Calcium Channel CaV2.3 Subunits Regulate Hepatic Glucose Production by Modulating Leptin-Induced Excitation of Arcuate Pro-opiomelanocortin Neurons

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**In Brief**
Smith et al. show that an R-type calcium channel containing the pore subunit Cacna1e mediates long-term depolarization of hypothalamic POMC neurons by leptin. Mice with POMC neuron knockdown of Cacna1e display elevated hepatic glucose production, demonstrating that leptin utilizes Cacna1e in POMC neurons to regulate glucose homeostasis.
Calcium Channel Ca\(_{\text{v}}\)2.3 Subunits Regulate Hepatic Glucose Production by Modulating Leptin-Induced Excitation of Arcuate Pro-opiomelanocortin Neurons

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Figure 1. Leptin Augments a Voltage-Gated Calcium Conductance

(A) Representative continuous whole-cell current-clamp trace (upper) and expanded sections (lower, as indicated by italic letters) from a POMC neuron recorded in intact slices. Locally applied leptin (50 nM; 2 min where indicated) depolarized and increased firing frequency of a sub-population of POMC neurons.

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Barium currents were evoked from dispersed POMC neurons by voltage steps to –32.2 ± 5.6 mV (n = 8; Figure 1B). While these properties are consistent with the activation of a Trpc5 subunit-containing conductance, an additional leptin-dependent inward current was evoked at potentials greater than –40 mV (Figure 1B). Given the dependence of the Trpc5 conductance on intracellular calcium, we therefore hypothesized that leptin may also activate a voltage-gated calcium (CaV) conductance.

To investigate the identity and properties of POMC CaV currents, whole-cell recordings were performed with a CsCl (130 mM) internal solution and extracellular divalent cations replaced with 10 mM BaCl2, to block K+ channels and enhance CaV currents. Recordings were also performed with tetrodotoxin and inhibitors of fast synaptic transmission. POMC neurons were voltage clamped at –100 to 0 mV (Figure S1A). CaV currents are classified as L, T, P, N, and R types based on molecular, biophysical, and pharmacological properties (Catterall et al., 2005). Thus, a pharmacological dissection of POMC CaV was performed using Ba2+ currents at 0 mV. Addition of mibebradil (10 μM), an R- and T-type (and partial L-type) inhibitor, followed by the N-type blocker ω-conotoxin-GVIA (200 nM) and L-type blocker nimodipine (10 μM), each inhibited evoked currents by similar magnitudes (Figures S1C and S1E). In the presence of all three CaV blockers, application of the P- and Q-type blocker ω-agatoxin-IVA (200 nM) reduced current amplitude further (Figures S1D and S1E). This cocktail of blockers did not completely suppress POMC CaV currents, indicating the presence of a resistant ("R-type") conductance. However, 50 nM SNX-484 (which blocks R currents in most but not all cells; Newcomb et al., 1998) did not affect the Ba2+ current amplitude (Figure S1F).

Next, we tested whether leptin modulates a specific CaV current. Following establishment of a stable current amplitude, leptin increased the Ba2+ current at 0 mV (Δ current, –27.5 ± 9.8 pA; n = 10; paired t test, t(9) = 2.81; p < 0.03) and by a greater magnitude at –30 mV (Δ current, –79.7 ± 30.4 pA; n = 10; paired t test, t(9) = 2.63; p < 0.03), which was sustained following leptin removal (Figures 1C–1F). Augmentation of the Ba2+ current was unaffected by 10 μM nimodipine (Δ current at 0 mV, –26.6 ± 8.6 pA; n = 10; paired t test, t(9) = 3.08; p < 0.02). However, leptin-induced augmentation was prevented by 10 μM mibebradil (Δ current at –30 mV, –8.0 ± 8.9 pA; n = 9; paired t test, t(8) = 0.90; p = 0.39) and a PI3K inhibitor TO-201 (1 μM; Δ current, –13.2 ± 23.4 pA; n = 13; paired t test, t(12) = 0.57; p = 0.58). We next examined leptin action on Ba2+ currents in acutely dissociated POMC neurons to exclude the possibility that changes in network activity were responsible for the augmentation of the CaV conductance. However, the Ba2+ current evoked at –40 mV was increased by leptin in dispersed POMC neurons by 24.9 ± 0.9% (n = 7; paired t test, t(6) = 2.65; p < 0.04; Figure 1G) and blocked by NiCl2 (100 μM; n = 3; Figure 1H), which inhibits T- and R-type conductances. Although the mibebradil-sensitive Ba2+ current has slow inactivation kinetics, we assessed leptin action on T-type calcium channels (also Ni2+ and mibebradil sensitive). Ba2+ currents, elicited at –50 mV in dispersed POMC neurons, displayed the rapid and transient current profile of T-type channels (Itrans, 3.1 ± 0.9 ms; n = 4; Figure 1G). Leptin did not alter the amplitude (Imax: control, 148.1 ± 62.7 pA, versus leptin, 166.6 ± 50.7 pA; n = 4; paired t test, t(3) = 0.68; p = 0.55) or steady-state inactivation (V50: control, –91.8 ± 2.5 mV, versus leptin, –91.4 ± 24.7 mV; n = 4; paired t test, t(3) = 0.12; p = 0.91; Figures S1G and S1H) of these currents.

Cell-Specific Knockdown of Cacna1e in POMC Neurons

These characteristics of the leptin-modulated CaV conductance are consistent with R type, where the pore-forming subunit is Cacna2.3 (Cacna1e). To investigate its role in POMC neurons, we used interference short hairpin RNA (shRNA) sequences directed toward Cacna1e (Figure 2A). Hypothalamic GT1-7 cells, which express R-type CaV, were independently transfected with each shRNA sequence directed toward Cacna1e or GFP as a control and Ba2+ currents evoked at 0 mV. For each shRNA sequence, peak Ba2+ currents were reduced by 75%–85% when compared to GFP-transfected cells (Figure 2B). To target POMC cells in vivo, we used the approach of Hitz et al. (2007), whereby a loxp flanked stop cassette was introduced between the U6 promoter and the 5′ end of the shRNA sequence. To identify expression, mCherry driven by the CMV promoter was inserted downstream of the shRNA (5′) sequence in an associated-adenovirus (AAV) expression vector (Figure 2C). To test the efficacy of this strategy, brains of wild-type (WT) (–/–) and nestin-cre-recombinase (–/+) expressing mice were injected
Figure 2. shRNA Knockdown of Cacna1e in POMC Neurons

(A) shRNA sequences targeting the mouse Cacna1e gene.

(B) Barium currents were evoked in GT1-7 cells by voltage steps to 0 mV from a holding potential of −100 mV. Cells were transfected with GFP or one of the four shRNA sequences (#1–4) shown in (A). Mean ± SEM; n = 9. One-way ANOVA (F(4,40) = 11.21, p < 0.0001); Bonferroni post hoc, ****p < 0.0001.

(C) Diagrammatic representation of the associated-adenoviral (AAV) expression vector before (top) and after cre recombination (bottom).

(D) Western blot analysis of CaV2.3 (Cacna1e) protein (top), Na+ /K+ ATPase loading control (bottom), and quantification (right bar chart; mean ± SEM) for brain lysates from wild-type (−/−) and nestinCre (+/+) mice injected with AAV particles containing the shRNA #2 sequence shown in (A).

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with viral particles containing the shRNA sequence directed to Cacna1e. Ca₂⁺ (Cacna1e) protein was reduced in AAV-injected brains in cre-positive mice compared to WT littermates (Figure 2D), demonstrating effective knockdown of Cacna1e. Viral particles were then injected into the MBH of WT and POMCCre mice to generate POMCCacna1eWT and POMCCacna1eKD mice, respectively. To control for potential off-target effects, we also injected a cre-dependent shRNA scramble virus into POMCCre mice to produce POMCshRNAscramble mice. Immunohistochemistry for POMC and mCherry confirmed expression in POMC neurons and the MBH (Figure 2E). Expression of mCherry was examined post mortem in all mice to confirm correct viral placement. For in vitro brain slice studies, as mCherry expression was cre-independent, we crossed POMCCre with POMCGFP mice to visualize POMC neurons. Current-voltage relationships of Ba²⁺ currents were recorded from mCherry and GFP-positive arcuate neurons in POMCCacna1eWT and POMCCacna1eKD mice. Consistent with knockdown of R-type Ca₉ in Cre-positive POMC neurons, peak current density was reduced with a shift in the current-voltage relationship to depolarizing potentials (Figure 2F). It is likely that the remaining barium currents are a mix of the other classes of CaV conductance present in POMC neurons (Figures S1C–S1H).

Resting Vm, firing frequency, and input resistance were unaltered between WT and Cacna1e knockdown POMC neurons (Figure 3A). During the initial 2-min application period, leptin (50 nM) equally depolarized POMCCacna1eWT (n = 12; paired t test, t(12) = 3.98; p < 0.003), POMCCacna1eKD (n = 15; paired t test, t(14) = 3.69; p < 0.003), and POMCshRNAscramble (n = 9; paired t test, t(8) = 2.59; p < 0.04) neurons (Figures 3B–3D). However, 10 min after leptin application, depolarization was only sustained in the POMCCacna1eWT and POMCshRNAscramble neurons (Figure 3D), suggesting that Ca₂⁺.3 subunit is required for the long-term depolarizing activity of leptin.

**Knockdown of Cacna1e Does Not Abolish Leptin Suppression of Feeding**

Prenatal leptin receptor deletion in POMC neurons increases body weight, fat mass, and serum leptin. However, knockdown of POMC neuron Cacna1e in adult mice did not alter body weight or fasted serum leptin compared to POMCCacna1eWT littermates (Figures 4A and 4B). Ad libitum or fast-refed food intake were also unaffected (Figures 4C and 4D), and exogenous leptin suppressed feeding and reduced body weight equally in POMCCacna1eWT and POMCCacna1eKD mice (Figures 4E and 4F).

**Knockdown of Cacna1e in POMC Neurons Leads to Increased HGP and Insulin Resistance**

Leptin action in the MBH regulates peripheral glucose homeostasis including HGP and insulin sensitivity, and deletion of signaling molecules in POMC neurons impairs peripheral glucose disposal and insulin sensitivity (Ruud et al., 2017). Thus, we measured fasted serum insulin and glucose levels in POMCCacna1eWT, POMCCacna1eKD, and POMCshRNAscramble littermate mice 8 weeks after viral injection. Serum insulin was increased in POMCCacna1eKD mice compared to POMCCacna1eWT littermates (Figure 4G) with glucose levels unaltered (Figure 4H). Similarly, serum insulin levels were increased (unpaired t test, t(8) = 3.14; p < 0.04) in POMCCacna1eKD (0.74 ± 0.03 ng/mL; n = 6) mice when compared to POMCshRNAscramble (0.48 ± 0.10 ng/mL; n = 4) littermate controls. This suggested the presence of insulin resistance, and so we undertook hyperinsulinemic-euglycemic clamp studies. Under basal and hyperinsulinemic conditions, glucose disposal (Rd) rate was unchanged (Figure 4I). However, HGP was significantly increased with a trend (unpaired t test, t(14) = 1.90; p = 0.078) to lower glucose infusion rates (GIRs) in POMCCacna1eKD mice (Figure 4J). 2-Deoxyglucose-phosphate (2DG-P) uptake into muscle, brown adipose tissue (BAT), and white adipose tissue (WAT) was unaltered between POMCCacna1eWT and POMCCacna1eKD mice (Figure 4K). Insulin-induced suppression of serum free-fatty acid (FFA) production was also unaffected by Cacna1e knockdown in POMC neurons (Figure 4K).

To investigate potential mechanism(s) for insulin resistance, we performed qPCR in hypothalamic and liver tissue from fasted mice. We observed no differences in hypothalamic expression of Pomc, glucokinase (Gck), or glucose transporter-2 (Slc2a2) in POMCCacna1eWT and POMCCacna1eKD mice (Figure 4L). However, liver Gck expression was reduced in POMCCacna1eKD mice, while interleukin-6 (Il6), phosphoenolpyruvate carboxykinase (Pck1), and glucose-6-phosphatase (G6pc) were unaltered (Figure 4M).

**DISCUSSION**

Our studies identify in POMC neurons a leptin-sensitive CaV conductance composed of Trpc subunits (Qiu et al., 2010). This activation was dependent on internal calcium, as found for Trpc5 conductance, including HGP and insulin sensitivity (Ruud et al., 2017). The sustained regulation of glucose homeostasis.

Leptin excites POMC neurons by activation of a non-selective conductance composed of Trpc subunits (Qiu et al., 2010). This activation was dependent on internal calcium, as found for Trpc5 subunits in heterologous expression systems (Blair et al., 2009). Consistent with Trpc activation, we also observed that leptin caused a reduction in input resistance at resting potentials and increased inward current at steady-state potentials. In hyperpolarizing voltage ramps, leptin induced a current that reversed close to the predicted reversal potential for a non-selective cation channel. Recently, Gao et al. (2017) deleted Trpc5 from POMC neurons, causing a loss of leptin-induced depolarization and reduced basal excitability. Many non-selective conductances, including Trpc5, are modulated by raised
Figure 3. Knockdown of Cacna1e Prevents Chronic Leptin-Induced Depolarization

(A) Bar charts showing resting membrane potential (Vm, left), spike firing frequency (middle), and input resistance (right) in POMC neurons from POMC/Cacna1eWT (red; n = 12), POMC/Cacna1eKD (blue; n = 15), and POMC/shRNAascramble (black; n = 9) mice. Mean ± SEM; one-way ANOVA (Vm: F(2,33) = 0.17, p = 0.85; spike: F(2,33) = 0.61, p = 0.55; input: F(2,33) = 0.06, p = 0.94).

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intracellular calcium, and since we also observed a leptin-activated voltage-dependent conductance, we postulated that this could facilitate leptin-induced POMC excitation. As Cacna1e channels are likely to be inactive at resting potentials (approximately ~50 mV), activation of Trpc5 and/or increased firing and excitatory synaptic input may be required to activate Cacna1e channels, allowing calcium entry to augment Trpc5 channel opening. Such a positive feedback mechanism could explain the sustained depolarization induced by brief exposure to leptin. Indeed, Cacna1e knockdown prevented chronic but not acute leptin-induced depolarization, suggesting that Trpc5 activation without calcium entry is insufficient to depolarize POMC neurons over longer periods. Given leptin’s dependence on uptake mechanisms to enter the brain (Banks and Farrell, 2003), it is not surprising that circulating leptin slowly activates POMC neurons in vivo (Beutler et al., 2017). Thus, it is far more probable that the loss of Cacna1e would impact on the overall magnitude of leptin response in vivo rather than altering the temporal pattern of activation.

Prenatal deletion of LepR in POMC neurons does not affect food intake or energy expenditure but causes obesity and reduces Pmc expression (Balasubramaniam et al., 2004). Overexpression of LepR in all POMC neurons in mice globally null for the LepR diminishes hyperphagia, whereas reintroduction of LepR into POMC neurons originally containing LepR minimally affects food intake (Berglund et al., 2012; Huo et al., 2009). Nevertheless, both models displayed improved glucose and insulin sensitivity. POMC neurons are important for hepatic parasympathetic nerve activity in response to leptin (Bell et al., 2018), but it is unclear whether POMC neurons regulate HGP directly through the autonomic nervous system, or indirectly by altering metabolic hormones. Recently, POMC neuron-specific deletion of the LepR in adult mice has been shown to impair insulin-induced suppression of HGP independent of changes in energy balance (Caron et al., 2018). These findings therefore show dissociation between the effects of leptin on glucose homeostasis and feeding and body weight regulation. Overexpression of suppressor of cytokine signaling-3 (Socs3) in POMC neurons increases adiposity and blocks leptin reduction in food intake (Reed et al., 2010) and POMC deletion of Socs3 reduces body weight, enhances leptin suppression of food intake, and improves peripheral glucose homeostasis (Kievit et al., 2006). Deletion of the LepR signaling molecule, signal transducer and activator of transcription-3 (Stat3), in POMC neurons causes mild obesity in female mice and reduces Pmc gene expression (Xu et al., 2007). Consistent with the idea of divergent outputs for leptin signaling in POMC neurons and with the differences between prenatal and adult-specific suppression of leptin signaling, knockout of Cacna1e only in arcuate POMC neurons did not alter food intake, leptin suppression of feeding, or body weight but caused defective regulation of HGP and insulin resistance in adult mice. The elevated fasted serum insulin may have occurred due to hepatic insulin resistance in order to maintain normal glucose levels. In contrast, deletion of Trpc5 in all POMC neurons leads to an age-dependent increase in body weight, with increased food intake, decreased energy expenditure, and attenuated leptin-mediated anorexia, although glucose homeostasis was unchanged (Gao et al., 2017). Our data indicate a subtle change in insulin sensitivity detected using highly sensitive hyperinsulinemic-euglycemic clamps. It is also possible that reduced resting excitability by Trpc5 deletion in POMC neurons increases body weight and feeding independent of leptin. Indeed, POMC Trpc5 expression is required, in part, for effective serotonergic action on feeding. Moreover, the mildly obese phenotype in Trpc5-deleted mice may contribute to leptin resistance attenuating leptin-induced anorexia and excitability.

In summary, the R-type (Ca$_{V}$2.3 or Cacna1e) channel is required for long-term leptin depolarization and excitation of POMC neurons in vitro. Furthermore, our findings suggest Cacna1e is an important component of the ion channel assembly in arcuate POMC neurons required for the maintenance of normal glucose homeostasis.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental Information includes one figure and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.09.024.

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AUTHOR CONTRIBUTIONS

DECLARATION OF INTERESTS
The authors declare no competing interests.
REFERENCES


inhibition of insulin-stimulated whole-body and muscle-specific glucose up-
take. Diabetes 50, 2585–2590.


Xu, A.W., Ste-Marie, L., Kaelin, C.B., and Barsh, G.S. (2007). Inactivation of signal transducer and activator of transcription 3 in proopiomelanocortin (Pomc) neurons causes decreased pomc expression, mild obesity, and de-
STAR METHODS

KEY RESOURCES TABLE

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to the Lead Contact, Professor Dominic J. Withers (d.withers@imperial.ac.uk).
EXPERIMENTAL MODEL AND SUBJECT DETAILS

**In-vivo animal studies**

POMCCre (Xu et al., 2005), NestinCre (B6.Cg-Tg(Nes-cre)1Kln/J), Z/EG (Tg(CAG-Bgeo/GFP21Lbe/J) and POMCGFP (B6.Cg-Tg(Pomc-MAPT/Topaz)1RCK/J) (Pinto et al., 2004) mice were bred on a C57BL/6J background and maintained on a 12 h light/dark cycle with free access to water and standard mouse chow (4.25% fat, RM3, Special Diet Services). Mice were housed in specific-pathogen free barrier facilities in individually ventilated cages of mixed genotypes. Male transgenic mice were age-matched (4-8 months) and studied with littermate controls. Mice were handled and all studies performed in accordance to the United Kingdom Animals (Scientific Procedures) Act (1986), amended regulations (2012), and approved by Imperial College and University of Cambridge Animal Welfare and Ethical Review Bodies. Findings and experiments described in this paper were designed and reported following the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines of animal experiment reporting (Kilkenny et al., 2010). Where possible, investigators were blinded to the genotype of both study animals and that of tissue and blood samples. For experiments involving treatments, mice were randomized by genotype to study groups or a cross-over design was used where indicated and study cohorts were matched for initial bodyweight where appropriate. Treatments were administered in random order. Mice were group housed (3-5 per cage) unless stated.

**Stereotactic surgery**

Male (4-6 month) POMCCre and NestinCre mice were anesthetized with isoflurane and placed in a KOPF stereotaxic frame. Analgesia was administered by topical bupivacaine (8mg/kg) and subcutaneous injection (5mg/kg) of carprofen during surgery, followed by the addition of carprofen to the drinking water (0.0272mg/ml) for 3-5 days post-surgery. Adeno-associated viral particles (AAV1 capsid) containing the cre-dependent shRNA construct (0.6-2.8 x 10^13 GC/ml) or shRNA scramble (2.0 x 10^13 GC/ml) were injected bilaterally with a Hamilton syringe (0.3 μl/min, 0.3 μl per inject site) into the arcuate nucleus at –1.2 mm posterior to bregma, ± 0.4 mm lateral to the midline and –6.1, –5.8 mm ventral to the surface of the skull. Co-ordinates for cortical injections into NestinCre mice were +0.5 mm posterior and ± 1.8 mm lateral from bregma and –2.0, –1.5 mm ventral from the surface of the skull. Mice were group housed and left for a minimum of 4-weeks post-surgery to recover before metabolic measurements or electrophysiological studies were performed. Viral expression of mCherry was used to confirm correct stereotaxic placement post-mortem.

**Cell Culture**

Hypothalamic GT1-7 cells (Mellon et al., 1990) were maintained in a high glucose Dulbecco’s Modified Eagle Medium (DMEM, Sigma) supplemented with fetal bovine serum (10% v/v), L-glutamine (8 mM), penicillin/streptomycin (2% v/v) and plated on poly-L-lysine coated flasks or glass coverslips. Cells were transfected 2 days following passage (1:6-1:8 density) with FuGENE (Promega) at a ratio of 2:1 reagent to cDNA. Cells were transfected with cDNA containing Eegfp in the pcDNA3.1 mammalian expression vector, or with the shRNA sequences in the pLK0.1 lentiviral expression vector. Cells expressing the puromycin selection marker in the pLK0.1 vector were treated with puromycin (5 µg/ml) for 2-3 days. All electrophysiological recordings were performed on GT1-7 cells 4-5 days post passage.

**METHOD DETAILS**

**Metabolic and food intake studies**

Studies were performed in the animal’s home cage unless indicated. Bodyweights from group-housed mice were measured weekly at 9-10am up to 5-6 weeks post-surgery. Fasted tail blood was analyzed for serum leptin (Millipore) and insulin (Crystal Chem) by ELISA 8 weeks post-surgery. For food intake studies, mice were group housed until 4 weeks post-surgery and then singly housed. Mice were allowed to acclimatize for 2 weeks and periodically fasted overnight. Ad-libitum food intake was measured over 3 consec-utive days and for 24 h following an overnight fast. Food intake was measured from singly housed mice injected with either vehicle or leptin (i.p. 1.5 mg/kg) at 9am and again at 6pm for 3 consecutive days. Treatments with either vehicle or leptin were crossed-over following a week wash-out period.

**Hyperinsulinemic-euglycemic clamps studies**

Clamps were conducted as previously described (Voshol et al., 2001). Animals were anesthetized by intraperitoneal injection of a combination of 6.25 mg/kg acetylpropromazine, 6.25 mg/kg midazolam and 0.31 mg/kg fentanyl. An infusion needle was placed into the tail vein and D-[3H] glucose (specific activity: 10-20Ci (370-740GBq/mm) was infused at a rate of 0.006 MBq/min for 80 min to achieve steady-state levels. Thereafter, insulin (Actrapid; Novo Nordisk) was infused at a constant rate of 0.11 μl/min after a bolus dose of 3.3 μl and D-[3H]-glucose was continued at a rate of 0.006 MBq/min. A variable infusion of 12.5% D-glucose was used to maintain blood glucose at euglycemic (basal) levels. Blood glucose was measured with an AlphaTRAK glucometer (Abbott Animal Health) every 5-10 minutes and glucose infusion adjusted accordingly. After 60 minutes from the start of the insulin infusion, ^14C-2-Deoxy-glucose-phosphate (Specific Activity: 250-350mCi (9.25– 13.0GBq)/mmol) was administered i.v. to assess tissue-specific glucose uptake. Steady-state was reached after 60 minutes and blood samples were taken at 10 minutes intervals over 30 minutes to determine steady-state levels of [3H]-glucose. Mice were then killed by cervical dislocation and the organs removed.
and frozen. To measure plasma [3H]-glucose, proteins were precipitated with trichloroacetic acid (final concentration 10%), centrifuged, and supernatant dried and re-suspended in water. The samples were counted using scintillation counting (Hydrox Scintillation counter, LabLogic). Muscle and brown adipose tissue samples were homogenized (~5%–10% wet wt/vol, depending on tissue) in 0.5% perchloric acid, centrifuged, supernatants neutralized, and 14C-2-Deoxy-glucose-phosphate precipitated using BaOH/ZnSO4. Total and precipitated counts of supernatants were subtracted and plasma 14C-2-Deoxy-glucose-phosphate counts were used to calculate tissue specific uptake. Protein content in homogenates was performed using DC protein assay (BioRad). For subcutaneous and epidemial white adipose tissue, 14C-2-Deoxy-glucose-phosphate was extracted using anion exchange columns as described previously (Kim, 2009). The glucose turnover rate (µmol/min) was calculated during the basal period and under steady-state clamp conditions as the rate of tracer infusion (dpm/min) divided by the plasma specific activity of [3H] glucose (dpm/µmol). Hyperinsulinemic hepatic glucose production was calculated as the difference between the tracer-derived rate of glucose appearance and the glucose infusion rate.

### Plasmids

Egfp was sub-cloned into the pcDNA3.1 expression vector. Four separate shRNA clones directed toward Cacna1e (#1: TRCN0000068910, #2: TRCN0000068912, #3 TRCN0000068908, #4: TRCN0000068911) were purchased from Thermo-Scientific (RMM4534) in the pLK0.1 lentiviral expression vector. A cDNA sequence comprising the U6 promoter, followed by a Loxp flanked stop cassette (Hitz et al., 2007) and the shRNA sequence (#2: TRCN0000068912) was synthesized by GeneArt (Life Technologies). Unique restriction sites SpeI and SvaI were inserted at the '3 end of the synthesized cDNA. The cDNA was subcloned into a pAAV expression vector (pAAV-EFα-DIO-hChR2-mCherry-WPRE) between MluI and EcoR1 removing the EFα-DIO-hChR2-mCherry sequence from the plasmid at the same time. A sequence containing the CMV promoter and mCherry was then synthesized (GeneArt) and inserted into the pAAV vector using the SpeI and SvaI sites to make the final construct (pAAV-U6-[LoxP-STOP-LoxP]-shRNA [Cacna1e]-CMV-mCherry-WPRE). Scrambled shRNA (GAGAATACCGACAAAGATACT, designed using Invivogen’s siRNA Wizard software) was synthesized by GeneArt (Life Technologies) and inserted into a pAM-FLEX-GFP vector (Murray et al., 2011).

### Genotyping

Generic Cre-recombinase (forward: 5'-AGCGATGAGTTCCGTACTT and reverse: 5'-CACCAGCTGGCATGATCCTC) and GFP (forward: 5'-AGCTAGCGCGAGGAG and reverse: 5'-ATCTCGAGGCTTACGAGCTGCTCATGCG) primers were used to genotype POMCCre, NestinCre and POMCGFP mice. Positive cre-recombinase bands were observed at approximately 200 bp and for GFP at 600 bp.

### Quantitative RT-PCR analysis

Tissues were lysed and homogenized in TRIzol reagent (Ambion) and total RNA was isolated using the RNeasy mini kit (QIAGEN). First-strand cDNA was generated using Taqman reverse transcription reagents (Applied Biosystems) and qPCR was performed using Taqman universal PCR mastermix in a 7900HT real-time PCR system (Applied Biosystems). mRNA quantities were normalized to Hprt after determination by the comparative Ct method. Primers used were: Gck (Mm00439129_m1), G6pc (Mm00839363_m1) Hprt (Mm00446968_m1), Pck1 (Mm01247058_m1), Pomc (Mm00435874_m1), Slc2a2 (Mm0044622_m1), Il6 (Mm00446190_m1).

### Western blot analysis

Cortices were removed and homogenized in lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% w/v Triton X-100) supplemented with Roche complete protease inhibitor cocktail and phosphatase inhibitors (1 mM sodium orthovanadate, 5 mM sodium fluoride and 2 mM β-glycerophosphate). 20–100 µg of total protein homogenates were run on 15% SDS-PAGE gels, transferred to nitrocellulose membranes and blotted with antibodies against Ca$_{2+}$2.3 (1:1000, #ACC-006 Alomone Labs) and Na$^+/K^-$ATPase α1 (1:1000, #05-369 Millipore). Detection was performed using enhanced chemiluminescence (Luminata Crescendo, Millipore) and exposed on films. The intensity of the bands was quantified by densitometry using Adobe Photoshop software and normalized to Na$^+/K^-$ATPase protein.

### Hypothalamic immunohistochemistry

Mice were perfused with paraformaldehyde (4% w/v) and frozen coronal sections (35 μm) were cut for immunohistochemistry as previously described (Choudhury et al., 2005). Arcuate sections were incubated with rabbit anti-POMC precursor (1:1000; Phoenix Pharmaceuticals Inc.) and detection performed using a secondary antibody coupled to Alexa Fluor-488 (1:200, Molecular Probes).

### Electrophysiology

Mice were killed by cervical dislocation and hypothalamic coronal slices (350 μm) were cut from aged matched (5-6 month old) transgenic mice expressing POMCCre and POMC-GFP or POMCCre and Z/EG. Slices were maintained at room temperature (22-25 °C) in an external solution containing (in mM) NaCl 125, KCl 2.5, NaH$_2$PO$_4$ 1.25, NaHCO$_3$ 25, CaCl$_2$ 2, MgCl$_2$ 1, D-glucose 10, D-mannitol 15, equilibrated with 95% O$_2$, 5% CO$_2$, pH 7.4. For intact slice studies, POMC neurons were visualized in the arcuate nucleus by video-enhanced differential interference contrast microscopy and by the expression and excitation of GFP. In addition, arcuate wedges from slices containing GFP labeled POMC neurons were dissected and incubated for 1 h in the standard external solution.
containing 0.5 mg/ml protease (type XIV) at room temperature. Following several washes with a HEPES (10 mM, pH 7.4) based external solution, individual neurons were dispersed by gentle trituration with fire polished glass Pasteur pipettes with decreasing tip aperture, as previously described (Spanswick et al., 1997). The cell suspension was evenly plated on concanavalin A (1 mg/ml) coated 35 mm culture dishes (Falcon) and left for 1 hour at 4°C allowing cell adhesion prior to use. Cells were visualized by phase contrast microscopy and POMC neurons identified by GFP expression and excitation.

Whole-cell current-clamp recordings were made at 33°C using borosilicate glass pipettes (4-8 MΩ) containing (in mM) Kgluconate 130, KCl 10, EGTA 0.5, NaCl 1, CaCl₂ 0.28, MgCl₂ 3, Na₂ATP 3, GTP 0.3, phosphocreatine 14 and HEPES 10 (pH 7.2). Following the establishment of a stable recording, leptin (R&D Systems) was applied for 2 minutes using a broken tipped pipette (~3 μm) positioned above the recording neuron. For qualitative descriptions only, responsive neurons were distinguished from non-responding neurons based on the criterion that the change in membrane potential by leptin challenge was ± three times the standard deviation prior to addition of the drug. In voltage-clamp studies, neurons were recorded in the standard external solution in the presence of 1 µM tetrodotoxin, 20 µM (+)-bicuculline and 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline-2,3-dione (NBQX, 5 µM) with D-2-amino-5-phosphonopentanoate (D-AP5, 50 µM). Neurons that changed series resistance (10-30 MΩ) were excluded from the analysis. Neurons were held at −70 mV and voltage-ramped (−10 to −100 mV over 2 s) every minute. In order to identify the conductances activated by leptin, the mean of 5 voltage ramps before leptin application was subtracted from mean of 5 ramps following leptin application. Voltage-gated calcium (Caᵥ) currents were recorded with a standard internal solution but with K⁺-gluconate replaced by 130 mM CsCl. The Caᵥ conductance was further isolated by an external solution containing in mM: NaCl 125, KCl 2.5, BaCl₂ 10, D-glucose 10, D-mannitol 15 and HEPES 10, pH 7.4. Series resistance and whole-cell capacitance were compensated (40%-70%) using an Axopatch 200B amplifier. Any uncompensated resistance and capacitance were digitally (IgorPro7) subtracted using an equivalent voltage-step protocol in which Caᵥ was inactivated by voltage-clamping the neuron at 0 mV for ≥ 2 minutes. Leptin was applied for 2 minutes following the establishment of a stable recording. An average of 5 consecutive trials before and immediately after leptin application was used to assess mean change in current.

Stock chemicals were dissolved and added to the appropriate external or internal solution prior to recording. Tetrodotoxin, SNX-482 and D-AP5 were from Tocris (UK); µ-conotoxin GVIA and µ-agatoxin IVA were purchased from Alomone Labs (Israel); mibefradil, (+)-bicuculline, NBQX and nimodipine were from Sigma-Aldrich.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical significance was calculated from all neurons (responsive and non-responsive) using repeated-measures (RM) two-way ANOVA and one-way ANOVA followed by Bonferroni post hoc analysis, Student’s two-tailed paired and unpaired t test, or one-sample t test where stated. Data are expressed as mean ± standard error of mean and degrees of freedom shown in brackets. A maximum of 2 recordings were made from any given mouse. Recordings and observations were repeated on at least 4 different mice. Study cohort sizes were determined by power calculations based on our previous data in mice with targeted hypothalamic mutations.