Leptin-inhibited PBN neurons enhance responses to hypoglycemia in negative energy balance

Jonathan N Flak1,10, Christa M Patterson1,10, Alastair S Garfield2,10, Giuseppe D’Agostino3,4, Paulette B Goforth5, Amy K Sutton6, Paige A Malec7, Jenny-Marie T Wong7, Mark Germani1, Justin C Jones1, Michael Rajala1, Leslie Satin5, Christopher J Rhodes8, David P Olson9, Robert T Kennedy7, Lora K Heisler3 & Martin G Myers Jr1,4

Hypoglycemia initiates the counter-regulatory response (CRR), in which the sympathetic nervous system, glucagon and glucocorticoids restore glucose to appropriate concentrations. During starvation, low leptin levels restrain energy utilization, enhancing long-term survival. To ensure short-term survival during hypoglycemia in fasted animals, the CRR must overcome this energy-sparing program and nutrient depletion. Here we identify in mice a previously unrecognized role for leptin and a population of leptin-regulated neurons that modulate the CRR to meet these challenges. Hypoglycemia activates neurons of the parabrachial nucleus (PBN) that coexpress leptin receptor (LepRb) and cholecystokinin (CCK) (PBN LepRbCCK neurons), which project to the ventromedial hypothalamic nucleus. Leptin inhibits these cells, and CCKER-mediated ablation of LepRb enhances the CRR. Inhibition of PBN LepRb cells blunts the CRR, whereas their activation mimics the CRR in a CCK-dependent manner. PBN LepRbCCK neurons are a crucial component of the CRR system and may be a therapeutic target in hypoglycemia.

Hypoglycemia and glucoprivation (which mimics low glucose availability by interfering with cellular glucose metabolism) activate a neurohormonal CRR that stimulates the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system (SNS) to promote glucose release into the bloodstream1–3. The SNS also acts on pancreatic islets to promote glucagon release and suppress insulin secretion4. The CRR serves to restore normoglycemia and protect the brain and body from damage due to hypoglycemia. An appropriately robust CRR is crucial to prevent cognitive impairment, unconsciousness or even death when blood glucose levels are too low. This response is especially crucial to counteract insulin-induced hypoglycemia (IIH) in patients with diabetes, for whom the risk of hypoglycemia is especially crucial during the night and other periods of fasting) is the most serious limitation to achieving tight glycemic control5–10. Hypothalamic LepRb neurons mediate many aspects of energy balance and metabolism11–13. Hypothalamic LepRb neurons project to the ventromedial hypothalamic nucleus. Leptin inhibits these cells, and CCKER-mediated ablation of LepRb enhances the CRR. Inhibition of PBN LepRb cells blunts the CRR, whereas their activation mimics the CRR in a CCK-dependent manner. PBN LepRbCCK neurons are a crucial component of the CRR system and may be a therapeutic target in hypoglycemia.

RESULTS

Electrical and structural properties of PBN LepRb neurons

Most effects of leptin are mediated by LepRb in the brain, especially the hypothalamic and brainstem, where most LepRb neurons reside15–17. Commensurate with the diverse processes controlled by leptin, various subtypes of LepRb neurons each contribute to distinct aspects of energy balance and metabolism18–20. Hypothalamic LepRb neurons in aggregate mediate most of leptin’s action on food intake and energy expenditure21–23. Although ablation of LepRb in the nucleus tractus solitarius (NTS) has revealed that NTS LepRb cells participate in the control of satiety24–26, roles for leptin and LepRb in most brainstem...
Figure 1 VMH-projecting PBN LepRb neurons are activated by low glucose levels and are inhibited by leptin. (a) Scheme demonstrating the experimental procedure in which LepRbCre+ mice were crossed (X) onto the Rosa26-eYFP background to generate LepRbCre+; eYFP in LepRb neurons and/or were injected with the Cre-inducible adeno viral tracing vector Ad-I-N-syn-mCherry to induce Syn-mCherry expression in LepRb neurons at the site of injection. IRES, internal ribosome entry site; CMV, cytomegalovirus. (b,c) Brain sections of LepRbCre+ mice that received PBN injection of Ad-I-N-syn-mCherry stained for dsRed (Syn-mCherry; purple) and/or GFP (eYFP; green). (b) Representative injection site in the PBN. (c) Representative section showing the main projection target, VMH. Scale bars, 100 µm. Images shown are representative of injections in eight separate animals. The inset shows a digital zoom of the indicated area (63×). Scp, superior cerebellar peduncle; 3v, third cerebral ventricle; ME, median eminence; Arc, arcuate nucleus; DMH, dorso medial hypothalamic nucleus; PAG, periaqueductal gray. (d–i) Current-clamp mode responses to glucose (G) and leptin in fluorescently detected PBN LepRb neurons in horizontal sections. (d) Representative trace of the membrane potential at baseline in 2 mM glucose and after switching to 0.5 mM glucose. (e,f) Membrane potential (P = 0.011, F(2,14) = 3.94; h) and action potential (AP) frequency (P = 0.042, F(2,14) = 4.012; f) of six individual neurons from four different animals. Wash indicates a return to 2 mM glucose. (g) Representative trace of membrane potential recorded in 0.5 mM glucose and in 0.5 mM glucose with the addition of leptin (+Lep; 10 mM). (h,i) Membrane potential (P = 0.011, F(5,26) = 3.94; h) and AP frequency (P = 0.037, F(5,26) = 2.83; i) of six individual neurons from four different animals. The data in e and f were analyzed by one-way repeated measures analysis of variance (ANOVA); the data in h and i were analyzed by paired two-tailed t test. The data in e and f are plotted as the mean ± s.e.m., and the data in h and i are plotted as quartile 1 (Q1), Q2 and Q3. 

sites remain essentially unstudied. Genetic markers such as enhanced YFP (eYFP) in LepRbCre+; Rosa26-eYFP (LepRbCre+; eYFP) mice (which express eYFP specifically in LepRb neurons), along with leptin-induced phosphorylation of STAT3 (pSTAT3; a marker of LepRb activity), reveal that the brainstem PBN contains a substantial number of LepRb neurons (Fig. 1a and Supplementary Fig. 1).13,18–20

To understand potential functions of PBN LepRb neurons, we examined their projections by injecting Ad-I-N-syn-mCherry into PBN of LepRbCre+ mice to reveal the location(s) of synaptic terminals from PBN LepRb neurons by the presence of mCherry immunoreactivity (IR) (Fig. 1a–c). This analysis revealed that synaptic terminals from PBN LepRb neurons target primarily the dorsomedial compartment of the ventromedial hypothalamic nucleus (dmVMH; a site that is important for SNS function, including the CRR to hypoglycemia).13,18–20

Because PBN LepRb neurons target dmVMH, we postulated that PBN LepRb neurons might respond to hypoglycemia or glucoprivation. Indeed, IHH and 2-deoxyglucose (2DG; which inhibits glucose metabolism to mimic cellular hypoglycemia)-induced glucopri vation both promoted cFos IR (a histochemical marker that often reflects increased neuronal activity) in many PBN LepRb neurons (Supplementary Fig. 2). The distributions of IIH- and 2DG-induced cFos IR in PBN LepRb neurons were similar, suggesting similar actions on these neurons by the two stimuli (Supplementary Fig. 3). Furthermore, decreased glucose concentrations depolarized and increased the firing frequency of approximately half (6/11) of the PBN LepRb neurons examined in electrophysiological slice preparations (Fig. 1d–f). Conversely, leptin hyperpolarized and decreased the firing rate of PBN LepRb neurons in low glucose conditions (Fig. 1g–i).

Together the projection of hypoglycemia-activated, leptin- inhibited PBN LepRb neurons to VMH suggests that these cells might participate in the CRR to glucoprivation, and the withdrawal of leptin-mediated inhibition from PBN LepRb neurons might enhance the CRR in low-leptin states. Such a system could overcome the limitations imposed by starvation, enabling an appropriate CRR despite decreased energy stores and baseline SNS tone. Indeed, leptin and energy balance modulate the amplitude of the CRR: a 12-h fast exaggerates the CRR to 2DG in mice, and exogenous leptin blunts this fasting-induced augmentation of the CRR (Supplementary Fig. 4). Hence, the fall in leptin levels during negative energy balance enhances the acute response to glucoprivation (thereby counteracting the inadequate CRR that might otherwise result).

**Exaggerated CRR in mice lacking LepRb in CCK cells**

To understand whether PBN LepRb neurons might enhance the CRR in low-leptin states, we sought a molecular marker to permit the manipulation of PBN LepRb neurons. Because CCK-containing PBN neurons project to VMH,25,36, we examined the potential expression of LepRb in PBN CCK-expressing neurons. We bred CCKCre+ mice (which express cre in CCK neurons) to the Rosa26-eYFP background to generate CCKCre+; eYFP mice and examined the induction of pSTAT3 IR in CCKCre+ cells, demonstrating that many PBN LepRb neurons express CCK (LepRbCre+; CCK) neurons (Fig. 2a,b). Although we observed some LepRbCre+ cells in other brainstem regions (including the Edinger-Westphal (EW) region, the periaqueductal gray region and NTS) (data
not shown), LepRbCCK neurons were absent from the hypothalamus and other brain areas. We observed increased cFos IR in PBN LepRb and PBN CCK neurons in LepRbYFP and CCKYFP mice, respectively, after treatment with 2DG (Supplementary Fig. 5), suggesting that PBN LepRbCCK cells are the subpopulation of PBN LepRb cells that are activated by glucoprivation.

To understand the function of these brainstem LepRb CCK cells in leptin action, we crossed LepRbCCK KO mice to generate mice lacking LepRb expression specifically in LepRb CCK neurons (LepRbCCK KO mice) (Fig. 2a). As we expected, leptin-stimulated pSTAT3 IR was absent from CCK-expressing PBN neurons in LepRbCCK KO mice, and overall leptin-stimulated PBN pSTAT3 IR was reduced by approximately 75% in the PBN of LepRbCCK KO animals compared to controls (Fig. 2b,c). Leptin-stimulated pSTAT3 IR was also significantly reduced in EW but not in other brain areas (Supplementary Fig. 6). Thus, Cckcre ablates LepRb expression in the LepRbCCK cells of brainstem PBN and EW nuclei.

We detected no alteration in body weight, endocrine function, glucose homeostasis or glucose tolerance in LepRbCCK KO mice compared to controls (Supplementary Fig. 7). Consistent with an enhanced CRR, however, LepRbCCK KO mice displayed blunted IIH (Fig. 2d). Indeed, as with fasted mice (Supplementary Fig. 3), LepRbCCK KO mice exhibited more robust glycemic excursions in response to 2DG than controls (Fig. 2e). Furthermore, although insulin levels were unchanged (data not shown), circulating glucagon, corticosterone and epinephrine concentrations were increased in LepRbCCK KO mice compared to controls 90 min after 2DG administration (Fig. 2f–h). The increased glycemic excursion in response to glucoprivation exhibited by LepRbCCK KO mice is similar to that observed in fasted control animals, and even prolonged fasting failed to further enhance this response in LepRbCCK KO mice, suggesting overlapping mechanisms for leptin action on LepRbCCK neurons and the augmented CRR observed with prolonged fasting (Supplementary Fig. 8). Furthermore, these cells are at least partly specific to hypoglycemia (as opposed to being sensitive to all stressors), as restraint stress provoked no increase in cFos IR in PBN LepRb neurons, and the hyperglycemic response to restraint stress was normal in LepRbCCK KO mice (Supplementary Fig. 9).
To examine IIH directly, we subjected the LeprBCK KO mice to hypoglycemic clamp analysis, in which high levels of insulin are continuously infused while the glucose infusion rate is varied to maintain the desired level of hypoglycemia, thereby examining counter-regulatory glucose production during IIH (Fig. 3). The glucose infusion rate required to prevent blood glucose from falling below the clamped level was decreased in LeprBCK KO mice compared to controls, which is consistent with increased counter-regulatory hepatic glucose production in these animals (Fig. 3a–c). Indeed, the hepatic expression of G6Pase (also called G6pc or glucose-6-phosphatase, which dephosphorylates glucose to permit its efflux from the cell) was increased in LeprBCK KO mice compared to controls at the end of the clamp (Fig. 3d). Of note, G6Pase expression, which is decreased by insulin, is augmented by counter-regulatory stimuli that increase cyclic AMP levels, including epinephrine and glucagon37, and thereby provides an indirect indication of the liver’s potential to release glucose. Thus, leptin action through LepRb CCK neurons does not contribute to baseline glucose tolerance but rather enhances the CRR by promoting increased counter-regulatory hormone release and hepatic glucose output.

Inhibition of PBN LepRb neurons blunts the CRR

To evaluate the function of PBN LepRb neurons specifically, we used stereotaxic injection of adeno-associated viruses (AAVs) that mediate the Cre-dependent expression of designer receptors exclusively activated by designer drugs (DREADDs; expressed as DREADD-mCherry fusion proteins). DREADDs are genetically engineered muscarinic receptor variants that are insensitive to endogenous ligands but are activated by the otherwise biologically inert clozapine-N-oxide (CNO)38,39; CNO activates neurons containing the G3-coupled DREADD hM3Dq and inhibits neurons that contain the Gq-coupled DREADD hM4D38,39.

To determine the role of PBN LepRb neurons in the endogenous CRR, we injected the inhibitory AAV-hM4Dq bilaterally into the PBN of Leprcre animals, allowed the animals to recover for several weeks and examined their response to 2DG-mediated glucoprivation in the presence or absence of CNO (Fig. 4a–c). We found that CNO significantly (P < 0.001) blunted the increase in blood glucose concentrations after 2DG treatment in mice that expressed hM4Dq in PBN LepRb neurons. Thus, inhibition of PBN LepRb cells impairs the CRR, demonstrating that PBN LepRb neurons have an integral role in the response to glucoprivation.

Activation of PBN LepRb neurons mimics the CRR

Our findings predict that activation of PBN LepRb neurons should mimic the CRR. To test this prediction, we injected the activating AAV-hM3Dq bilaterally into the PBN of Leprcre animals and, after a several-week recovery period, treated them with CNO (Fig. 5). CNO treatment increased cFos IR in PBN and VMH of these animals compared to vehicle (Supplementary Fig. 10), as would be expected after activation of the PBN Lepr→VMH circuit. CNO also increased blood glucose concentrations (Fig. 5b,c), along with glucagon and insulin concentrations.
corticosterone levels, and tended to decrease insulin levels compared to vehicle (Fig. 5d–f). Because the effect of increased glucose levels on pancreatic islets would cause glucagon levels to fall and insulin levels to rise, the opposite finding (that glucagon levels rose while insulin levels tended to fall) implies increased SNS outflow to the pancreatic islets after activation of PBN LepRb neurons. Activation of PBN LepRb neurons also increased hepatic G6Pase expression (Fig. 5g). Thus, activation of PBN LepRb neurons increases circulating concentrations of counter-regulatory hormones and hepatic glucose production to raise blood glucose levels, similar to the native CRR.

To determine the role of CCK in the PBN LepRb neuron–mediated stimulation of blood glucose levels, we injected AAV-hM3Dq bilaterally into PBN of Lepr<sup>cre</sup> animals, allowed them to recover for several weeks and treated them with CNO in the presence or absence of the CCK receptor inhibitor proglumide (Fig. 6a,b). Pretreatment with proglumide blocked the increase in blood glucose levels and hepatic G6pase expression during CNO-activated mediation of PBN LepRb neurons. This requirement for CCK signaling in the hyperglycemic response to activated PBN LepRb neurons suggests that the LepRb<sup>CCK</sup> subpopulation of PBN LepRb neurons mediates this response, which is consistent with the exaggerated CRR displayed by the LepR<sup>B</sup>C<sup>CK</sup> KO mice. To determine whether CCK is also required for the normal response to glucoprivation, we also examined the effect of proglumide on the hyperglycemic response to 2DG in normal C57BL/6 mice (Fig. 6c). Although proglumide did not lower baseline blood glucose levels, it blunted the response to 2DG (similar to the DREADD hM4D<sup>+</sup>–mediated inhibition of PBN LepRb neurons), revealing the importance of CCK neurotransmission for the endogenous response to glucoprivation.

**DISCUSSION**

Our findings reveal that low leptin levels enhance the CRR, identify a previously unknown component of the neural circuitry that mediates the CRR (along with the neuropeptide by which it acts, CCK) and demonstrate that leptin acts through these cells to modulate the CRR (Supplementary Fig. 11).

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**Figure 5** Activation of PBN LepRb neurons mimics the CRR to glucoprivation. (a) Scheme showing the experimental design of bilateral injection of the Cre-inducible AAV-hM3Dq into the PBN of male Lepr<sup>cre</sup> mice, which induces the expression of the activating DREADD hM3D<sup>q</sup> in PBN LepRb neurons. Animals were injected with vehicle or CNO (0.3 mg per kg body weight, i.p.) and monitored for 120 min. (b) Blood glucose at the indicated times; *P < 0.001 (30 min), *P < 0.001 (45 min), *P < 0.001 (60 min), *P < 0.001 (90 min), *P = 0.002 (120 min), F(6,30) = 8.23, n = 16 (vehicle) and 16 (CNO) animals. (c) AUC for the data in b; *P < 0.001, t(42) = −4.28, n = 22 (vehicle) and 22 (CNO) animals. (d–f) Plasma glucagon (*P = 0.002, t(35) = −3.03, n = 18 (vehicle) and 19 (CNO) animals; d), insulin (*P = 0.049, t(40) = 1.69, n = 21 (vehicle) and 21 (CNO) animals; e) and corticosterone 30 min after CNO administration (*P = 0.034, t(40) = −1.87, n = 21 (vehicle) and 21 (CNO) animals; f). (g) Hepatic G6Pase mRNA expression (fold over control) at the end of the experiment; *P < 0.001, t(17) = −6.64, n = 10 (vehicle) and 9 (CNO) animals. The data in b were analyzed by two-way repeated measures ANOVA with Fisher LSD post-hoc test; the data in c were analyzed by two-tailed t test, consistent with our directional hypothesis. The data in d are plotted as the mean ± s.e.m., and the data in e–g are shown as Q1, Q2 and Q3 with whiskers indicating the 10th and 90th percentiles.

**Figure 6** CCK dependence of the hyperglycemic responses to activation of PBN LepRb neurons and glucoprivation. (a) Blood glucose levels at the indicated times in male Lepr<sup>cre</sup> mice that were injected bilaterally with the activating DREADD hM3Dq and allowed to recover for at least 2 weeks, after which time they were injected with proglumide (PG; 20 mg per kg body weight, i.p.) or vehicle (Veh) and then injected with vehicle or CNO (0.3 mg per kg body weight, i.p.) 30 min later; *P = 0.006 compared to PG/Veh, *P = 0.001 compared to PG/CNO, and *P = 0.031 compared to Veh/Veh, F(3,16) = 6.19, n = 3 (Veh/Veh), 3 (PG/Veh), 7 (Veh/CNO) and 7 (PG/CNO) animals. (b) Hepatic G6Pase mRNA expression (fold over control) measured at the end of the experiment; *P < 0.001 compared to all other groups, F(3,13) = 21.839, n = 5 (Veh/Veh), 4 (PG/Veh), 4 (Veh/CNO) and 4 (PG/CNO) animals. (c) Blood glucose levels at the indicated times in C57BL/6 mice that were injected with proglumide (100 mg per kg body weight, i.p.) or vehicle and then injected with vehicle or 2DG (500 mg per kg body weight, i.p.) 30 min later; *P < 0.001 compared to all other groups; F(3,25) = 43.47, n = 6 (Veh/Veh), 7 (PG/Veh), 8 (Veh/2DG), 8 (PG/2DG) animals. All data are plotted as the mean ± s.e.m. The data in a and c were analyzed by two-way repeated measures ANOVA with Fisher LSD post-hoc test; the data in b were analyzed by one-way ANOVA with Fisher LSD post-hoc test.
We report that low glucose levels activate PBN LepRb cells in electrophysiologic slice preparations. These data suggest that PBN LepRb neurons either sense low glucose levels directly or receive inputs from other glucose-sensing cells within the slice preparations. It is also possible that PBN LepRb cells receive additional input from other PBN-projecting neurons that sense hypoglycemia. Furthermore, neurons that convey information relevant to other physiologic emergencies that demand an appropriately robust response, even when energy stores are depleted, might also activate these neurons to promote the CRR and permit its enhancement by low leptin levels. However, gut peptides such as amylin that activate the NTS→PBN→nucleus of the amygdala anorexia circuit do not activate PBN LepRb neurons (data not shown). Furthermore, restraint stress fails to activate PBN LepRb neurons by the criterion of cFos accumulation, and the hyperglycemic response to restraint is normal in LepRbCck KO mice, suggesting some specificity of PBN LepRb neurons for the CRR to hypoglycemia.

Leptin inhibits PBN LepRb neurons in electrophysiologic slice preparations, suggesting that decreased leptin action on PBN LepRb neurons would augment the CRR. Indeed, deletion of LepRb from LepRbCck cells (~75% of PBN LepRb neurons) enhances the CRR without altering energy balance or other parameters of glucose homeostasis. Thus, our findings reveal a mechanism by which low leptin levels can enhance the allostatic response to acute hypoglycemia (as is required in the face of depleted energy stores and decreased baseline SNS tone) without altering baseline energy and glucose homeostasis. Similar to the attenuation of overall energy expenditure during caloric restriction, diminished leptin action undermines the enhanced CRR in fasted animals. Thus, low leptin enhances the CRR, in addition to increasing appetite and blunting baseline energy expenditure. These findings not only reveal a previously unrecognized neural system that regulates the CRR but also demonstrate a broader and previously undescribed role for (low) leptin levels in adapting allostatic physiology appropriately for limited nutritional reserves. Fasting increases the risk for hypoglycemia, and this system presumably ensures the adequacy of the response to hypoglycemia under these conditions. Notably, as PBN LepRb neurons do not alter baseline glucose homeostasis but rather enhance the CRR, in addition to increasing appetite and blunting baseline energy expenditure, these findings not only reveal a previously unrecognized neural system that regulates the CRR but also demonstrate a broader and previously undescribed role for (low) leptin levels in adapting allostatic physiology appropriately for limited nutritional reserves. Fasting increases the risk for hypoglycemia, and this system presumably ensures the adequacy of the response to hypoglycemia under these conditions. Notably, as PBN LepRb neurons do not alter baseline glucose homeostasis but rather enhance the CRR during hypoglycemia, agents that sensitize these cells could be therapeutically useful to mitigate hypoglycemia while not disrupting tight glycemic control.

Low glucose levels or glucoprivic stimuli activate PBN LepRb cells that project to dmVMH. The pharmacogenetic activation of these cells promotes cFos in dmVMH and mimics the CCK in a CCK-dependent manner, increasing the levels of blood glucose, counter-regulatory hormones and markers of hepatic glucose production. Furthermore, pharmacogenetic inhibition of PBN LepRb neurons blunts the response to glucoprivation (as does inhibition of CCK signaling), demonstrating the role of PBN LepRbCCK neurons in the normal glucoprivic response. The CCK-responsive cells that lie downstream of PBN LepRb neurons presumably represent crucial effectors of the CRR, and it will be important to identify these neurons and understand their function. Our identification of a discrete neural system that mediates and modulates the CRR provides a potential target for therapeutic intervention to mitigate iatrogenic IH and improve the safety and efficacy of insulin therapy in diabetes mellitus.

METHODS

Methods and any associated references are available in the online version of the paper.
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ONLINE METHODS

Animals. All of the procedures listed in this manuscript were approved by the University of Michigan (UM) Committee on the Use and Care of Animals or were in accordance with the UK Animal (Scientific Procedures) Act 1986. C57BL/6 males were from Jackson Laboratories. Leprcre mice on the C57BL/6 background have been described19 and were bred in our colony, CCKtm1.1(cre)Zjh mice were purchased from Jackson laboratories and crossed to Leprfl/fl or Rosa26YFP mice from our colony23,41. LepRB GK KO study animals (along with Cc kre, Lep r fl /fl and wild-type controls) were generated in our colony by crossing Cc kre, Lep r fl /fl and Lep r fl /fl animals; these animals were on the segregating C57BL/6; 129Sv background. All of the procedures listed in this manuscript were approved by the University of Michigan (UM) Committee on the Use and Care of Animals (UM Animal Phenotyping Core). Samples for the determination of epinephrine concentrations were obtained from animals with an arterial catheter (placed by the UM Animal Phenotyping Core). Animals were allowed for 5 h of recovery before each challenge. Mice were fasted for 4 hours before each challenge: food was removed after lights on, and testing followed 4 hours later. Tail vein blood was collected for the measurement of glucose levels (OneTouch Ultra 2 glucometer (Johnson and Johnson)). Plasma or serum were prepared from larger volume samples and stored at −20 °C for later assay. Samples for the determination of epinephrine concentrations were obtained from animals with an arterial catheter (placed by the U M Animal Phenotyping Core (APC)). Glucagon (Millipore) and glucocorticoid (MP Biomedicals) levels were determined by radioimmunoassay, and insulin and leptin levels were determined by multiplex assay (Millipore).

Determination of plasma epinephrine concentrations. 5 μL of plasma was spiked with 1.25 μL of D6-epinephrine as an internal standard. Proteins were removed by the addition of 25 μL of ice-cold acetoneitrile, followed by centrifugation for 10 min at 13,400 r.p.m. 20 μL of the supernatant was removed and benzoylated42 by sequential addition of 10 μL of 100 mM sodium tetraborate, 10 μL of benzylo chloride (2% in acetoneitrile, v/v) and 10 μL of sulfuric acid (1% in dimethyl sulfoxide (DMSO), v/v). Standard solutions of epinephrine were prepared in AC5F, which is similar in composition to plasma without protein43, to create a calibration range of 0.1–20 nM. Standards were spiked with the internal standard, diluted with acetoneitrile and derivatized as described above. Calibration curves were prepared based on the peak area ratio of the standard to the internal standard by linear regression. All samples and standards were analyzed in triplicate using an Acquity HSS T3 C18 chromatography column (1 mm × 100 mm, 1.8 μm, 100-Å pore size) in a nanoAcquity ultra performance liquid chromatograph (UPLC) (Waters) interfaced to an Agilent 6410 triple quadrupole mass spectrometer. Multiple phase A was 10 mM ammonium formate with 0.15% (v/v) formic acid in water. Mobile phase B was acetonitrile. The gradient used was as follows: initial, 0% B; 0.01 min, 23% B; 2.51 min, 23% B, 3 min, 50% B; 5.3 min, 60% B; 6.46 min, 65% B; 6.47 min, 100% B; 7.49 min, 100% B; 7.5 min, 0% B; 8.5 min, 0% B at 100 μL/min. Benzoylated epinephrine eluted at 6.85 min. The sample injection volume was 5 μL in partial loop injection mode. The autosampler was kept at ambient temperature, and the column was held at 27 °C. Electrospray ionization was used in positive mode at 4 kV. The temperature was 350 °C, gas flow was 11 L/min, and the nebulizer was at 15 psi. Ions were detected in tandem mass spectrometry (MS-MS) mode with precursor ions of 496 and 502 m/z for benzoylated epinephrine and D6-epinephrine, respectively, and product ion 105 m/z for both. The following voltage settings were used: fragmentor = 120 V, collision energy = 15 V, cell accelerator = 4 V. Automated peak integration was performed using Agilent MassHunter Workstation Quantitative Analysis for QQQ, version B.05.00. All peaks were visually inspected to ensure proper integration.

Perfusion and immunohistochemistry. Where indicated, mice were treated with leptin (5 mg per kg body weight; the generous gift of AstraZeneca Pharmaceuticals) 2 h before perfusion to label leptin-responsive neurons through the induction of pSTAT3 IR. The mice were anesthetized with sodium pentobarbital, and a lobe of liver was removed before transcardial perfusion and collection of the brain. The brains were sectioned coronally at 30 μm on a freezing microtome and distributed into four series for analysis. The series were pretreated with 1% hydrogen peroxide followed by individual washes of PBS with 0.3% glycine and 0.03% sodium dodecyl sulfate (SDS). The sections were incubated overnight at room temperature in rabbit anti-cFos (Santa Cruz, sc-52; 1:1,000), chicken anti-GFP (Abcam, ab12970; 1:1,000), rabbit anti-dsRed (living colors, 632496; 1:1,000) and/or rabbit anti-pSTAT3 (Cell Signaling Technology, 91455; 1:500) and exposed the next day with either biotinylated (1:200 followed by avidin-biotin complex (ABC) amplification and 3,3-diaminobenzidine (DAB) reaction) or fluorescent secondary antibody (Molecular Probes, 1:200) to visualize proteins. Each of these antibodies is included in the Journal of Comparative Neurology Antibody database (http://onlinelibrary.wiley.com/journal/10.1002%2F CNSN91906-9861/homepage/jcn_antibody_database.htm). The sections were mounted on glass slides and coverslipped with Vectashield mounting medium (Vector labs) for later image capture. Images were collected on an Olympus BX-51 microscope, and immunoreactive cells and colabeled cells were counted manually using Adobe Photoshop. Total eYFP IR cells were counted within the area, as well as cocolocalized eYFP IR cells with nuclear marker (cFos IR or pSTAT3). Cells were counted bilaterally and are expressed as raw counts from each 1:1 series.

mRNA analyses. mRNA was extracted from liver samples using TRIzol (Invitrogen); 1 μg was converted to cDNA using a SuperScript Reverse Transcriptase kit (Invitrogen). Using the CDNA, GAPDH and target genes were analyzed using TaqMan kits (Applied Biosystems) on an Applied Biosystems 7500 Real Time PCR system. Relative mRNA expression was calculated using the 2−△△Ct method.
**Stereotaxic injection of viral constructs.** After the induction of isoflurane anesthesia and placement in a stereotaxic frame, the skulls of adult *Leprcre* mice were exposed. After bregma and lambda were leveled, a guide cannula with injector was lowered into the approximate PBN coordinates from bregma (anteroposterior: −4.85, mediolateral: 1.2, dorsoventral: −3.3). 100 nL of Ad-iN-Syn-mCherry\(^{29}\), AAV-hM4Di or AAV-hM3Dq (University of North Carolina Vector Core) was injected using a 500-nl Hamilton syringe at a rate of 20 nL/minute. After 5 min following injection to allow for adequate dispersal and absorption of the virus, the injector and cannula were removed from the animal; the incision site was closed and sutured. The mice received analgesics before and after surgery. The mice injected with Ad-iN-Syn-mCherry were allowed 1 week to recover before being euthanized, and the mice injected with AAV-hM3Dq or hM4Di were allowed at least 2 weeks to recover from surgery before experimentation.

**Proglumide treatment.** *Leprcre* mice were pretreated with AAV-hM3Dq bilaterally injected into the PBN (as described above) and at least 2 weeks later were food deprived from 9:00 a.m. to 1:00 p.m., at which time the study commenced. Baseline blood glucose levels were determined, and mice were pretreated with 20 mg per kg body weight of the pan-specific CCK-receptor antagonist proglumide sodium salt (Tocris Biosciences) or 0.9% sterile saline. After 30 min, blood glucose levels were assessed, and mice were administered 0.3 mg per kg body weight CNO or 0.9% sterile saline. Blood glucose levels were monitored for a further 120 min. The study was repeated in C57BL/6 mice pretreated with 100 mg per kg body weight proglumide, followed by treatment with 500 mg per kg body weight 2DG or 0.9% sterile saline 30 min later. Blood glucose levels were monitored for a further 180 min.

**Statistics.** No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in a previous publication from our group\(^{23}\). Sample sizes for experiments with stereotaxic injections of AAVs were doubled, with the prediction of a 50–60% bilateral hit rate of PBN. The data presented include many groups of animals and therefore do not have the same \(n\) values for each experiment. Blood glucose data over multiple time points was analyzed by two-way repeated measures ANOVA with Fisher LSD post-hoc test. AUC (two sided) and hormone data (one sided) were analyzed by Student’s t test; AUC data in the fasting 2DG challenge experiment were analyzed by one-way ANOVA with Fisher LSD post-hoc test. No animals were removed from analysis unless there was evidence of either sickness or injury (loss of >10% body weight or malaise). Data that did not fall in a normal distribution or equal variance were log transformed and then reanalyzed. Variance was similar among all groups. Data were deemed significant when \(P \leq 0.05\). All statistics were performed with SigmaStat (Systat).

A Supplementary Methods Checklist is available.

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Corrigendum: Leptin-inhibited PBN neurons enhance responses to hypoglycemia in negative energy balance

Jonathan N Flak, Christa M Patterson, Alastair S Garfield, Giuseppe D'Agostino, Paulette B Goforth, Amy K Sutton, Paige A Malec, Jenny-Marie T Wong, Mark Germani, Justin C Jones, Michael Rajala, Leslie Satin, Christopher J Rhodes, David P Olson, Robert T Kennedy, Lora K Heisler & Martin G Myers Jr
Nat. Neurosci. 17, 1744–1750 (2014); published online 10 November 2014; corrected after print 10 December 2014

In the version of this article initially published, the anteroposterior and dorsoventral stereotaxic injection coordinates in the Online Methods were transposed. The error has been corrected in the HTML and PDF versions of the article.