Leptin’s hunger-suppressing effects are mediated by the hypothalamic–pituitary–adrenocortical axis in rodents

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Contributed by Gerald I. Shulman, May 20, 2019 (sent for review February 4, 2019; reviewed by Roger D. Cone and Richard D. Palmiter)

Leptin informs the brain about sufficiency of fuel stores. When insufficient, leptin levels fall, triggering compensatory increases in appetite. Falling leptin is first sensed by hypothalamic neurons, which then initiate adaptive responses. With regard to hunger, it is thought that leptin-sensing neurons work entirely via circuits within the central nervous system (CNS). Very unexpectedly, however, we now show this is not the case. Instead, stimulation of hunger requires an intervening endocrine step, namely activation of the hypothalamic–pituitary–adrenocortical (HPA) axis. Increased corticosterone then activates AgRP neurons to fully increase hunger. Importantly, this is true for 2 forms of low leptin-induced hunger, fasting and poorly controlled type 1 diabetes. Hypoglycemia, which also stimulates hunger by activating CNS neurons, albeit independently of leptin, similarly recruits and requires this pathway by which HPA axis activity stimulates AgRP neurons. Thus, HPA axis regulation of AgRP neurons is a previously underappreciated step in homeostatic regulation of hunger.

Significance

Low levels of leptin, a hormone secreted by adipocytes that signals the body as to the availability of fuel stores, are known to increase food intake. Here, we demonstrate a mechanism by which low leptin stimulates food intake in rodents: Under conditions of hypoleptinemia, stress hormone (glucocorticoid) production is increased, and in turn stimulates AgRP neurons to promote appetite.


Reviews: R.D.C., University of Michigan; and R.D.P., University of Washington.

Conflict of interest statement: G.I.S. is on the Scientific Advisory Boards for Merck, NovoNordisk, AstraZeneca, Aegerion, iMBP, and Jansen Research and Development, and receives investigator-initiated support from Gilead Sciences and Merck. R.J.P. and G.I.S. receive investigator-initiated support from AstraZeneca. All other authors declare no competing interests.

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This article contains supporting information online at www.pnas.orglookup/supp/doi/10.1073/pnas.1901795116/-/DCSupplemental.

Published online June 18, 2019.
receptor antagonist, mifepristone, suppressed caloric intake to that measured in recently fed animals without altering plasma leptin concentrations. Plasma adrenocorticotropic hormone (ACTH) concentrations mirrored plasma corticosterone, with the exception of mifepristone-treated rats, which exhibited the expected increase in ACTH resulting from glucocorticoid receptor antagonism. Taken together, these data suggest that hypercorticosteronemia mediates the majority of fasting-induced hyperphagia (Fig. 1 A–C and SI Appendix, Fig. S1 A–F). Next, we performed similar studies in insulin-deficient, poorly controlled type 1 diabetic rats, as we and others have shown that poorly controlled diabetes is a state of severe hypoleptinemia (25–33). In this model, we again found that hypoleptinemia caused hypercorticosteronemia in TID rats as replacement leptin normalized plasma corticosterone concentrations and reversed hyperglycemia without affecting plasma insulin concentrations. Hypoleptinemia caused hyperphagia in TID rats, an effect mediated through hypercorticosteronemia: Animals with poorly controlled TID consumed twice as many calories within a 2-h span compared with nondiabetic rats, an observation corrected by replacement leptin infusion and restored by coinfusion of corticosterone.

**Fig. 1.** Hypoleptinemia-mediated hypercorticosteronemia causes hyperphagia in fasted and type 1 diabetic rats. (A and B) Plasma leptin and corticosterone in healthy rats after 4 h of infusion, before refeeding. (C) Food intake measured over a 2-h period. (D and E) Plasma leptin and corticosterone in healthy and type 1 diabetic rats after 4 h of infusion, before refeeding. (F) Food intake measured over 2 h. In all panels, *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 by one-way ANOVA with Bonferroni’s multiple-comparisons test. Data are the mean ± SEM.
in rats treated with leptin. Mifepristone treatment abrogated the effect of poorly controlled diabetes to cause hyperphagia, suppressing food intake after an overnight fast to rates measured in nondiabetic controls (Fig. 2 A–C and SI Appendix, Fig. S2 A–F).

HPA Axis Activation Causes Hyperphagia in Hypoglycemia. We next hypothesized that HPA axis activation would drive hyperphagia during acute, insulin-induced hypoglycemia (34). Consistent with this, following a 4-h hyperinsulinemic–hypoglycemic clamp, rats consumed 4 times as many calories as euglycemic rats infused with the same dose of insulin, despite unchanged plasma leptin concentrations. The majority of this hyperphagia was driven by hypercorticosteronemia, as evidenced by the fact that infusing euglycemic rats with corticosterone to increase plasma corticosterone concentrations to levels measured in hypoglycemic rats recapitulated the majority of the effect of hypoglycemia to cause hyperphagia. In contrast, mifepristone treatment reduced food intake in hypoglycemic rats to rates measured under euglycemic conditions (Fig. 3 A–C and SI Appendix, Fig. S3 A–F), thereby demonstrating that hypercorticosteronemia, and not central hypoglycemia, is ultimately responsible for the majority of hypoglycemia-induced hyperphagia.

**Elevated Leptin Abrogates HPA Axis-Mediated Hyperphagia.** To directly test the role of corticosterone in modulating food intake, we studied adrenalectomized (ADX) mice infused with low (0.75 mg/d) or high (2 mg/d) doses of corticosterone s.c. Corticosterone drove food intake: Both total caloric intake and caloric intake following a 24-h fast were reduced in the ADX-low corticosterone group, and increased to control rates in ADX-high corticosterone-treated mice (SI Appendix, Fig. S4 A–D). We then placed the ADX mice on a high-fat diet (HFD) for 2 wk to test whether increased adiposity may prevent hyperphagia after a fast. High-fat feeding more than doubled fat mass and reduced caloric intake upon refeeding by 40% only in sham-operated mice (SI Appendix, Fig. S4 E–H). Given the decreased food intake after a fast in sham-operated mice fed an HFD, these data suggest that obesity generates a negative-feedback signal dependent on glucocorticoid activity to suppress fasting-induced hyperphagia.

We tested this hypothesis in ADX rats, which, unlike mice, afford the ability to measure plasma glucocorticoid concentrations in the unrestrained, awake state. In ADX rats, fasting lowered leptin and refeeding increased it independent of adrenal function. However, corticosterone drove fasting-induced hyperphagia: Upon refeeding, 24-h–fasted ADX-low corticosterone-treated rats exhibited a 50% reduction in caloric intake compared with both sham-operated and high-corticosterone treated ADX rats. After 10 d of high-fat feeding, plasma leptin concentrations increased: After a 24-h fast, plasma leptin concentrations dropped only to ~2 ng/mL, compared with 0.5 ng/mL in chow-fed rats. This increase in plasma leptin concentrations prevented the fasting-induced increase in plasma corticosterone in sham-operated rats, and consequently reduced food intake after a 24-h fast to levels measured in ADX-low corticosterone-treated rats (Fig. 3 A–F and SI Appendix, Fig. S5 A–D).

**Correcting Body Weight in Obese Rodents Restores Fasting-Induced Hyperphagia.** Because these data demonstrate that elevated leptin reduced fasting-induced hypoleptinemia and consequently abrogated hypercorticosteronemia-mediated hyperphagia in the fasted–refed state, we next sought to determine whether these alterations could be reversed by normalizing body weight in rats with diet-induced obesity. To that end, we placed 4-wk HFD rats on a very-low–calorie diet (VLCD) (10.4 kcal/d, ~20% of their typical daily caloric intake) to reduce their body weight to that of healthy rats. This intervention lowered plasma leptin and increased plasma corticosterone during a 48-h fast to those of lean rats, resulting in a 3.5-fold increase in food intake upon refeeding (Fig. 3 G–I and SI Appendix, Fig. S6 A–C), suggesting a threshold for plasma leptin at which the HPA axis and, consequently, hyperphagia are activated.
Corticosterone Increases the Activity of AgRP Neurons. Because genetic tools in mice more readily allow for a mechanistic examination of neural processes, we next set out to confirm a similar role for corticosterone in modulating feeding behavior in mice. We therefore assessed the effect of elevated corticosterone on food intake in mice. To do so, slow-release corticosterone or placebo pellets were implanted subcutaneously 2 h prior to dark cycle onset. As a consequence, mice that received corticosterone pellets should have elevated corticosterone in the following light period, compared with placebo-implanted animals, in which corticosterone is at its circadian nadir (35–38) (Fig. 4A). Indeed, corticosterone implants significantly increased plasma corticosterone to levels similar to previous reports in fasted mice (36) and our starvation, T1D, hypoglycemia, and corticosterone infusion studies in rats (Fig. 4B; see Figs. 1–3). Food intake in the early light period was also significantly increased (Fig. 4C), while food intake in the preceding dark cycle, when corticosterone is naturally high, was comparable to placebo-implanted animals (Fig. 4D). Thus, corticosterone administration did not have an effect on the firing of AgRP neurons, which persisted in the presence of synaptic blockers (Fig. 4E and F). This effect persisted in the presence of blockers of AMPA, kainate, NMDA, and GABA-A receptor-mediated synaptic transmission, suggesting a cell-autonomous component to their firing under caloric deficit. Second, we assessed whether administering exogenous corticosterone to mice would increase the firing rate of AgRP neurons. We therefore implanted placebo or corticosterone pellets into Npy-hrGFP mice (as in Fig. 4G), and mice were killed for electrophysiology recordings 2 h into the following light period when corticosterone levels are naturally low. AgRP neuron firing rates were significantly higher in mice that received corticosterone pellets, which persisted in the presence of synaptic blockers (Fig. 4G and H). Exogenous corticosterone administration did not have an effect on the firing of Npy-hrGFP-negative neurons in the arcuate (Fig. 4I).

To further examine the effect of corticosterone on AgRP neuron firing, we assessed whether corticosterone increases the activity of AgRP neurons in vivo. To do so, we virally expressed the Ca2+ sensor, GCaMP6s, in AgRP neurons using Agrp-IRESCre mice (45) and performed fiber photometry recordings in the early light cycle in awake, behaving mice (46) (Fig. 4J). Subcutaneous injection of corticosterone (2 mg/kg) significantly increased AgRP neuron activity compared with vehicle injection (Fig. 4K and L and SI Appendix, Fig. S7B). Thus, corticosterone not only causes persistent increases in AgRP neuron firing in ex vivo cell-attached recordings from AgRP neurons under 2 conditions in which corticosterone levels are high, concomitant with an increase in appetite. AgRP neurons were identified in ex vivo brain slices for electrophysiology studies by using Npy-hrGFP mice (44) (Fig. 4D). First, we confirmed previous findings that AgRP neurons fire with higher frequency in the fasted state (41–43) (Fig. 4E and F). This effect persisted in the presence of blockers of AMPA, kainate, NMDA, and GABA-A receptor-mediated synaptic transmission, suggesting a cell-autonomous component to their firing under caloric deficit. Second, we assessed whether administering exogenous corticosterone to mice would increase the firing rate of AgRP neurons. We therefore implanted placebo or corticosterone pellets into Npy-hrGFP mice (as in Fig. 4G), and mice were killed for electrophysiology recordings 2 h into the following light period when corticosterone levels are naturally low. AgRP neuron firing rates were significantly higher in mice that received corticosterone pellets, which persisted in the presence of synaptic blockers (Fig. 4G and H). Exogenous corticosterone administration did not have an effect on the firing of Npy-hrGFP-negative neurons in the arcuate (Fig. 4I).

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Finally, because corticosterone has many targets within the brain and may activate AgRP neurons through modulation of their neural afferents, we sought to determine whether direct action of corticosterone on AgRP neurons was necessary to augment their activity. To do so, we overexpressed the glucocorticoid-inactivating enzyme 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2) in AgRP neurons. 11β-HSD2 is a key regulator of active glucocorticoid levels as it converts corticosterone to its biologically inert isoform, dehydroxycorticosterone, thus preventing GR-mediated action of corticosterone on AgRP neurons. To determine whether direct action of corticosterone on AgRP neurons is necessary for the hyperphagia caused by elevated glucocorticoid levels, we overexpressed 11β-HSD2 in AgRP neurons, which blunted both the fasting-induced and corticosterone-mediated increase in AgRP neuron firing rate (Fig. 5 E and F). Together, these data suggest that corticosterone promotes appetite by directly increasing the firing of orexigenic AgRP neurons.

**Corticosterone Promotes Fasting- and Hypoglycemia-Induced Hyperphagia through AgRP Neurons.** To examine whether corticosterone signaling in AgRP neurons is necessary for the hyperphagia caused by elevated corticosterone, we bilaterally injected Cre-dependent AAV-DIO-GCaMP6s expressing AAV-DIO-GCaMP6s into the arcuate of Agrp-IRES-Cre mice. 11β-HSD2 overexpression in AgRP neurons completely abolished the hyperphagia effect of corticosterone pellets during the early light cycle (Fig. 6 A and B). Given that our work in rats demonstrated that corticosterone drives fasting and hypoglycemia-induced hyperphagia, we assessed whether food intake under these conditions is mediated by action of corticosterone on AgRP neurons. Indeed, expression of 11β-HSD2 in AgRP neurons significantly reduced food consumption following a fast (Fig. 6 C and D), while hypoglycemia-induced feeding was entirely abolished by 11β-HSD2 expression in AgRP neurons (Fig. 6 E and F). Furthermore,
Despite their differing etiologies, hypoglycemia (51–59), poorly controlled diabetes (17, 55, 60–69), and starvation (70–74) are all well-known triggers of hyperphagia. Our current study demonstrates that hyperphagia driven by these states is dependent upon hypercorticosteronemia and that the effect of leptin to reduce food intake in starvation and poorly controlled diabetes is mediated through suppression of the HPA axis. Furthermore, we show that activation of appetite-promoting AgRP neurons in the hypothalamic arcuate nucleus by corticosterone underlies these effects. While surprising, these results are in line with prior work linking elevated corticosterone to hyperphagia, weight gain, and increased expression of the orexigenic neuropeptides NPY and AgRP in the arcuate (22–24, 75). Furthermore, most prior studies (76–81), but not all (82), have found that adrenalectomy moderates the development of obesity in leptin-deficient rodents; however, these studies attributed the finding that adrenalectomy reduced food intake in leptin-deficient rodents to multiple alternative mechanisms and did not implicate stimulation of AgRP neuron firing by glucocorticoids.

It is generally believed that leptin does not regulate the HPA axis in humans because some leptin-deficient humans, unlike leptin-deficient rodents, do not have elevated glucocorticoids (83, 84). However, evidence to the contrary exists: Patients with congenital leptin deficiency (85, 86) or endocrine dysfunction concomitant with hypoleptinemia (87) present with elevated cortisol, which can be rescued with leptin treatment (87). Furthermore, humans with low glucocorticoids have reduced hunger and body weight (88, 89), and increased cortisol stimulates appetite in humans (90, 91). Our current findings and the discrepancies regarding HPA axis regulation of hyperphagia in humans with leptin deficiency strongly suggest that further examination of this system is warranted.

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ventrolateral medulla, which are activated by hypoglycemia and are viewed as playing a key role in transducing the vital counterregulatory responses (92), stimulate both glucocorticoids and feeding (34). This is likely mediated through their projections to the paraventricular nucleus of the hypothalamus, suggesting that these neurons directly regulate the HPA axis (34, 92). Previously, insulin or 2-deoxyglucose–induced glucoprivation was shown to increase \( \text{Agrp} \) and \( \text{Npy} \) transcripts in the arcuate (93, 94), suggesting that \( \text{AgRP} \) neurons are likely involved in driving the hypoglycemia-induced hyperphagia. Strikingly, \( \beta \)-HSD2 expression in AgRP neurons blocked hypoglycemia-induced hyperphagia (93, 94), indicating that hypoglycemic activation of the HPA axis and subsequent corticosterone-mediated stimulation of AgRP neurons is necessary for the induction of hypoglycemia-driven feeding.

Inhibition of glucocorticoid signaling in AgRP neurons blocked hyperphagia induced by hypercorticosteronemia and hypoglycemia, while prominently reducing food intake after fasting. However, the molecular mechanism underlying corticosterone activation of AgRP neurons is unknown. Glucocorticoids primarily regulate gene expression, but the genomic effects of corticosterone signaling are dependent upon many factors, including cell type, and are not fully characterized (95). Given that AgRP neuron action potential firing caused by fasting or hypercorticosteronemia persists ex vivo in the presence of synaptic blockers, corticosterone signaling may drive expression of genes that promote cell-autonomous activity. We have previously proposed similar actions by aldosterone on neurons that drive sodium appetite in the hindbrain (96). In addition, non-genomic effects of corticosterone signaling, which are often mediated through modulation of synaptic inputs (97), may contribute to driving AgRP neuron activity. Indeed, excitatory inputs to AgRP neurons are strengthened during fasting (15, 42), and corticosterone was previously reported to regulate synaptic input organization and excitability of AgRP neurons (98). Our findings that corticosterone signaling in AgRP neurons is necessary for hyperphagia contrast with a previous report that mice lacking glucocorticoid receptors specifically in AgRP neurons show little to no reduction in food intake or body weight (99). However, compensatory actions of mineralocorticoid receptors, which bind corticosterone with high affinity, may account for these findings. In addition, compensation for AgRP neuron loss of function has been reported in other studies including neonatal ablation (100, 101) and developmental deletion of leptin receptors (17, 102). Future studies leveraging RNA-sequencing technology to examine gene targets and recordings of AgRP neuron activity in the presence and absence of corticosterone signaling are needed to decipher which genomic and nongenomic mechanisms are involved in hypercorticosteronemia-mediated stimulation of AgRP neurons.

In summary, these findings reveal that hunger driven by hypoleptinemia or hypoglycemia is not entirely mediated via CNS circuits. Unexpectedly, both hypoleptinemia and hypoglycemia recruit and require an HPA axis–AgRP neuron pathway to fully induce hunger. We further demonstrate that the majority of leptin-mediated suppression of hyperphagia occurs through its ability to suppress hypercorticosteronemia in the transition from low to physiologic leptin concentrations (25, 26, 103). Finally, this study provides evidence for stimulation of a glucocorticoid–AgRP neuron axis that promotes food intake and suggests that AgRP neurons may be an attractive therapeutic target to suppress hyperphagia under conditions of leptin deficiency or glucocorticoid excess including Cushing’s disease.

**Methods**

**Animals.** All protocols were approved by the Institutional Animal Care and Use Committees of Yale University or Beth Israel Deaconess Medical Center. In the rat studies, healthy male rats weighing 250–300 g were ordered from Charles River Laboratories and maintained on regular chow (Harlan Teklad, 2018). Where indicated, adrenalectomy was performed by Charles River, and ADX rats were maintained on drinking water containing 0.9% NaCl and 2%
sucrose. Upon arrival, they were housed in a 12-h light/dark cycle at ~25 °C, and underwent surgery under isoflurane anesthesia to place catheters in the jugular vein and carotid artery, and a femoral artery catheter and Alzet pump to deliver low-dose (7.5 mg/dl) or high-dose (20 mg/dl) corticosterone. Arterial catheters were used for all rat infusions, while venous catheters were used for blood sampling. To avoid any effects of diurnal variation on food intake, all acute food intake measurements were obtained between 2:00 and 4:00 PM following the fasting times listed in the figure legends. At 10:00 AM, treatment with leptin, corticosterone, and/or mifepristone, as described below, was begun following 42 h of food withdrawal or at the time of food withdrawal as designated in the figure legends (final fasting time, 6 or 48 h). ADX rats were given access to NaCl/sucrose-containing water throughout the fast, but food was removed 48 h before the study. After a fast–refeeding study, ADX rats were placed on HFD (Research Diets; 12492) for 2 wk, after which the fast–refeeding study was repeated. In all rat studies, food intake was measured by a blinded investigator, who weighed food before and after refeeding.

To induce poorly controlled T1D, rats were injected with 65 mg/kg streptozotocin after an overnight fast at 9:00 AM the morning before the study, and then refed. At 7:00 PM, food was removed. Plasma insulin concentrations were measured after a 15-h fast, and those with plasma glucose concentrations <160 mg/dl were later removed from analysis. Beginning at 10:00 AM, rats were injected with leptin, corticosterone, and/or mifepristone, as described below. Food was provided at 2:00 PM, and rats were allowed to eat ad libitum until they were killed at 4:00 PM.

Four-hour euglycemic and hypoglycemic clamps were performed beginning at 10:00 AM after an overnight fast. In both cases, insulin was infused intraperitoneally (prime 40 μU/kg, continuous infusion 4 μU/kg/min), and a variable infusion of 20% dextrose was administered to maintain euglycemia (~150 mg/dl). The Jackson Laboratory and singly housed before experiments. For injections into the arcuate nucleus, mice were anesthetized with xylazine (5 mg per kg) and ketamine (75 mg per kg) diluted in saline (350 mg/kg), and placed into a stereotaxic apparatus (KOPF; model 963). For postoperative care, mice were injected s.c. with sustained release meloxicam (4 mg/kg). After exposing the skull via a small incision, a small hole was drilled for injection. A pulled-glass pipette with a 20- to 40-μm tip diameter was inserted into the brain and virus was injected by an air pressure system. A micromanipulator (Grass Technologies; model S48 stimulator) was used to deliver the injection at 25 nL/min and the pipette was withdrawn 5 min after injection. For behavior experiments pAAV8-Ishy-DIO-mCherry (UNC Vector Core) or pAAV8-Ishy-DIO-Hsd11b2-t2a-mCherry was injected into Agrp-IRES-Cre mice at 6 sites to cover the anterior-posterior extent of the arcuate (50 nl per site; bregma: anteroposterior [AP], −1.30 and −1.45 mm; dorsorostral/ventral [DV], −5.85 and −5.40 mm; lateral [ML], 51.80 and 50.30 mm) as verified by post hoc histological analysis of XFP reporters. All subjects determined to be surgical “misses” based on little or absent reporter expression were removed from analyses.

**Optic Fiber Implantation.** For fiber photometry recordings, an optic fiber was implanted in the same surgery as virus injection. A metal ferrule optic fiber (400 μm diameter; BFH37-400 Multimode; NA 0.48; Thor Labs) was implan- ted unilaterally over the arcuate (AP, −1.45 mm; DV, −5.8 mm; ML, −0.25 mm from bregma). Fibers were fixed to the skull using dental acrylic. Following experiments and histology of the brain tissue, the location of the fiber tips was identified.

**in Vivo Fiber Photometry Recordings.** Fiber photometry was performed on a rig constructed as follows: A 465-nm LED (PlexBright LED Module and LD-1 Driver; Plexon) was used as the excitation source, which was passed through a fluoride optic fiber cable (1 m long, 400 μm diameter; N.A., 0.48; Doric Lenses) and transmitted onto the sample via a fiber optic cable to the detector (2151; Newport). The signal was digitized at 1 kHz with a National Instruments data acquisition card and collected with a custom MATLAB (MATLAB 2016a; MathWorks) script. To reduce photobleaching during fiber photometry recordings, the LED was pulsed for 1 s every 10 s by a pulse generator (Arduino) as in ref. 46.

Recordings were performed within-subject, with animals receiving 2 vehicle (10% polyethylene glycol 4000 millipore) or 2 corticosterone (Sigma) trials (2 mg/kg in vehicle). The trial order was counterbalanced with 1 rest day between each recording session. Mice were habituated to s.c. injections and tethering to the optic fiber cable for 2 d before the first day of recording. Recordings were performed in the home cage early in the light cycle with food removed from the cage during the recordings. Data were analyzed using a custom Python script (Python 3.6) script. The median fluorescence value of each LED pulse was taken to condense each pulse into a single data point. The average fluorescence change was calculated as ΔF/F = (F − F0)/F0, where F0 was the mean of all data points from the 15-min baseline before injection, for each recording session.

**Biochemical Analysis.** Plasma glucose was measured using the YSI Glucose Analyzer. Plasma insulin, leptin, corticosterone, and ACTH concentrations were measured by ELISA (Enzo Life Sciences, R&D Systems, Alpco, and MyBioSource, respectively), with the exception of the hypoglycemic clamps in which insulin concentrations were measured by radioimmunossay by the Yale Diabetes Research Core. To assess corticosterone levels in mice implanted with
slow-release pellets, trunk blood was taken following sacrifice and centrifuged for plasma collection. Plasma was run in duplicate in a 96-well plate ELISA kit for corticosterone (Enzo Life Sciences) according to the manufacturer's protocol.

Electrophysiology Studies. Loose-seal, cell-attached recordings were performed as described previously with minor modifications (96). Briefly, brains were quickly removed and placed into ice-cold cutting solution consisting of the following (in mM): 92 choline chloride, 10 Hepes, 2.5 KCl, 1.25 NaH2PO4, 0.1 MgCl2, 2 MgSO4, 1 CaCl2, and 10 glucose. After 20 min at 24 °C, sections were washed and incubated at room temperature (20–24 °C) for at least 60 min before recording. Loose-seal, cell-attached recordings (seal resistance, 20–50 MΩ) were made in voltage-clamp mode with a 0.2-mA current injection as a control condition. Data were filtered at 2 kHz and digitized at 10 or 20 kHz before analysis offline using Clampfit 10.

Histology. Immunofluorescence was performed as described previously (96). Briefly, mice were terminally anesthetized with 7% choral hydrate (500 mg kg−1; Sigma-Aldrich) diluted in saline and transcardially perfused with 0.1 M PBS followed by 10% neutral-buffered formalin solution (NBF) (Thermo Fisher Scientific). Brains were extracted and postfixed overnight at 4 °C in NBF, cryoprotected with 20% sucrose, and sectioned coronally at 30 μm on a freezing microtome (Leica Biosystems). The following primary antibodies were used overnight at room temperature: rabbit anti-HSD2 (H-145; Santa Cruz Biotechnology), 1:300; rat anti-mCherry (Life Technologies), 1:3,000. The following day, the sections were washed and incubated at room temperature in donkey Alexa Fluor secondary antibody (Life Technologies; 1:1,000). Fluorescent images were captured using an Olympus VS120 slide-scanning microscope.

Data Analysis. Statistical analyses were performed using Prism 7 (GraphPad software) and are described in the figure legends. No statistical method was used to predetermine sample size, nor were randomization and blinding methods used, and statistical significance was defined as P < 0.05. All data presented met the assumptions of the statistical test employed. Experimental animals were excluded if histological validation revealed poor or absent reporter expression. N values reflect the final number of validated animals per group.

ACKNOWLEDGMENTS. We thank J. Dong, W. Zhu, A. Nasiri, Y. Li, J. Berrios, Z. Yang, and J. Yu for their invaluable technical contributions. This study was funded by grants from the US Public Health Service (RO1 DK-113984, P30 DK-59637, RO1 DK-101019, K99/R00 CA-215315, R15 NS078568, UL1TR000142, T32 DK-007058, RO1 DK-075632, RO1 DK-089044, RO1 DK-068010, RO1 DK-114101, K99/R00 HL-144932, P30 DK-06200, and P30 DK-075521) as well as an investigator-initiated award from AstraZeneca and a fellowship from Naomi Berrie Diabetes Center.
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