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High glucose increases action potential firing of catecholamine neurons in the nucleus of the solitary tract by increasing spontaneous glutamate inputs

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Roberts BL, Zhu M, Zhao H, Dillon C, Appleyard SM. High glucose increases action potential firing of catecholamine neurons in the nucleus of the solitary tract by increasing spontaneous glutamate inputs. Am J Physiol Regul Integr Comp Physiol 313: R229–R239, 2017. First published June 14, 2017; doi:10.1152/ajpregu.00413.2016.—Glucose is a crucial substrate essential for cell survival and function. Changes in glucose levels impact neuronal activity and glucose deprivation increases feeding. Several brain regions have been shown to respond to glucoprivation, including the nucleus of the solitary tract (NTS) in the brain stem. The NTS is the primary site in the brain that receives visceral afferent information from the gastrointestinal tract. The catecholaminergic (CA) subpopulation within the NTS modulates many homeostatic functions including cardiovascular reflexes, respiration, food intake, arousal, and stress. However, it is not known if they respond to changes in glucose. Here we determined whether NTS-CA neurons respond to changes in glucose concentration and the mechanism involved. We found that decreasing glucose concentrations from 5 mM to 2 mM to 1 mM, significantly decreased action potential firing in a cell-attached preparation, whereas increasing it back to 5 mM increased the firing rate. This effect was dependent on glutamate release from afferent terminals and required presynaptic 5-HT3R activity, and 5-HT3R agonist-induced increase in the frequency of spontaneous glutamate inputs onto NTS-CA neurons. Low glucose also blunted 5-HT-induced inward currents in nodose ganglia neurons, which are the cell bodies of vagal afferents. The effect of low glucose in both nodose ganglia cells and in NTS slices was mimicked by the glucokinase inhibitor glucosamine. This study suggests that NTS-CA neurons are glucosensing through a presynaptic mechanism that is dependent on vagal glutamate release, 5-HT3R activity, and glucokinase.

Glucose; catecholamine; serotonin; vagus; NTS

Glucose is essential for cell survival and plays a critical role in metabolic processes. Multiple brain regions contain glucose-sensing neurons, including the hypothalamus and hindbrain (9, 30–33, 66). The nucleus of the solitary tract (NTS) is one brain region identified as responsive to glucoprivation, as when the glucose metabolism inhibitor 5TG is injected into the NTS, it elicits a feeding response (4, 13, 32). The NTS impacts multiple homeostatic functions and is the primary site by which visceral afferent information concerning cardiovascular, respiratory, and gastrointestinal systems enters the brain (22, 25, 40, 53, 55, 58). This provides a mechanism by which changes in glucose could impact these homeostatic responses; however, the neuronal phenotype that responds to changes in glucose, and the cellular mechanisms involved, are not well understood.

The A2/C2 group of catecholamine (CA) neurons is located in the NTS and is directly activated by incoming visceral afferents of the solitary tract (ST) (1). They are also activated by signals that inhibit food intake and inhibited by compounds that stimulate food intake, suggesting they lie downstream of a reflex satiety pathway (1, 10–12, 21, 26, 38, 44, 68, 69). Furthermore, optogenetic and chemogenetic activation of these neurons inhibits food intake, with prolonged activation reducing body weight (50). NTS-CA neurons form extensive projections, including to the hypothalamus, amygdala, nucleus accumbens, ventral tegmental area, parabrachial nucleus (PBN), and other brain stem nuclei (3, 42, 43, 50, 54, 61, 63, 67), making them ideally suited to send visceral afferent information in a coordinated manner to multiple brain regions. NTS-CA neurons express norepinephrine, epinephrine, and glutamate and the release of these neurotransmitters at these target nuclei can affect many behaviors, including stress, arousal, anxiety, reward, food intake, and cardiovascular function (8, 22, 25, 28, 40, 44, 50, 58–60).

Glucose plays a critical role in metabolic processes, and hindbrain catecholaminergic neurons are involved in hypoglycemia-induced food intake and other homeostatic functions modulated by glucose concentrations (18, 47, 52). Strong evidence exists suggesting that A1/C1 catecholamine neurons of the ventral lateral medulla (VLM) are glucosensing, but the role of the A2/C2 neurons is less clear and it is not known whether they respond to changes in glucose concentration (32, 48).

The goal of these studies was to determine whether NTS-CA neuronal activity is altered by changes in glucose concentration. We report here that the majority of NTS-CA neurons are glucose excitatory and that glucose has presynaptic effects on visceral afferent terminals to alter 5-HT-induced glutamate release. Furthermore, inhibition of glucokinase mimics the effects of glucose on 5-HT signaling, suggesting that glucokinase is involved in the glucosensing mechanism.

MATERIALS AND METHODS

NTS slices. All animal procedures were conducted with the approval of the Animal Care and Use Committees at Washington State University (Pullman, WA) and in accordance with the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals (PHS Policy) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH). Eight to sixteen-week-old mice (both males and females) were used for these studies. No difference was noted between male and female mice for the...
parameters studied and so the data were pooled. The hindbrain was removed and placed for 1 min in cold (0–4°C) artificial cerebral spinal fluid (aCSF). The cutting plane was aligned vertically, sectioning caudal to rostral when mounted in a vibrating microtome (Lieva VT-1000S). Coronal slices (250 μm thick) from the TH-EGFP mice (Matsushita et al. 2002) were cut with a sapphire knife (Delaware Diamond Knives) and preserved the raphe nucleus and caudal NTS. This allowed us to conserve serotonergic inputs into the NTS. Slices were submerged in a perfusion chamber and all recording were performed at 31–35°C.

Whole cell recordings were made using an external aCSF bath solution containing (in mM): 125 NaCl, 3 KCl, 1.2 KH2PO4, 1.2 MgSO4, NaHCO3, 10, 5, 2, or 1 dextrose, and 2 CaCl2, bubbled with 95% O2-5% CO2; 30–34°C; pH 7.3, adjusted to 300–310 mosmol using sucrose. Internal recording solution contained (in mM) 10 NaCl, 125 KCl, 11 EGTA, 1 CaCl2, 2 MgCl2, and 10 HEPES, pH 7.3, 295–300 mosmol. External bath solution was used in patch electrodes for cell-attached recordings. Neurons were recorded from NTS within 200 μm rostral or caudal from obex and medial to the ST. Patch electrodes, 3–5 MΩ, were guided to neurons using both fluorescence (FITC) and differential interference contrast (DIC) optics (Olympus BX51). Voltage clamp and current clamp recordings were made with an Axopatch 700B and pClamp10 software (Axon Instruments). Only neurons not exceeding holding currents of 100 pA at holding potential (Vh) = −60 mV for the 10 min control period (input resistance > 120 MΩ) were studied further. Current clamp recordings were made at resting membrane potentials, and current injections were not used to hold the membrane at set potentials. Only neurons with an access resistance less than 20 MΩ were used for analysis. All membrane potentials reported were for junction potential (~14 mV). All drugs were obtained from Tocris Cookson or Sigma Aldrich.

Cell cultures. Nodose ganglion neurons were dissected from male and female mice (6–16 wk) and cultured similar to previously reported protocols (24). Cells were maintained in Neurobasal A media (Invitrogen). Nodose cells were recorded on days 1–2 at room temperature. Locally administered drugs were applied to nodose cells for 2 s with a pressure of 2–3 psi using a picospritzer III (Parker). Input resistance ranged from 0.9 to 18.5 MΩ. The response rate of neurons to 5-HT was highly variable, ranging from 10 to 70% of the neurons tested, depending on the culture.

Statistics. All data are presented as means with errors bars as SE. Differences in drug effects were tested by repeated measured ANOVA, using Tukey’s post hoc analysis unless otherwise noted. Differences were considered statistically significant for P values <0.05 unless otherwise stated (SigmaPlot 11.2).

Mice. TH-EGFP mice were on a C57Bl/6J background. Transgenic mice were housed on a 12-h light/12-h dark cycle at room temperature in the Department of Integrative Physiology and Neuroscience animal vivarium. Mouse chow and water were provided ad libitum. Genotyping and breeding of mice were as described previously (1). All animal procedures were conducted with the approval of the Institutional Animal Care and Use Co mIteet at WSU in accordance with the US Public Health Service Policy on Humane Care and Use of Laboratory Animals (PHS Policy) and the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH).

RESULTS

Low glucose decreases action potential firing of TH-EGFP neurons in NTS. TH-EGFP neurons were easily identified and visualized for recordings (Fig. 1A). We have previously demonstrated a greater than 88% colocalization of EGFP with TH in the medial and caudal NTS of these mice (1, 10). To determine whether changes in glucose concentration alter action potential (AP) firing rates in NTS TH-EGFP neurons, we used cell attached recordings to preserve the internal conditions of the neurons and measured AP firing rate in different glucose concentrations. Decreasing the aCSF glucose concentration from 5 mM (1.87 ± 0.74 Hz) to 2 mM (1.23 ± 0.45 Hz) to 1 mM (0.43 ± 0.27 Hz) gradually decreased the action potential firing rate in TH-EGFP neurons (n = 7). This effect was...
reversible, as action potential firing rate increased again when glucose concentrations were returned to 5 mM glucose (2.85 ± 0.31 Hz; Fig. 1, B and C).

**Effect of glucose on NTS TH-EGFP neurons is presynaptic.**
To determine whether the effects of glucose were direct or indirect (e.g., pre- or postsynaptic), we tested the effect of glucose after blocking GABA<sub>A</sub>, ionotropic glutamate (α-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid (AMPA)/kainate and N-methyl-d-aspartate (NMDA) and glycine receptors using GABA<sub>zi</sub>ne (0.5 μM), 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX, 50 μM), (2R)-amino-5-phosphonopentanoate (AP-5, 50 μM), and strychnine (1 μM), respectively (Cocktail). Using a cell-attached preparation, we again recorded the AP firing rate of NTS TH-EGFP neurons, at a starting glucose concentration of 5 mM (6.45 ± 1.05 Hz), to 5 mM + Cocktail (5.92 ± 1.94 Hz) to 2 mM + Cocktail (6.32 ± 2.17 Hz), to 1 mM + Cocktail (4.87 ± 1.5 Hz), and then raised to 2 mM + Cocktail (6.81 ± 2.35 Hz) to 5 mM + Cocktail (3.71 ± 1.34 Hz; Fig. 1D). In contrast to the control experiment described above, in the presence of GABA<sub>A</sub>, AMPA/kainate, NMDA, and glycine receptor antagonists, lowering glucose concentration no longer decreased AP firing rate (n = 5; one-way ANOVA).

**Effect of glucose on NTS TH-EGFP neurons is mediated by changes in presynaptic glutamate release.** We have previously shown that the firing rate of NTS TH-EGFP neurons is dependent on glutamate inputs (10). As glucose has been shown to alter both the excitability of vagal afferents and nodose ganglion cells (17) and the release of glutamate from presynaptic terminals (64, 65), we tested whether the effects of altering glucose concentration were dependent on presynaptic glutamate release. Using a cell-attached preparation, we again recorded the AP firing rate of NTS TH-EGFP neurons, at a starting glucose concentration of 5 mM (1.97 ± 1.06 Hz) to 2 mM (1.4 ± 1.09 Hz) to 1 mM (1.03 ± 0.86 Hz), verifying that the cells were glucose excitatory. We then bath applied the non-NMDA ionotropic glutamate receptor antagonist NBQX (50 μM) before raising the glucose concentration back from 1 mM (0.99 ± 0.81 Hz) to 2 mM (0.06 ± 0.04 Hz) and finally 5 mM (0.03 ± 0.01 Hz; n = 6). In contrast to the control experiments (Fig. 1, B and C), AP firing rate did not return after raising glucose concentrations in the presence of NBQX, suggesting that the effects of glucose depend on glutamate inputs. The effects of NBQX were not rapidly washed out in 5 mM glucose (0.02 ± 0.01 Hz; Fig. 2, A and B), likely due to the high affinity of NBQX for AMPA receptor.

**Effect of glucose on NTS TH-EGFP neurons requires 5-HT<sub>3</sub>Rs.** The 5-HT<sub>3</sub>R is a potent modulator of presynaptic glutamate release in the NTS, and the size of 5-HT<sub>3</sub>R-mediated 5-HT currents are dependent on glucose concentration (2, 12, 65). To test whether glucose effects are dependent on 5-HT<sub>3</sub>R function we lowered the glucose concentration from 5 mM (1.17 ± 0.28 Hz) to 2 mM (0.76 ± 0.27 Hz) to 1 mM (0.18 Hz ± 0.06 Hz; n = 6/8) and then bath applied the 5-HT<sub>3</sub>R antagonist ondansetron (ODT, 0.5 μM) and raised the glucose concentration from 1 mM (0.44 ± 0.32 Hz), 2 mM (0.02 ± 0.02 Hz), to 5 mM (0.05 ± 0.04 Hz) in the presence of ODT. ODT blocked the return of AP firing after increasing glucose concentrations suggesting that glucose actions on NTS TH-EGFP neurons require 5-HT<sub>3</sub>Rs. After ODT was washed out for 15 min in 5 mM glucose concentration, the firing rate of the neurons began to increase again (0.25 ± 0.11 Hz; ~21% of control. Fig. 2, C and D).

**5-HT<sub>3</sub>R-stimulated increase in sEPSC frequency in NTS TH-EGFP-positive neurons is blunted in low glucose concentrations.** As we had determined that the effects of glucose were indirect we next used whole cell patch-clamp techniques in the coronal brain slice to determine whether glucose affects basal 5-HT<sub>3</sub>R modulation of spontaneous excitatory postsynaptic current (sEPSC) frequency in NTS TH-EGFP neurons as this has been demonstrated to be a potential mechanism for changes in glucose to alter glutamate inputs onto unidentified NTS neurons (65).

We have previously shown that 5-HT increases sEPSC frequency in horizontal slices, but that there was no effect of the 5-HT antagonist alone in that orientation, suggesting that we had severed the serotonergic inputs (12). In contrast, 5-HT has been shown to provide a basal tone in coronal slices to increase sEPSCs frequency (65), suggesting that the serotonergic inputs are intact in the coronal slice. To confirm that we also see an endogenous basal 5-HT tone in our coronal slices we applied ODT (0.5 μM) in 10 mM glucose; sEPSC frequency decreased from 4.29 ± 1.01 Hz during the control period to 3.27 ± 0.63 Hz in the presence of ODT. After the wash, sEPSC frequency returned to 4.30 ± 0.08 Hz (Fig. 3, A and B). In the presence of ODT, sEPSC amplitude did not change (~33.2 ± 6.23 pA) compared with control (~34.91 ± 6.15 pA; D < 0.05, one-way ANOVA; Fig. 3C).

To determine whether the effect of 5-HT on the sEPSC frequency of NTS TH-EGFP neurons is dependent on glucose concentration, we used whole cell patch clamp techniques to record from NTS TH-EGFP neurons. In 5 mM glucose, bath application of SR57227 (30 μM), a 5-HT<sub>3</sub>R agonist, increased sEPSC frequency from 7.14 ± 1.75 Hz to 18.42 ± 4.6 Hz (Fig. 3, D and E). After a 10-min wash, sEPSC frequency returned to control (15.58 ± 5.63 Hz). In 2 mM glucose, bath application of SR57227 did not change sEPSC frequency (5 mM: 5.5 ± 1.62 Hz, 2 mM: 6.76 ± 1.98 Hz, 2 mM + SR: 13.5 ± 6.29 Hz, 2 mM: 10.15 ± 4.19, 5 mM: 8.02 ± 4.03 Hz; Fig. 3E). sEPSC amplitude did not change after application of SR57227 in 5 mM (Control: ~31.8 ± 3.8 pA; SR: ~30.4 ± 4.4 pA; Wash: ~27.7 ± 3.2 pA) or 2 mM glucose concentrations (Control: ~35.48 ± 5.38 pA; SR: ~33.77 ± 3.22 pA; Wash: ~34.5 ± 5.25 pA; P < 0.05, one-way ANOVA; Fig. 3F).

**Low glucose blunts 5-HT inward current in dissociated nodose ganglion neurons.** To determine the mechanism by which glucose acts we performed whole cell patch-clamp recordings in dissociated nodose ganglion neurons, which are the cell bodies of the presynaptic afferents providing inputs onto the TH-EGFP neurons in our NTS slices. We started by testing the effect of 5-HT on the holding current of nodose ganglion neurons. We locally applied 5-HT from a glass pipette with a 2-s puff from a picospritzer. To determine the effective 5-HT concentration, we tested 5-HT at two concentrations, 100 μM and 300 μM. The 100 μM concentration of 5-HT induced a large inward current (~312.54 ± 132.73 pA; n = 5) with it being further increased at 300 μM (~1063.77 ±...
concentrations, decay time was 10.92 ± 2.69 s and 43.04 ± 9.19 s, respectively (data not shown). To control for cell size and capacitance differences, we also calculated the current density induced by both 100 μM 5-HT as −5.63 ± 2.88 pA/pF [membrane capacitance (Cm): 62.52 ± 3.9 pF] and 300 μM 5-HT as −23.68 ± 7.0 pA/pF (Cm: 52.44 ± 3.38 pF). We used 100 μM 5-HT for the remainder of our nodose recordings as this produced an optimal response.

We next tested 100 μM 5-HT in 10 mM, 5 mM, and 2 mM glucose concentrations. In 10 mM glucose, 5-HT induced a large inward current (−585.88 ± 131.17 pA, Fig. 4A), which was significantly larger than at 2 mM glucose (−15.25 ± 4.42 pA; Fig. 4A) where 5-HT-induced inward currents were minimal. The effect was intermediate at 5 mM (trace not shown, −159.5 ± 55.26 pA). Current density was −8.91 ± 2.03 pA/pF (Cm: 66.49 ± 3.0 pF; n = 9) at 10 mM, −4.04 ± 1.22 pA/pF (Cm: 38.72 ± 2.93 pF; n = 6) at 5 mM, and −0.3 ± 0.07 pA/pF (Cm: 48.6 ± 6.03 pF; n = 8) at 2 mM glucose concentrations (Fig. 4C). Decay times at 10, 5, and 2 mM were 33.2 ± 10.95 s, 10.3 ± 1.82 s, and 6.36 ± 1.91 s, respectively (Fig. 4D).

To determine whether glucose had nonselective effects on all ligand gated ion channels in nodose neurons, we also tested the response of the TrpV1 ligand capsaicin (CAP). In contrast to 5-HT, altering glucose concentration did not significantly change CAP-induced inward currents (100 μM) at 10 mM glucose (−1605.8 ± 450.37 pA; CD: −30.31 ± 8.29 pA/pF; Cm: 51.64 ± 6.93 pF; n = 6) and 2 mM glucose (−1187.26 ± 352.65 pA; CD: −20.54 ± 4.61 pA/pF; Cm: 51.55 ± 8.24 pF; n = 8; Fig. 4E).

Glucokinase inhibition blunts 5-HT-induced inward current in dissociated nodose ganglia. Glucokinase has been shown to be required for the effects of glucose on nodose neuronal excitability (17). To determine whether it is also required for glucose effects on 5-HT3R activity, we tested the glucokinase inhibitors; if glucokinase is required, inhibiting it should mimic the effect of lowering glucose concentrations. We tested the effects of the inhibitors in 10 mM glucose concentration, as the 5-HT response is largest at this concentration.

Pretreating neurons with glucosamine (GSM, 100 μM) significantly decreased the amplitude of 5-HT-induced inward currents (−333.48 ± 186.42 pA; n = 6) compared with control (−1237.98 ± 565.79 pA; n = 6) as fold change (one-way ANOVA, Tukey post hoc, P < 0.01; Fig. 5, A and B). Fold change of current density was significantly less in GSM (−8.56 ± 4.46 pA/pF) compared with control (−35.32 ± 18.79 pA/pF; Fig. 5, A and B). These data suggest that glucose actions on 5-HT signaling are dependent on glucokinase ac-

Fig. 2. Effect of glucose on NTS TH-EGFP neurons is mediated by presynaptic glutamate release and the 5-HT3R. A: average AP firing rate from a representative cell-attached recording of a TH-EGFP neuron in lowered glucose concentrations after application of the non-N-methyl-D-aspartate (NMDA) ionotropic glutamate receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX, 50 μM), binned into 60-s periods. B: average fold change of cell-attached AP firing rate in lowered glucose concentrations from 5 mM (1.0 ± 0.5 intrinsic variance), 2 mM (0.5 ± 0.2) 1 mM (0.4 ± 0.2), and after application of NBQX (50 μM) in 1 mM (0.3 ± 0.2); 2 mM (0.1 ± 0.05), 5 mM (0.03 ± 0.02) followed by a 5 mM wash (0.02 ± 0.01; n = 6). C: AP firing rate from a representative cell-attached recording of a TH-EGFP neuron in lowered glucose concentrations with ondansetron (ODT; 0.5 μM) treatment, binned into 60-s periods. D: average fold change of cell-attached AP firing rate in lowered glucose concentrations from 5 mM (1.0 ± 0.3 intrinsic variance), 2 mM (0.4 ± 0.1), 1 mM (0.13 ± 0.04), and after application of ODT (0.5 μM) in 1 mM (0.08 ± 0.02), 2 mM (0.05 ± 0.02), and 5 mM (0.1 ± 0.05) followed by a 5 mM wash (0.3 ± 0.1; n = 6/8). Error bars indicate SE; *P < 0.05 denotes a significant change compared with baseline (aCSF/control), one-way ANOVA.
Fig. 3. 5-HT3R activity on NTS TH-EGFP-positive neurons is blunted in low glucose concentrations. A: representative (single) trace from a TH-EGFP-positive neuron in a coronal brain slice following bath application of the 5-HT3R antagonist ODT (0.5 μM) in 10 mM glucose. Average fold change of sEPSCs frequency (control: 1 ± 0.2 intrinsic variance; ODT: 0.8 ± 0.1; Wash: 0.9 ± 0.1) (B) and change in amplitude (C) following bath application of ODT (0.5 μM) in 10 mM glucose (n = 8) are shown. D: representative (single) trace from a TH-EGFP-positive neuron in a coronal brain slice following bath application of the 5-HT3R antagonist SR57227 (30 μM) in 5 mM and 2 mM glucose. Fold change of sEPSC frequency in 5 mM (5 mM + SR: 4.9 ± 2.4; Wash: 2.9 ± 0.8) and 2 mM glucose concentrations (2 mM + SR; 1.6 ± 0.4; 2 mM Wash: 1.1 ± 0.3) (E) and change in amplitude (F) following bath application of SR57227 (30 μM) in 5 mM (n = 8) compared with 2 mM glucose (n = 6) after SR57227 application in 5 mM compared with 2 mM glucose concentration. Error bars indicate SE. *P < 0.05 denotes a significant change compared with baseline (aCSF/control), one-way ANOVA.

Glucokinase inhibition blunts 5-HT effects on sEPSCs in NTS TH-EGFP-positive neurons. The glucokinase inhibitor GSM blunted the effect of 5-HT-induced currents in nodose ganglion neurons. We next tested whether the effect of glucose involved AMPK activity. As a control we again tested CAP, a TRPV1 agonist, in 10 mM glucose before (~20.45 ± 9.52 pA/pF; ~1091.27 ± 572.0 pA, n = 6) and after pretreatment of GSM (~19.8 ± 8.76 pA/pF; ~1042.62 ± 527.26 pA n = 6; Fig. 5B) and saw no significant change. The decay time was also not significantly different (CAP in control: 36.25 ± 14.48 s, n = 6; CAP in GSM: 31.53 ± 12.93 s, n = 6 cells from 3 cultures; Fig. 5D).

To confirm this finding we also tested another glucokinase inhibitor, alloxan. Pretreating neurons with alloxan (400 μM) for 10 min significantly decreased the amplitude of 5-HT-induced inward currents (~57.06 ± 24.05 pA; n = 5) compared with control (~749.14 ± 211.24 pA; n = 5) as fold change (one-way ANOVA, Tukey post hoc, P < 0.01). 5-HT current density was significantly less with pretreatment of alloxan (~1.07 ± 0.52 pA/pF) compared with control (~13.34 ± 3.63 pA/pF; Fig. 5B). Decay time was also significantly different between control (37.39 ± 15.5 s) and GSM (15.65 ± 6.4 s) or alloxan (17.37 ± 8.44 s) pretreatment preceding local application of 5-HT (Fig. 5D).

K<sub>ATP</sub> channels, but not AMPK, influence 5-HT currents in dissociated nodose ganglion neurons. We next determined whether the effects of glucose involved AMP kinase (AMPK) as AMPK activity is positively correlated with the AMP-to-ATP ratio. As AMPK is activated by low glucose, if AMPK were required, activating it should decrease 5-HT-induced currents. Bath application of the AMPK activator 5-aminomimidazole-4-carboxamide ribonucleotide (AICAR, 1 mM) onto dissociated nodose ganglion neurons did not alter 5-HT-induced currents. Bath application of the AMPK activator 5-aminomimidazole-4-carboxamide ribonucleotide (AICAR, 1 mM) onto dissociated nodose ganglion neurons did not alter 5-HT-induced currents, but 5-HT-induced inward currents (~331.22 ± 111.17 pA) when compared with control (~280.44 ± 86.75 pA; n = 5). There was also no change in 5-HT current density before (~6.73 ± 1.37 pA/pF; Cm: ± 5.63 ± 3.61 pF; n = 5) or after AICAR application (~7.86 ± 1.74 pA/pF; Fig. 5C) or decay time (control: 45.76 ± 10.73 s and AICAR: 42.44 ± 9.7 s; n = 5 cells from 3 cultures; P < 0.05, one-way ANOVA). This suggests that the effect of glucose concentration on 5-HT-induced inward currents is independent of AMPK.

Next, we determined whether ATP-sensitive potassium channel (K<sub>ATP</sub>) channels were involved in the glucose-sensing mechanism. K<sub>ATP</sub> channels are open when glucose concentrations, and thus intracellular ATP levels, are low. The K<sub>ATP</sub> channel activator diazoxide (300 μM), which would be expected to mimic low glucose, significantly decreased the amplitude of the inward current induced by 5-HT by ~40% (Control: ~1217.1 ± 680.48 pA; diazoxide: ~810.92 ± 560.93 pA; 0.54 ± 0.08-fold control; n = 5 from 3 cultures; P < 0.05, one-way ANOVA; Fig. 5C).

As AMPK activity is positively correlated with the AMP-to-ATP ratio, and thus intracellular ATP levels, are low. The K<sub>ATP</sub> channel activator diazoxide (300 μM), which would be expected to mimic low glucose, significantly decreased the amplitude of the inward current induced by 5-HT by ~40% (Control: ~1217.1 ± 680.48 pA; diazoxide: ~810.92 ± 560.93 pA; 0.54 ± 0.08-fold control; n = 5 from 3 cultures; P < 0.05, one-way ANOVA; Fig. 5C).

Glucokinase inhibition blunts 5-HT effects on sEPSCs in NTS TH-EGFP-positive neurons. The glucokinase inhibitor GSM blunted the effect of 5-HT-induced currents in nodose ganglion neurons. We next tested whether this effect was also observed in the brain slice (Fig. 5, A and B). In 5 mM glucose, 5-HT (30 μM) increased control sEPSC frequency by 10.220.33.1 on September 21, 2017 http://ajpregu.physiology.org/ Downloaded from
attenuated the 5-HT-induced increase in sEPSCs frequency (2.97 ± 0.05 pA vs. 3.53 ± 0.96 pA, respectively; n = 7) or without GSM pretreatment (−31.23 ± 3.8 pA vs. −36.86 ± 4.41 pA, respectively, data not shown).

While there was a trend for GSM to inhibit 5-HT effects on sEPSC frequency in 5 mM glucose, which is apparent on the scatter plot, it did not reach statistical significance (Fig. 6A). To increase our ability to detect an inhibition we recorded 5-HT responses in 10 mM glucose as they are larger. In 10 mM glucose 5-HT (30 μM) increased control sEPSC frequency from 4.18 ± 1.21 Hz to 16.64 ± 5.43 Hz (n = 8). GSM treatment (300 μM; 2.43 ± 0.52 Hz) had no effect on control sEPSCs frequency (2.97 ± 1.92 Hz; n = 8), but significantly attenuated the 5-HT-induced increase in sEPSCs frequency (7.38 ± 2.68 Hz; n = 8) when normalized to control (Fig. 6, D and E). sEPSC amplitude was not altered after 5-HT application with (−24.26 ± 2.79 pA vs. −31.51 ± 7.38 pA, respectively) or without GSM pretreatment (−23.93 ± 3.53 pA vs. −25.31 ± 3.96 pA, respectively; Fig. 6F). 5-HT application compared with 5-HT application after pretreatment was significantly decreased in 10 mM glucose (P < 0.05, one-way ANOVA). A comparison of GSM effects in 5 mM versus 10 mM glucose concentrations is shown in Fig. 6, A and B.

**DISCUSSION**

Catecholamine neurons in the NTS are implicated in a broad number of homeostatic functions including the control of food intake, reward, stress, and cardiovascular reflexes (22, 23, 25, 38, 44, 45, 50, 56, 58, 59, 69). Changes in glucose concentration can have a large impact on neuronal activity (27, 64) and also affect homeostatic functions (15). Hindbrain catecholamine neurons are critical for glucoprivic responses and A1/C1 catecholamine neurons respond to changes in glucose (46). However, it was not known whether changes in glucose concentration alter the activity of NTS-CA neurons. Here we report four key new findings. First, the majority of NTS-CA neurons fire more action potentials in high glucose (5 mM) concentrations than in low glucose (1 mM) concentrations. Second, the effect of glucose on action potential firing rate is dependent on glutamate inputs. Third, the effect of glucose on glutamate release requires presynaptic 5-HT3R. Fourth, inhibition of glucokinase mimics the effect of low glucose to inhibit both 5-HT3R-stimulated currents in nodose neurons and glutamate release in the NTS.

**Majority of NTS TH-EGFP neurons are excited in higher glucose concentrations.** Here we show for the first time that glucose concentration has a direct relationship with action potential firing in NTS-CA neurons. The finding that 86% of NTS-CA neurons are excited by high glucose shows a remarkably homogenous response. In comparison, a survey of all NTS neurons (of unidentified phenotype) showed that 35% were excited and 21% inhibited by higher glucose concentrations, with the rest being nonresponsive (36). The response of GABAergic NTS neurons to changes in glucose concentration were also very heterogeneous, with 40% being glucose excited (GE), 33% glucose inhibited (GI), and 27% nonresponsive (7). Our results therefore identify NTS-CA neurons as a unique population of neurons predominately excited in high glucose concentrations. The fact that changes in glucose increase the firing rate of almost all of these neurons provides a mechanism for the coordinated modulation of the majority of downstream targets of NTS-CA neurons, which include multiple brain regions (3, 42, 43, 54, 61, 63, 67). It is possible that the 14% of CA neurons that decrease their firing in higher glucose, or are nonresponsive, represent a specific subpopulation that underlie a discrete function and project to different brain regions than glucose-excitatory CA neurons.
Our finding that NTS-CA neurons decrease their firing rate in lower glucose concentrations is also consistent with the fact that glucoprivation does not increase fos-ir in the majority of A2/C2 neurons, in contrast to the large activation of A1/C1 neurons (48). If A2/C2 neurons are glucose excitatory (albeit indirectly), their activity would be predicted to decrease in a glucoprivic state, and therefore c-fos is unlikely to be activated in these neurons.

Glucose excites NTS-CA neurons by increasing glutamate inputs onto NTS TH-EGFP-positive neurons through the 5-HT3R. Our results show that the effect of glucose concentration on NTS-CA neuronal firing is dependent on glutamate inputs as it is blocked by the ionotropic AMPA/kainate glutamate receptor antagonist NBQX. This suggests that the effects of glucose are indirect on glutamate terminals in the NTS, with higher concentrations of glucose leading to a higher probability of glutamate release. Consistent with this model we found that spontaneous glutamate inputs onto NTS-CA neurons are also dependent on glucose concentration, with glucose inputs being increased in higher glucose. This finding is consistent with previous reports that glucose concentration impacts the frequency of glutamate inputs onto unidentified rat NTS neurons (64) and that the firing rate of NTS-CA neurons is dependent on spontaneous glutamate inputs (10, 12).

The effect of glucose on both NTS-CA neuronal firing rate and spontaneous glutamate inputs is also dependent on 5-HT3Rs as it was blocked by the 5-HT3R antagonist ODT.
The size of the effect of the 5-HT3R agonist SR57227 on sEPSC frequency in NTS-CA neurons was also positively correlated with aCSF glucose concentrations. This suggests that increasing glucose concentrations increases serotonin 5-HT3R signaling in vagal afferent terminals to increase glutamate release onto NTS-CA neurons. Consistent with this we show that the size of the 5-HT3R-mediated current in mouse vagal afferent nodose neurons is dependent on glucose concentration, as has been reported previously for the rat (2). Browning and her colleagues showed that this is likely due to changes in the surface expression of 5-HT3R, as surface expression is dependent on glucose concentration (2, 65). Here we extend those findings to the mouse and identify NTS-CA neurons as a critical downstream population of neurons that are excited by glucose in a 5-HT3R-dependent manner.

NTS neurons receive a basal serotonergic tone onto 5-HT3Rs that contributes to the normal frequency of release of glutamate as ODT application decreases sEPSC frequency in the general population of NTS neurons (65). This effect is positively correlated with aCSF glucose concentrations. Our findings suggest that this basal serotonin tone also exists on afferent inputs specifically onto NTS-CA neurons. One implication of this result is that it suggests that serotonergic terminals release 5-HT onto vagal afferent terminals to control glutamate activation of NTS-CA neurons independently of vagal afferent firing. Furthermore, as this tone is lost in lower glucose concentrations it suggests a mechanism by which changes in glucose can modulate the firing rate of NTS-CA neurons, and presumably the catecholaminergic tone at downstream target nuclei, again independently of changes in vagal afferent firing. Interestingly, we do not see this basal serotonin tone in a horizontal slice preparation, which preserves the connections in the rostral caudal orientation, but doesn’t retain the dorsal ventral connections (12). This suggests that the basal tone of serotonin might originate from the raphe obscurus, which lies ventral to the NTS, is present in the coronal, but not the horizontal slice, and is an endogenous source of 5-HT producing neurons located ventral to the NTS (41). Interestingly, stimulation of the raphe obscurus produces significant 5-HT release into the dorsal vagal complex (DVC; i.e., the NTS and dorsal motor nucleus of the vagus), in fed, but not fasted rats (37, 65). In addition, high-fat diet blunts the ability of glucose to increase 5-HT3R responses (62). Therefore, different energy states could potentially influence excitation of this pathway by altering both the size of serotonin release and activation of 5-HT3Rs at the level of the NTS.

**Glucokinase activity contributes to presynaptic 5-HT signaling.**

Here we report that the reduction in 5-HT-3R function in vagal afferents seen in low glucose is mimicked by two different glucokinase inhibitors. Glucokinase is the enzyme responsible for converting glucose to glucose-6-phosphate and acts as a glucose sensor, shifting cellular function and metabolic processes based on fluctuating glucose concentrations (29). Glucokinase has been shown to respond to the glucose concentrations we use here in other brain regions (4, 13, 16, 17). It is present in nodose ganglia neurons and knockdown of its expression by short hairpin RNAs abolishes glucose excitation of nodose neurons (17). It is also required for glucose sensing by GABAergic neurons in the NTS (7) and its expression decreases following chronic hyperglycemia induced by streptozotocin-induced diabetes (20). Taken together these results suggest that glucokinase provides one mechanism by which vagal afferents “sense” changes in glucose concentration to alter serotonin receptor function. Critically, inhibition of glucokinase did not alter the current mediated by the vallinoid receptor VR1; suggesting the effect is selective to the 5-HT3R and it is not a global effect of the drugs on excitability.

The glucokinase inhibitor GSM also blunted 5-HT3R-mediated glutamate release in the coronal brain slice, suggesting that glucokinase affects 5-HT3R signaling in vagal afferent terminals to modulate glutamate release onto NTS-CA neurons. This effect was most robust at high concentrations of glucose (10 mM), likely because 5-HT3R expression is already decreased in lower glucose concentrations, making it hard to measure a further reduction by glucokinase inhibition.

**$K_{\text{ATP}}$ channel influences 5-HT currents in vagal afferents.**

$K_{\text{ATP}}$ channels, which are inhibited by high levels of intracellular ATP, are also found in the NTS (34, 70) and in nodose ganglia neurons (17). High concentrations of glucose increase intracellular ATP (6) and this has been shown to inhibit $K_{\text{ATP}}$ channels to increase neuronal firing rate in some neurons (14, 17). We found that the $K_{\text{ATP}}$ channel activator diazoxide, which mimics low glucose concentrations and activates $K_{\text{ATP}}$ channels, significantly decreased the 5-HT-induced inward currents even in 10 mM glucose concentrations. This suggests that $K_{\text{ATP}}$ channels also play a role in modulating 5-HT currents in vagal afferents. $K_{\text{ATP}}$ channels have previously been shown to be important for glucose-induced firing of gastric afferents (16, 17) and for mediating the GE effect in an unidentified subpopulation of caudal NTS neurons (14) as well as the GE response of medial NTS GABA neurons (7). Our data suggests that at least some of these GE neurons could be NTS-CA neurons that respond to changes in glucose concentration.

Our predicted model is that in high glucose, a combination of activation of glucokinase and inhibition of $K_{\text{ATP}}$ channels depolarizes vagal afferent terminals and increases glutamate

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**Fig. 7. A model of how different glucose concentrations impact NTS-CA neuronal activity.** Our findings suggest that changes in glucose concentration alter presynaptic vagal afferent glutamate release onto NTS-CA neurons through modulation of the 5-HT-R. In high glucose concentrations (bottom), 5-HT-R activity increases glutamate release onto NTS-CA neurons, which increases action potential firing in these neurons. Low glucose concentrations (top) decrease 5-HT-R activity and blunt the effects of 5-HT on NTS-CA neuron activity. Glucokinase (GK) appears to be important for the effects of changes in glucose.
release onto NTS-CA neurons. In contrast, we did not find evidence that AMPK, whose activity is also regulated by changes in the AMP-to-ATP ratio in neurons, plays a role in the expression of the 5-HT3R on nodose ganglion neurons. This is contrary to other neuronal populations, including in the hypothalamus, hippocampus, and hindbrain, where AMPK has been shown to be critical (5, 39, 51). However, this finding is consistent with the finding that glucoprivation does not activate AMPK in A2/C2 neurons, in contrast to the A1/C1 neurons (33).

Physiological implications. NTS-CA neurons have been widely implicated in the control of homeostatic functions, including the control of food intake. The finding that alterations in glucose concentrations impacts NTS-CA neuronal activity identifies one potential mechanism by which changes in glucose levels can modulate feeding behavior (4, 46, 49, 52). Brain glucose levels don’t fluctuate as much as blood glucose; with brain glucose concentrations reported to range from ~0.2 mM in a state of hypoglycemia to ~4 mM in a state of hyperglycemia, with normoglycemic levels at ~2.5 mM in rats (51, 57). In contrast, blood glucose concentrations in rats can range from ~3 mM to ~16 mM (51, 57). For our brain slice studies we see changes in glutamate inputs and NTS-CA neuronal activity with changes in glucose concentrations from 5 mM to 2 mM, within these physiological ranges. In addition, increasing the glucose concentration to 10 mM further increases the size of the 5-HT3R response (as was also shown by Refs. 2 and 64); suggesting that if the NTS sees higher glucose concentrations the neurons will further increase their firing rate. Our data suggests that hyperglycemia will increase the effect of serotonin to increase NTS-CA neuronal firing rate through increased glutamate inputs, presumably leading to increased transmitter release at their downstream targets. In contrast, during low-to-normal glucose conditions this drive will be blunted and the activity (firing rate) of these neurons is likely to be lower. The exact concentration of glucose that NTS-CA neurons are exposed to, in vivo is not known. The NTS lies directly below the area postrema, a circumventricular organ that lies outside of the blood brain barrier (BBB) and portions of the medial/commissural NTS have been reported to contain some fenestrated capillaries (19, 35). If NTS-CA neurons are exposed to glucose concentrations closer to blood glucose levels, then there is likely a serotonergic drive that increases glutamate inputs onto these neurons, even during normal glucose concentrations. However, parts of the NTS are not exposed to the BBB and likely see glucose concentrations closer to those in other brain regions. The nodose ganglia, which contain the cell bodies of vagal afferents, lie outside the BBB and are therefore exposed to blood glucose concentrations. It is possible that prolonged high blood glucose could influence the long-term expression of 5-HT3Rs on vagal afferents.

Perspectives and Significance

In summary, we show that the firing rate of NTS-CA neurons is impacted by changes in glucose concentration, with their firing rate being higher in higher glucose concentrations. We show that this is an indirect (presynaptic) mechanism through potentiation of 5-HT3R activation of vagal afferent terminals to increase glutamate release. Additionally, we show that glucose effects on 5-HT3R function are dependent on glucokinase activity and KATP channels and are independent of AMP kinase. Blood and brain glucose concentrations are altered in hypo- and hyperglycemia. Given the crucial role of NTS-CA neurons in the control of food intake, stress responses, and motivational behaviors, these studies identify a potential mechanism by which high glucose concentrations could influence these behaviors (Fig. 7). Thus they contribute to our understanding of how glucose can impact neuronal circuits involved in the control of food intake and the mechanisms by which physiological glucose levels can influence neuronal activity.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

B.L.R., M.Z., and S.M.A conceived and designed the experiments; B.L.R., M.Z., H.Z., and C.D. performed experiments; B.L.R. and M.Z. analyzed data; B.L.R. and S.M.A. interpreted results of experiments; B.L.R. prepared figures; B.L.R. and S.M.A. drafted manuscript; B.L.R. and S.M.A. edited and revised manuscript; B.L.R., M.Z., H.Z., C.D., and S.M.A. approved final version of manuscript.

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