Melanocortin 3 receptor-expressing neurons in the ventromedial hypothalamus promote glucose disposal

Amy K. Sutton1,2, Paulette B. Goforth3,4, Ian E. Gonzalez5, James Dell’Orco6, Hongjuan Pei7, Martin G. Myers Jr4,8, and David P. Olson2,9,10

1Department of Molecular and Integrative Physiology, University of Michigan Medical School, Ann Arbor, MI 48109; 2Department of Pharmacology, University of Michigan Medical School, Ann Arbor, MI 48109; 3Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, MI 48109; and 4Division of Endocrinology, Department of Pediatrics, University of Michigan Medical School, Ann Arbor, MI 48109

Edited by Susan G. Amara, National Institutes of Health, Bethesda, MD, and approved March 9, 2021 (received for review February 20, 2021)

The ventromedial hypothalamus (VMH) is a critical neural node that senses blood glucose and promotes glucose utilization or mobilization during hypoglycemia. The VMH neurons that control these distinct physiologic processes are largely unknown. Here, we show that melanocortin 3 receptor (Mcr3r)-expressing VMH neurons (VMHmc3r) sense glucose changes both directly and indirectly via altered excitatory input. We identify presynaptic nodes that potentially regulate VMHmc3r neuronal activity, including inputs from proopiomelanocortin (POMC)-producing neurons in the arcuate nucleus. We find that VMHmc3r neuron activation blunt glucose excursion following a glucose load. Overall, these findings demonstrate that VMHmc3r neurons are a glucose-responsive hypothalamic subpopulation that promotes glucose disposal upon activation; this highlights a potential site for targeting dysregulated glycemia.

Results

VMHmc3r Neurons Sense Glucose Changes Both Directly and Indirectly and Receive Input from Glucose-Sensing Regions. Mc3r-2a-Cre mice (8) were crossed to the Rosa26R14tdTomato reporter for electrophysiological analysis of VMHmc3r neurons (Fig. 1 A–C). Application of the Mc3r-selective agonist d-trp8-MSH (10 nM) depolarized VMHmc3r neurons (Fig. 1 D and E) (9). Decreasing glucose from 2 to 0.1 mM suppressed the activity of VMHmc3r neurons; returning glucose to 2 mM depolarized VMHmc3r neurons and restored their firing (Fig. 1 F, G, and I). Increasing glucose to 5 mM did not significantly affect VMHmc3r membrane potential or firing (Fig. 1 F, H, and I). The hyperpolarization of VMHmc3r neurons in low glucose persisted during blockade of action potential (AP) firing and synaptic input in four of seven neurons, indicating direct glucose regulation (Fig. 1J).

Likewise, low glucose activated an outward current in five of nine cells (Fig. 1 K and L).

Indirect regulation of VMHmc3r neurons is suggested by the observation that hypoglycemia inhibits nearly all VMHmc3r neurons, yet only a subset sensed glucose directly. Indeed, lowering glucose without synaptic blockade decreased the frequency and amplitude of spontaneous excitatory postsynaptic currents (sEPSCs) on VMHmc3r neurons, thus demonstrating indirect glucose-dependent regulation through presynaptic input (Fig. 1 M–O). There was no change in spontaneous inhibitory postsynaptic currents (sIPSCs).

Given these glucose-dependent alterations in excitatory input, we used monosynaptic retrograde tracing to inputs to VMHmc3r neurons. Projection-specific modified rabies virus tracing was performed in the bed nucleus of the stria terminalis (BNST), a site targeted by Mc3r axons relevant for VMH-mediated blood glucose regulation (Fig. 1P) (2). BNST-projecting VMHmc3r neurons receive inputs from several brain areas relevant to glucose regulation including POMC cells of the ARC (Fig. 1S), and cells in the lateral parabrachial nucleus (LPB) (Fig. 1W).

VMHmc3r Neurons Regulate Glucose Disposal. To directly test the capacity of VMHmc3r neurons to promote glucose disposal, we chemogenetically activated VMHmc3r neurons using the Cre-dependent modified human muscarinic receptor hM3Dq (AAV8-hSyn-Flex-hM3Dq-mCherry; Fig. 2A–D) 15 min prior to a glucose tolerance test (GTT) (Fig. 2E). Precactivation of VMHmc3r neurons blunts glucose excursion in comparison to controls (Fig. 2 F and G). This effect appears to be largely independent of insulin secretion, as insulin is unchanged 5 min after glucose administration (Fig. 2 H and I).

To test the potential benefit of activating VMHmc3r neurons in a glucose-intolerant state, we chemogenetically activated VMHmc3r neurons prior to a GTT in mice fed a 60% high-fat diet (HFD) (Fig. 2I). Precactivation of VMHmc3r neurons promotes glucose disposal in HFD conditions (Fig. 2 K and L), suggesting that driving VMHmc3r neuron activity may be sufficient to promote glucose disposal in glucose-intolerant states. Since VMHmc3r activation without a glucose load does not significantly alter peripheral glucose levels in mice on either chow or HFD conditions (Fig. 2 M and N), the effects of VMHmc3r neuronal activation may be most impactful during a glucose load.

To examine the contribution of VMHmc3r neurons to glucose homeostasis, we silenced these neurons using a Cre-dependent
tetanus toxin (AAV8-hSyn-Flex-TetTox-GFP; Fig. 2 O and P). Loss of VMHMC3R neuronal activity does not alter blood glucose levels in the basal state (Fig. 2Q) but does increase blood glucose during a GTT (Fig. 2 R and S). VMHMC3R neuron silencing did not affect body weight or food intake (body weight at t = 45 d post injection [mean ± SEM]: TetTox, 30.4 ± 1.9 g; control, 29.6 ± 1.0 g; two-way ANOVA), suggesting that the impaired glucose handling was not secondary to obesity alone.

**Discussion**

The VMH regulates both glucose mobilization during the counterregulatory response and glucose disposal in response to hormonal factors (1–4). However, the cellular mechanisms coordinating these seemingly opposing physiologic functions are largely unknown. Here, we show that VMHMC3R neurons respond to melanocortin agonists and their activation promotes glucose disposal in both chow and HFD conditions. Silencing of VMHMC3R neurons disrupts normal glucose handling, underscoring an important role for these neurons in glucose homeostasis. These neurons sense glucose in low glucose was measured simultaneously recording EPSC and IPSCs (M). Low glucose decreased amplitude and frequency of sEPSCs, but not sIPSCs (N and O). Identification of presynaptic inputs to VMHMC3R neurons projecting to the BNST using modified rabies virus tracing (P–R) identifies ARC inputs (S, purple) containing αMSH (S, green). Additional inputs include the PVH (U), MeA (V), IPBN (W), and BMP (X). Data are represented as mean ± SEM (G, H, and O), or box plots ± maximum/minimum (E, I, and J). Significance was determined using a repeated-measures one-way ANOVA (E, G, H, J, K, and O), or mixed-effects analysis followed by Tukey’s post hoc if applicable (I) with *P < 0.05, **P < 0.01, and ***P < 0.001.

**Methods**

**Animals.** Animals were bred and housed according to guidelines approved by the University of Michigan Committee on the Care and Use of Animals. Male mice (8–23 wk) were used for all behavioral studies. Mice were provided ad libitum food (standard chow, Purina Lab. Loberto Diet 5001; 60% HFD, Research Diets D12492) and water, unless otherwise noted, and acclimatized to intraperitoneal (i.p.) injections 3 d prior to experimentation. Following experimentation, mice were perfused, immunohistochemistry (IHC) performed as previously described (11), and data points excluded if extra-VMH viral transduction occurred. IHC was performed for dRed (Clontech), GFP (Invitrogen), Fox (Cell Signaling 96F), and αMSH (Phoenix Pharmaceuticals).

**Stereotaxic Injections.** Surgeries were performed as previously described (11). VMH-directed injections (25 nL/side) were performed relative to bregma (anteroposterior [AP]: −1.0; mediolateral [ML]: ± 0.25; dorsoventral [DV]: −5.5). Mice recovered for at least 2 wk before testing. For barbs tracking, helper virus (11) was injected followed by EnvA-dB19G-rabies-mCh injections (BNST) (AP: +0.65; ML: +0.4; DV: −3.8) 3 wk later. Mice were perfused and IHC performed 5 d after virus injection.

**Electrophysiology.** Coronal slices (250 μm) were prepared from 4- to 12-wk-old Mc3R-2a-Cre, Td mice of either sex in oxygenated ice-cold artificial cerebrospinal fluid (ACSF) solution containing the following (in mM): 125 NaCl, 3 KCl, 1.25 NaH2PO4, NaHCO3, 2 glucose, 1 MgCl2, and 2 CaCl2, plus 0.2 mM ascorbic acid and 1 mM kynurenic acid, pH 7.4, 300–305 mOsm, and allowed to recover ≥ 1 h (kynurenic acid) at 33–34 °C. Patch-clamp recordings from VMHMC3R neurons were made using borosilicate electrodes (3–7 MΩ) filled with either perforated patch internal (in mM): 130 K gluconate, 10 KCl, 1 EGTA, 10 Hepes, 2 MgCl2, amphotericin B (0.15 mg/mL) for current-clamp recordings of Vm/AP firing in response to glucose (Fig. 1 F–J) or whole-cell internal solution (in mM): 130 K gluconate, 10 KCl, 1 EGTA, 10 Hepes, 0.6 NaGTP, 2 MgATP, and 8 phosphocreatine, pH 7.2, 285–295 mOsm, for all other experiments. Slices were perfused with ACSF solution (33–34 °C, 1.5–2.0 mL/min) and bubbled with 5% CO2/95% O2. When stimulated, synaptic input and AP firing were blocked by inclusion of 1 μM tetrodotoxin, 20 μM α-APV, 10 μM CNQX, and 50 μM picrotoxin.
Spontaneous EPSCs and IPSCs were measured from neurons voltage clamped to −50 mV, producing inward sEPSCs and outward sIPSCs, and analyzed using Synaptosoft software. Data were not corrected for a junction potential of ∼15 mV. Analyzed neurons had uncompensated stable series resistances (<30 MΩ).

GTI. Following a 4-h fast, mice were injected with glucose (2 g/kg, i.p.), and blood glucose monitored by tail vein (OneTouch Ultra 2 glucometer). For hM3Dq experiments, mice received vehicle (10% glucose) or CNO (0.3 mg/kg) 15 min prior to glucose administration counterbalanced across at least 1 wk. Insulin analysis was performed in a separate experiment using blood collected via tail vein; insulin levels were measured by enzyme-linked immunosorbent assay (Crystal Chem). Separate experiments were performed with 60% HFD (Research Diets).

Statistical Analyses. Paired and unpaired t tests, one-way, two-way, or three-way ANOVAs followed by Tukey’s multiple comparisons (if applicable), or mixed model analyses were calculated using GraphPad Prism 8 as appropriate, with significance denoted at P < 0.05.

Data Availability. All study data are included in the article and/or supporting information.

1. O. Chan, R. Sherwin, Influence of VMH fuel sensing on hypoglycemic responses. Trends Endocrinol. Metab. 24, 616–624 (2013).
2. T. H. Meek et al., Functional identification of a neurocircuit regulating blood glucose. Proc. Natl. Acad. Sci. U.S.A. 113, E2073–E2082 (2016).
3. J. N. Flako et al., Ventromedial hypothalamic nucleus neuronal subset regulates blood glucose independently of insulin. J. Clin. Invest. 130, 2943–2952 (2020).
4. Z. Song, B. E. Levin, J. J. Mc Ardle, N. Bakhos, V. H. Routh, Convergence of pre- and postsynaptic influences on glucose sensing neurons in the ventromedial hypothalamic nucleus. Diabetes 50, 2673–2681 (2001).
5. C. Toda et al., Distinct effects of leptin and a melanocortin receptor agonist injected into medial hypothalamic nuclei on glucose uptake in peripheral tissues. Diabetes 58, 2757–2765 (2009).
6. C. K. Gavini, W. C. Jones 2nd, C. M. Novak, Ventromedial hypothalamic melanocortin receptor activation: Regulation of activity energy expenditure and skeletal muscle thermogenesis. J. Physiol. 594, 5285–5301 (2016).
7. K. Begriche et al., Genetic dissection of the functions of the melanocortin-3 receptor, a seven-transmembrane G-protein-coupled receptor, suggests roles for central and peripheral receptors in energy homeostasis. J. Biol. Chem. 286, 40771–40781 (2011).
8. H. Pei et al., Lateral hypothalamic Mc3R-expressing neurons modulate locomotor activity, energy expenditure, and adiposity in male mice. Endocrinology 160, 343–358 (2019).
9. P. Grieco, P. M. Balse, D. Weinberg, T. Maclachlin, V. J. Hruby, α-Amino acid scan of γ-melanocyte-stimulating hormone: Importance of Trp(8) on human MC3 receptor selectivity. J. Med. Chem. 43, 4998–5002 (2000).
10. J. W. Hill et al., Direct insulin and leptin action on pro-opiomelanocortin neurons is required for normal glucose homeostasis and fertility. Cell Metab. 11, 286–297 (2010).
11. A. K. Sutton et al., Paraventricular, subparaventricular and periventricular hypothalamic i5δ4-expressing neurons are required for normal energy balance. Sci. Rep. 10, 5546 (2020).