fresh-frozen tissue (Advanced Cell Diagnostics). Briefly, sections were pretreated with protease IV, followed by hybridization with target probes, which contained 20 double Z oligo probe pairs for the specific RNA target of interest (Agtr1a, 404001; AgRP, 400711; and POMC, 314081). Subsequent hybridization was completed with RNAscope detection reagents to amplify the fluorescent signal. All images were captured using a Leica STED 3× at ×20 to ×40 magnification.

**Statistics.** All quantitative data were analyzed using the 2-tailed Student’s t test, ANOVA, or analysis of covariance (ANCOVA), followed by Tukey’s multiple comparisons procedures. All experimental results passed tests of normal distribution and equal variance, so only parametric analyses are reported. Gene expression data were analyzed using the Livak method (47). Data are reported as the mean ± SEM throughout, and a P value of less than 0.05 was used as the threshold for statistical significance.

**Study approval.** All animal experiments were approved by the IACUC of the University of Iowa.

**Author contributions**

KEC and JLG conceptualized the project and drafted and revised the manuscript. KEC, JAS, AML, BJW, NKL, CMLB, NAP, DAM, KNGC, and KR collected and analyzed data and revised the manuscript.

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