Primary VMH Neuron Culture:
Coronal brain slices (250 µm) from WT and nNOS KO mice (5-7 weeks old) were prepared as previously described (8; 22). After brain slices have been incubated in aCSF (containing in mM: 126 NaCl, 1.9 KCl, 1.2 KH2PO4, 26 NaHCO3, 2.4 CaCl2, 1.3 MgCl2, 2.5 glucose; osmolarity adjusted to 300-310 mOsM, pH 7.4) for 1 hour, VMH (VMN + ARC) were dissected in cold Hibernate A media (Brain Bits) (containing 2.5 mM glucose, 0.5 mM L-glutamine, 1 mM lactic acid, 0.23 mM sodium pyruvate, 100 U/ml penicillin/streptomycin, pH 7.4). VMH were then digested in Hibernate A with 30 U/ml papain (Worthington Biochem) for 35 minutes at 37°C, 100 rpm. Tissues were rinsed and triturated in Hibernate A containing 0.05 KU/ml of DNAse I (Sigma). Single cells were separated from the suspension by layering onto an 8% BSA gradient and centrifugation at 1200 g for 5 minutes at room temperature. Cell pellets were resuspended in astrocyte-conditioned Neurobasal media (see below). Cells will be plated into cloning cylinders on poly-d-lysine (0.1 mg/ml; Sigma) coated glass coverslips. After 30 minutes, cylinders were removed and astrocyte-conditioned Neurobasal media (described below) was added for 12-24 hours before imaging.

Preparation of astrocytes-conditioned Neurobasal media:
VMH slices from WT mice were obtained as described above. VMH were triturated in Neurobasal A (Invitrogen; containing 2.5 mM glucose, 0.5 mM L-glutamine, 1 mM lactic acid, 0.23 mM sodium pyruvate, 100 U/ml penicillin/streptomycin, pH 7.4) with 10% FBS. Dissociated cells were plated in 25 cm^2 flasks containing 5 ml Neurobasal A with 10% FBS until confluence. Astrocytes were separated by shaking at 250 rpm for 18 hours at 37°C. The media containing all cell types except astrocytes was discarded. Astrocytes attached to the flask were trypsinized with 0.05 % Trypsin-EDTA (Sigma) and centrifuged 1200 rpm for 5 minutes at room temperature. The pellet was rinsed, resuspended in Neurobasal A with 10% FBS, and plated in 100 cm^2 dishes. Astrocytes were grown in Neurobasal A plus 10% FBS until confluence. Astrocyte-conditioned media was prepared by incubating astrocytes in Neurobasal A (containing 2.5 mM glucose, 0.5 mM L-glutamine, 1 mM lactic acid, 0.23 mM sodium pyruvate, 100 U/ml penicillin/streptomycin, 2% B27 without insulin, pH 7.4) overnight. Astrocyte-free media was decanted, filtered and added to dissociated VMH neurons (see above).

Membrane Potential Dye Imaging:
Coverslips containing VMH dissociated neurons were assembled into a closed perfusion chamber (Warner RC-43C) and positioned under an upright microscope (Olympus BX61 WI microscope) and perfused with an oxygenated recording solution (in mM: 132 NaCl, 5 KCl, 1.2 CaCl2, 0.5 MgCl2, 0.4 MgSO4, 0.45 KH2PO4, 0.45 Na2HPO4, 10 Hepes, 10 sucrose, pH 7.3) containing 0.5% membrane potential dye (FLIPR-MPD blue, Molecular Devices) at 0.6 ml/min. Neurons were visualized on an Olympus BX61 WI microscope with a X10 objective equipped with a red filter (excitation 545 nm, emission 563–647 nm) for visualization of FLIPR-MPD dye. Fluorescence images were captured every 30 seconds using a charge-coupled device camera (Cool Snap, Photometrics). Images were acquired and analyzed using MetaMorph software (Molecular Device). Cells were exposed to 2.5 mM glucose for 10 minutes (baseline), 0.1 mM glucose for 15 minutes (challenge), and 2.5 mM glucose for 15 mintues (reversal). Cells with background-subtracted fluorescence increasing greater than 25% with challenge and reversing upon return to 2.5 mM glucose were considered as GI neurons.

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