A voltage-dependent depolarization induced by low external glucose in neurons of the nucleus of the tractus solitarius of rats: interaction with K_{ATP} channels regulated by external glucose.

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Abstract

The brain demands constant supply of glucose-derived energy for cell metabolism and survival, and brain hypoglycemia may compromise synaptic functions. The brainstem nucleus of the tractus solitarius (NTS) is an integrative center for autonomic counterregulatory responses to hypoglycemia, and recent evidences have demonstrated that NTS neurons can also sense fluctuations in glucose levels in the extracellular milieu. Glucose-sensing neurons rely on glucose metabolism to respond to changes in glucose availability, but the ionic mechanisms underlying the low-glucose sensitivity are not well elucidated in NTS neurons. Therefore, this work aimed to investigate the effect of low extracellular glucose (0.5 mM) on the electrophysiological properties of NTS neurons of rats, by whole-cell patch-clamp. We showed that in NTS neurons maintained in 5 mM extracellular glucose, low glucose induces a depolarization in most neurons, which was correlated with membrane potential, decreased with depolarization. ATP-sensitive potassium (K_{ATP}) channels contribute to the hyperpolarized resting membrane potential (RMP) of NTS neurons, and blockage of K_{ATP} channels produced depolarization of the membrane and occludes the effect of low glucose, thus acting as modulators of low-glucose sensing in NTS neurons. However, this effect is caused by depolarization, since membrane hyperpolarization after K_{ATP} blockage restores the effect of low glucose. Additionally, we demonstrated that the incubation of NTS neurons in high glucose (10 mM) prior to recordings leads to more depolarized RMP in these neurons, what contributes to increase the number of neurons unresponsive to a low-glucose challenge, possibly via depolarization by inhibition of K_{ATP} channels following increased amounts of high glucose-derived intracellular ATP. We conclude that NTS neurons depolarize the membrane in response to the application of a low-glucose solution, but this effect is occluded by membrane depolarization triggered by K_{ATP} blockage. This suggests a homeostatic regulation of the membrane potential by glucose.

Keywords: Glucose-sensing neurons, Glucose metabolism, ATP-sensitive potassium channels, Nucleus of the tractus solitarius.

Introduction

The brain primarily relies on constant glucose supply to meet its energy demand for sustaining cell metabolism and functioning (Mergenthaler *et al.*, 2013). Following uptake at the blood-brain barrier, glucose enters brain cells via facilitated diffusion by glucose transporters (GLUTs) to be subsequent phosphorylated by hexokinase to produce glucose-6-phosphate, which can be driven to different metabolic routes: (1) mainly glycolysis, leading to lactate production or mitochondria metabolism to generate energy; (2) pentose phosphate pathway, generating power for biosynthesis and antioxidation, and; (3) glycogenesis (particularly in astrocytes), storing glucose in the form of glycogen to be mobilized when needed (Dienel, 2012). Due to high levels of energy expenditure for neuronal activity and low content of brain glycogen, the human brain consumes up to 20% of the glucose-derived energy under physiological conditions (Magistretti & Allaman, 2015). Brain hypoglycemia, a condition of limited energy availability, can cause neuronal death and may lead to cognitive impairments and conscience loss (Cryer, 2007). Therefore, several peripheral and central components act on energy homeostasis regulation in order to maintain adequate levels of circulating glucose (Marty *et al.*, 2007; Verberne *et al.*, 2014).

The brainstem of the nucleus of the tractus solitarius (NTS) is a key integrative center for glucose homeostasis regulation. The NTS is the primary central site for viscerosensory afferent fibers arising from peripheral glucose-sensing neurons (Marty *et al.*, 2007; Donovan & Watts, 2014). Taste receptors in sensory neurons located in tongue and oral cavity activates NTS neurons following glucose consumption (Oliveira-Maia *et al.*, 2011). Hypoglycemia induces c-Fos immunoreactivity in NTS neurons, and portal-mesenteric deafferentation suppressed this effect (Bohland *et al.*, 2014). Additionally, low glucose increases firing activity in carotid sinus nerve (Gao *et al.*, 2014), which mostly propagates signals into the NTS. NTS neurons are also connected within a neural circuitry involving the corticolimbic system, and densely project to the dorsal motor nucleus of the vagus, where the cell bodies of the preganglionar parasympathetic branch of the vagus are located (Marty *et al.*, 2007; Verberne *et al.*, 2014). Furthermore, local nutrient and metabolic signals (including glucose and metabolites) in the NTS, as well as direct modulation by receptor agonists/antagonists in NTS neurons, can also induce physiological responses regarding blood glucose regulation (Ritter *et al.*, 2000; Lam *et al.*, 2010; Zhao *et al.*, 2012). Therefore, in response to food ingestion and signals related to glucose levels, NTS neurons integrate a variety of synaptic inputs and mediate a plethora of autonomic counter regulatory mechanisms driven to ensure adequate levels of glucose, including feeding behavior, gastric motility, and hormonal secretion (Marty *et al.*, 2007; Donovan & Watts, 2014; Hermann *et al.*, 2014).

Several reports have highlighted the ability of NTS neurons to detect fluctuations in glucose levels in the extracellular milieu (McDougal *et al.*, 2013; Lamy *et al.*, 2014; Roberts *et al.*, 2017), as do peripheral glucose-sensing neurons mentioned above. These neurons respond to changes in glucose level by altering its electrical excitability. Glucose-excited neurons increase firing activity in response to an increase in glucose levels, while others neurons increase firing activity when glucose levels drop, namely glucose-inhibited neurons. Both subtypes of glucose-sensing neurons are found in the NTS, and mostly neurons rely on glucose metabolism for their glucose sensitivity (Thorens, 2012). Inhibition of glucokinase activity suppresses the response of NTS neurons to changes in extracellular glucose levels (Boychuk *et al.*, 2015; Roberts *et al.*, 2017). Additionally, AMPK is required for low-glucose sensing in GLUT2-expressing NTS neurons, and high amounts of intracellular glucose blunts this response (Lamy *et al.*, 2014). These findings demonstrate that intracellular molecules acting on glucose metabolism as well as glucose-derived metabolites are used as signals to control firing activity in glucose-sensing neurons.

A significant component that links metabolic status and electrical excitability is the ATP-sensitive potassium (K_{ATP}) channel (Nichols, 2006), as better described in pancreatic beta-cells (Ashcroft & Rorsman, 2013). This channel is a hetero-octameric complex comprised of four pore-forming Kir6.x subunits and four regulatory sulfonylurea receptor (SURx) subunits, and it conducts an inwardly rectifying potassium current that is inhibited by ATP binding to Kir6.x subunits and stimulated by ADP interaction with nucleotide-binding sites within SURx subunits, according to the ATP:ADP ratio (Hibino *et al.*, 2010). In the NTS, the subunits Kir6.2 and SUR1 are expressed in glucose-sensing neurons (Balfour *et al.*, 2006; Halmos *et al.*, 2015). Glucose-excited NTS neurons indeed appear to respond to changes in glucose levels using a mechanism similar to pancreatic beta-cells, in a GLUT/glucokinase/ K_{ATP} channels system (Thorens, 2012; Ashcroft & Rorsman, 2013), since K_{ATP} channel antagonists blunts the responsive of these neurons to increased glucose levels (Balfour *et al.*, 2006; Boychuk *et al.*, 2015), mimicking the effect of high content of intracellular ATP levels. On the other hand, the ionic mechanisms of response in neurons activated by low glucose are more heterogeneous, and the role of K_{ATP} channels is less known. Therefore, we aimed to investigate the effect of a low external glucose (0.5 mM) on the membrane properties of NTS neurons of rats, and its interaction with K_{ATP} channels.

Materials and Methods

Brainstem slices preparation

Animal procedures were performed according to protocol approved by the Committee on Ethics in Animal Experimentation (CEUA) from the School of Medicine of Ribeirão Preto, University of São Paulo (protocol # 149/2015). Male Wistar rats (3- to 11-week-old) were decapitated following isoflurane anesthesia, and the brainstem quickly removed and placed in a dish

containing ice-cold artificial cerebrospinal fluid (aCSF) modified for slicing, containing (in mM): 87 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 75 sucrose, 25 D-glucose, 0.2 $CaCl_2$, and 7 $MgCl_2$ [330 mOsm/kg H_2O, pH 7.4 when bubbled with carbogenic mixture (95% O_2 and 5% CO_2)]. The specimen was glued to the sectioning stage, and submerged in ice-cold slicing aCSF, in the vibratome´s (1000 Plus, Vibratome) chamber. Then, three to four coronal brainstem slices (250 µM) containing the NTS near the level of the area postrema (i.e., ± 500 µm rostral and caudal) were sectioned and incubated at 32 – 33ºC for 45 min in aCSF, containing (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 5 D-glucose, 2 $CaCl_2$, and 1 $MgCl_2$ [298 mOsm/kg H_2O, pH 7.35 when bubbled with carbogenic mixture (95% O_2 and 5% CO_2)]. In some experiments, the aCSF contained 10 mM glucose. After this period, the slices were stored into the same solution at room temperature until use for electrophysiological recordings. Low-glucose (0.5 mM) aCSF, as well as 10 mM glucose aCSF, were made using equimolar amouts of NaCl or sucrose to maintain osmolality equal of the 5 mM glucose aCSF.

Electrophysiological recordings

Single brainstem slices were transferred to a chamber mounted on a stage of an upright microscope (BX51WI; Olympus, Japan), and continuously perfused with aCSF at 30 – 33ºC using an inline heating system (TC-324B; Warner Instruments, Hamden, CT, USA) at a rate of ~2 mL/min using a gravity-driven perfusion system. Neurons were then visualized under DIC optics with a 60 x immersion objective, and patched with eletctrodes made with thick-walled borosilicate glass (BF150-86-10; Sutter Instruments, Novato, CA, USA) pulled using a horizontal puller (P-87; Sutter Instruments). The electrodes were filled with a internal solution, containing (in mM): 128 K-gluconate, 8 KCl, 10 HEPES, 0.5 EGTA, 4 Mg_2ATP, 0.3 Na_2GTP, and 10 Na-phosphocreatine (295 mOsm/kg H_2O, pH 7.3), resulting in pipette tip resistance between 3 and 7 MΩ.

Whole-cell patch-clamp recordings were performed with an EPC-10 patch-clamp amplifier (HEKA Eletronik, Lambrecht, Germany) using the PatchMaster acquisition software (HEKA Eletronik), in current-clamp mode. Data was acquired at 20 kHz and low-pass filtered at 5 kHz (Bessel). Cells with series resistance larger than 30 MΩ or showing large variations during whole-cell recording were discarded. After entering in whole-cell configuration, neurons were allowed to stabilize for ~10 min. Membrane potential was monitored for 10 min in aCSF with 5 or 10 mM glucose prior to switch to low glucose (0.5 mM) solution, or with drug, for 10 min before returning to 5 or 10 mM glucose aCSF. Hyperpolarizing pulses (ranging from -30 to -60 pA; 1000 ms) were applied every 15 seconds to monitor input resistance and membrane time constant. All conditions were recorded during 10 min, and we just recorded one neuron per slice. Based on previous reports regarding glucose sensing in NTS neurons (Balfour *et al.*, 2006; Lamy *et al.*, 2014; Boychuk *et al.*, 2015), and the response profile to a low-glucose challenge observed in the current investigation, we assumed that a neuron was responsive to low glucose when they showed a clear membrane depolarization above the oscilation of the baseline induced by low-glucose solution and/or drug. Thus, we observed that neurons considered responsive presented a depolarization of at least 3 mV.

Drugs

Tetrodotoxin (0.3 – 0.5 µM; Alomone Labs, Jerusalen, Israel) was used to block voltage-dependent sodium channels. Tolbutamide (100 µM; Sigma, St. Louis, MO, USA) was used to block ATP-sensitive potassium channels.

Data Analysis

Electrophysiological data were analyzed with IGOR Pro 6.37 (WaveMetrics, Portland, OR, USA), MiniAnalysis 6.0 (Synaptosoft, Fort Lee, NJ, USA), and ClampFit 10.6 (Molecular Devices, Sunnyvale, CA, USA). Voltages were corrected for measured liquid junction potential of +10 mV. Data are showed as mean ± SEM. All data values were determined after obtained a plateau response. Resting membrane potential was analyzed as the mean of all points histogram of the recording taken in a segment of the last 3 - 4 min. Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA), conducted with paired and unpaired two-tailed t-test, and one-way repeated measures ANOVA with Fisher's LSD test. Correlations were determined using a linear regression. Significance level was set at $p < 0.05$.

Results

Most NTS neurons depolarize in response to low-glucose aCSF

Lamy *et al.* (2014) reported that GLUT2-expressing GABAergic neurons in the NTS of mice responded to solution simulating hypoglycemic conditions by depolarization of the resting membrane potential (RMP). We tested if the same effect could be observed in non-identified NTS neurons. Perfusion of unidentified NTS neurons (n = 37, 23 animals) with a low-glucose (0.5 mM) solution resulted in three types of neuronal responses (according to described in Materials and Methods section). In 30 cells (81%), the RMP was depolarized by 9.3 ± 1.0 mV, from a mean of -74.3 ± 1.6 mV to -65.0 ± 1.7 mV in low glucose ($p < 0.0001$; **Figure 1A**), and took on average 382.7 ± 18.4 s to peak. This effect was reversible in most neurons (70%), and the RMP after returning to 5 mM glucose was -71.3 ± 1.9 mV ($p < 0.0001$).

Depolarization induced by low glucose triggered action potential (AP) firing in 16 of 28 silent neurons (57%; 0.6 ± 0.2 Hz, $p = 0.0069$), and an increase in AP frequency in the only two active neurons (from a mean of 3.3 ± 2.7 Hz to 8.4 ± 2.0 Hz in low glucose; $p = 0.4696$). Additionally, a single cell (3%) was hyperpolarized by -8 mV during perfusion with low-glucose solution, from -60 mV to -68 mV, and this effect was partially reversed

(~40%) on reinstatement of 5 mM glucose. A third subgroup, composed by six neurons (16%), was considered unresponsive to low glucose (1.0 ± 0.7 mV), from a mean RMP of -64.1 ± 2.0 mV to -63.1 ± 1.6 mV ($p = 0.2163$; **Figure 1B**).

To test the dependence on activation of voltage-gated sodium channels for low-glucose sensing, we performed the low-glucose challenge in the presence of tetrodotoxin (TTX; 0.3 – 0.5 μM) in a second set of NTS neurons (n = 23 cells, 11 animals). Again, three types of responses were identified in lowered glucose, such as mentioned above. Fourteen cells (61%) were depolarized in low glucose by 8.8 ± 1.0 mV, from a mean of -79.1 ± 1.6 mV to -70.3 ± 1.3 mV ($p < 0.0001$; **Figures 2A***i* and **2A***ii*), and took, on average, 348.6 ± 18.7 s to peak. This effect was reversible in most neurons (71%), and the RMP after returning to 5 mM glucose was -77.0 ± 1.8 mV ($p < 0.0001$). A single cell (4%) was hyperpolarized by -5.5 mV, from -72.1 mV to -77.6 mV in low glucose, and this effect was reverted (~65%) after returning to 5 mM glucose. Lastly, eight cells (35%) were unresponsive to low glucose solution (1.1 ± 0.5 mV; from a RMP of -66.9 ± 1.6 mV to -65.8 ± 1.7 mV; $p = 0.0667$; **Figures 2B***i* and **2B***ii*). Both depolarizing effect and latency to peak response were not different from what was observed in experiments conducted with no TTX ($p = 0.7537$, and $p = 0.2608$, respectively), and as in normal aCSF most neurons depolarized in low-glucose solution (84% in normal aCSF, and 65% in TTX). We conclude that the depolarization triggered by low glucose is not dependent on voltage-gated sodium channels. Most of the experiments shown from now on were performed in the presence of TTX.

Lamy *et al*. (2014) found that the membrane depolarization induced by low glucose in GABAergic NTS neurons was accompanied by an increase in the membrane input resistance (R_{input}) caused by inhibition of a potassium leak conductance. Surprisingly, we verified that NTS neurons depolarized by low glucose showed a significant decrease in R_{input} (-101.1 ± 37.8 MΩ, from a mean of 440.3 ± 61.6 MΩ to 339.2 ± 40.0 MΩ in low glucose; $p = 0.0192$; **Figure 2A***iii*), but a non-significant decrease in τ_m (-4.6 ± 2.7 ms, from a mean of 18.6 ± 3.8 ms to 14.0 ± 2.0 ms in low glucose; $p = 0.1302$). Differently from the observed with the membrane potential, these effects did not revert on reinstatement of 5 mM glucose aCSF ($p = 0.7589$, and $p = 0.8995$, respectively). Additionally, neurons unresponsive to low glucose showed a decrease in both R_{input} (-80.3 ± 13.2 MΩ, from a mean of 522.9 ± 71.1 MΩ to 442.6 ± 61.6 MΩ in low glucose; $p = 0.0005$; **Figure 2B***iii*) and τ_m (-3.2 ± 1.0 ms, from a mean of 21.9 ± 1.9 ms to 18.7 ± 1.8 ms in low glucose; $p = 0.0126$), suggesting that the decrease in R_{input} caused by low-glucose solution is not related to its effect on RMP. Like in responsive neurons, both R_{input} and τ_m did not revert after returning to 5 mM glucose aCSF ($p = 0.5205$, and $p = 0.9604$, respectively) in non-responsive cells.

In order to understand the relationship between the effect on membrane resistance and depolarization by low glucose, we correlated the effect of low glucose on RMP with the effects on R_{input} and τ_m. Intriguingly, we found an inverse weak correlation of low glucose effect on RMP and R_{input} ($r^2 = 0.1815$, $p = 0.0427$; **Figure 3A***ii*), and a not significant correlation with τ_m ($r^2 = 0.1535$, $p = 0.1199$; **Figure 3A***iii*), indicating that both decreases in R_{input} and τ_m are not casually related to the low glucose-induced membrane depolarization. However, the depolarization caused by low glucose was strongly correlated with neuronal RMP ($r^2 = 0.5272$, $p < 0.0001$; **Figure 3A***i*), with the more negative the RMP, the greater the membrane potential change in response to low glucose. We then compared the RMP between responsive and non-responsive neurons, and found that the RMP of non-responsive cells were significantly more depolarized than responsive cells (-66.9 ± 1.6 mV vs. -78.6 ± 1.6 mV, respectively; $p = 0.0001$; **Figure 3B**).

Inhibition of K_{ATP} channels depolarize RMP in most NTS neurons

K_{ATP} channels are traditionally associated to coupling cell energy status and electrical activity, triggering depolarization in high ATP/ADP ratio, and hyperpolarization in low ATP/ADP ratio (Nichols, 2006; Hibino *et al.*, 2010). NTS neurons express K_{ATP} channels, which are more responsive to metabolic ATP than to ATP provided by the whole-cell pipette solution (Balfour *et al.*, 2006). Curiously, our findings of low glucose-induced depolarization in NTS neurons contrast with what is expected from the effect caused by reducing extracellular glucose, which should lead to a drop in metabolic ATP levels, increased K_{ATP} conductance, and cell membrane hyperpolarization. Thus, maybe the ATP (4 mM) present in the pipette solution of our experiments could be blocking K_{ATP} channels in our neurons.

We then tested if the K_{ATP} channels in the NTS neurons were active, by the application of their antagonist tolbutamide (100 μM; n = 15, seven animals). We verified that in 10 neurons (67%), tolbutamide triggered a strong and fast depolarization (16.7 ± 2.7 mV, from a mean of -76.1 ± 2.6 mV to -59.4 ± 1.2 mV; $p = 0.0002$; **Figures 4A***i* and **4A***ii*), accompanied with robust increased R_{input} (190.5 ± 55.2 MΩ, from a mean of 406.9 ± 79.2 MΩ to 597.4 ± 73.4 MΩ; $p = 0.0072$; **Figure 4A***iii*), in accordance to its effect in blocking K_{ATP} channels. Additionally, five neurons (33%) did not change their RMP in response to tolbutamide (1.0 ± 0.5 mV, from a mean of -65.1 ± 2.4 mV to -64.1 ± 2.2 mV; $p = 0.1168$; **Figures 4B***i* and **4B***ii*) as well as R_{input} changes (14.8 ± 11.0 MΩ, from a mean of 525.8 ± 121.8 MΩ to 540.6 ± 129.1 MΩ; $p = 0.2501$; **Figure 4B***iii*). Interestingly, similar to what found in low-glucose aCSF, we verified that neurons unresponsive to tolbutamide were significantly more depolarized than neurons responsive to tolbutamide ($p = 0.0188$; **Figure 4C**). Therefore, we conclude that NTS neurons present active K_{ATP} channels, which can control RMP. Because tolbutamide-insensitive neurons had more depolarized RMP, it is possible that K_{ATP} channels are already closed by endogenous ATP in these neurons.

Depolarization by blocking of K_{ATP} channels inhibits the effect of low-glucose aCSF

We showed that NTS neurons have K_{ATP} channels opened at rest, and that these channels can strongly affect RMP if inhibited, as demonstrated by the depolarization triggered by its antagonist tolbutamide. Additionally, we found that perfusion with low glucose aCSF depolarized the RMP of these neurons, inconsistent with increased activation of K_{ATP} channels by reduced metabolic ATP. It is surprising that two opposite signals produce a similar effect. It is possible that low-glucose aCSF activates glycogen degradation in astrocytes, leading to lactate production which would be transported to neurons and enter the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (Nortley & Attwell, 2017), producing ATP and blocking K_{ATP} channels. To test this hypothesis we measured the effect of low-glucose aCSF after the application of tolbutamide to see if the effect of low glucose could be occluded by K_{ATP} blockage.

First, we tested the responsiveness of the neuron to low glucose, washed it, and then applied tolbutamide. We found that all neurons responsive to low glucose were responsive to tolbutamide (**Figures 5A** and **5B***i*). Low glucose reversibly depolarized the RMP by 9.1 ± 2.2 mV (p = 0.0141; n = 5), and subsequent application of tolbutamide was able to depolarize the RMP by 18.5 ± 3.0 mV (p = 0.0036), showing that neurons responsive to low glucose have K_{ATP} channels opened at rest. In these neurons, application of low glucose aCSF in the presence of tolbutamide induced only a small, but significant, membrane depolarization (2.0 ± 0.3 mV, p = 0.0019), which was smaller than the depolarizing response before tolbutamide application (p = 0.0274; **Figure 5B***ii*).

This result could lead to the conclusion that the effect induced by low glucose could be mediated by K_{ATP} channels. However, since the effect of low-glucose aCSF was dependent on the membrane potential, we tested if this response was indeed caused by neuronal depolarization. We then repeated this protocol, but hyperpolarized the neurons by injecting DC current after the application of tolbutamide, and tested the effect of low-glucose aCSF (n = 5; **Figure 5C***i*). Again, we found that the membrane potential is depolarized by both low glucose (4.4 ± 0.7 mV, p = 0.0029) and tolbutamide (12.2 ± 2.8 mV, p = 0.0119). Next, the injection of -30 to -50 pA of DC current led the membrane potential from a mean of -60.5 ± 0.8 mV to -76.9 ± 1.3 mV, and in this condition, low-glucose aCSF was able to depolarize the membrane of NTS neurons (6.0 ± 2.4 mV, p = 0.0678). This depolarization was similar to the observed in the absence of tolbutamide (p = 0.6346; **Figure 5C***ii*), showing that tolbutamide is not able to occlude the effect of low glucose if the membrane is hyperpolarized to values similar to the RMP. Interestingly, all neurons unresponsive to low glucose aCSF (0.6 ± 0.5 mV, p = 0.2960; n = 5) were also not responsive to tolbutamide (1.0 ± 0.5 mV, p = 0.1168; **Figure 5D**). These neurons also had more depolarized RMP in comparison to responsive neurons (-65.7 ± 1.9 mV vs. -73.1 ± 1.9 mV, respectively; p = 0.0304; **Figure 5E**), showing that responses to both low glucose and tolbutamide are regulated by membrane potential.

Incubation with 10 mM glucose aCSF increases RMP and reduces the number of neurons responsive to low glucose and tolbutamide

We then asked if this depolarizing effect could be sensitive to the initial concentration of glucose, i.e., if starting from a higher glucose concentration could induce higher changes in membrane potential. Additionally, we intended to know if the membrane potential could be sensitive to external glucose, by producing more metabolic ATP and consequently blocking of K_{ATP} channels. For this, the slices were incubated in an aCSF solution containing twice the concentration of glucose than we used (i.e., 10 mM glucose). We found that NTS neurons incubated in high glucose aCSF (n = 12, eight animals) had more depolarized RMP than neurons incubated in 5 mM glucose aCSF (-68.7 ± 2.1 mV vs. -74.5 ± 1.7, respectively; p = 0.0438; **Figure 6G***i*), and increased latency for peak response (435.0 ± 38.6 ms vs. 348,6 ± 18.7 ms in 5 mM glucose aCSF, **Figure 6D**). Of all tested neurons, four cells (33%) were reversible depolarized by 11.2 ± 0.8 mV in response to changing the solution to a low-glucose aCSF (from -74.0 ± 3.7 mV to -62.8 ± 3.3 mV (p = 0.0008; **Figure 6B**), and took 435.0 ± 38.6 s to peak. After washout, the RMP returned to -72.7 ± 5.3 mV (p = 0.0583). These neurons were also strongly responsive to tolbutamide (depolarization of 18.4 ± 1.6 mV, p = 0.0074; n = 3), and similar depolarizations were triggered by both low glucose (**Figure 6G***ii*) and tolbutamide in comparison to what observed in 5 mM glucose (p = 0.2117, and p = 0.4919, respectively). Low-glucose solution was able to depolarize NTS neurons following K_{ATP} occlusion and membrane hyperpolarization (2.8 ± 3.2 mV, p = 0.4748; **Figure 6B**). On the other hand, eight neurons (67%) were considered unresponsive to low glucose, although they produced a small significantly depolarization of 1.4 ± 0.5 mV, from a mean of -66.0 ± 2.2 mV to -64.7 ± 2.0 mV (p = 0.0286; **Figure 6C**), and had more depolarized RMP compared to the responsive cells. (p = 0.0764; **Figure 6E**). Additionally, tolbutamide was applied in four of these cells and did not induce significant membrane potential change (0.8 ± 0.4 mV, p = 0.0804). Like in neurons incubated with 5 mM glucose, we found a positive correlation between the effect of low glucose and RMP (r^2 = 0.3840, p = 0.0316; **Figure 6F**).

We conclude that incubating NTS neurons in a high glucose aCSF decreases the number of low glucose-responsive neurons, probably due to RMP depolarization, but did not change the magnitude of the response to low glucose or tolbutamide. We conclude that the depolarizing effect is independent of the magnitude change in external glucose, being related to the presence of low-glucose aCSF only. The more depolarized RMP of these neurons suggests that the higher extracellular glucose can increase metabolic ATP, which blocks K_{ATP} channels and depolarize neuronal membrane.

Discussion

Glucose is the primary energy source for brain metabolism and survival, and brain hypoglycemia may short-term compromise neural functions (Cryer, 2007; Mergenthaler *et al.*, 2013). Recent evidences have demonstrated that glucose-sensing neurons located in the brainstem nucleus of the tractus solitarius (NTS) can sense glucose levels in the extracelullar milieu (Balfour *et al.*, 2006; Lamy *et al.*, 2014; Roberts *et al.*, 2017), but the electrophysiological mechanisms underlying the low-glucose response are not well elucidated. In the current investigation, we showed that low glucose (0.5 mM glucose aCSF) induces a voltage-dependent depolarization in most NTS neurons of rats, and ATP-sensitive potassium (K_{ATP}) channels contribute to establish the resting membrane potential (RMP) of neurons, thus acting as modulators of low-glucose sensing in NTS neurons. Additionally, we showed that the more depolarized RMP in NTS neurons incubated in high glucose (10 mM glucose aCSF) could contribute to the increased number of neurons unresponsive to a low-glucose challenge, possibly via inhibition of K_{ATP} channels induced by increased levels of glucose-derived intracellular ATP.

Glucose-sensing neurons in the NTS depends on glucose metabolism for their glucose sensitivity, and K_{ATP} channels mostly underlie the activation of glucose-excited neurons (Balfour *et al.*, 2006; Boychuk *et al.*, 2015; Halmos *et al.*, 2015). Since these channels closes when ATP levels increases (Hibino *et al.*, 2010), this mechanism may explain why we observed that NTS neurons incubated (for at least 90 min) in high-glucose aCSF (10 mM) had more depolarized RMP in comparison to normal aCSF (5 mM glucose). The period of NTS neurons exposed to a solution containing high amounts of glucose led to a proportional increase in its metabolism and subsequent increased intracellular ATP levels, causing inhibition of K_{ATP} channels, and thus establishing more positive RMP in these cells. Therefore, we expected that the depolarization of the RMP induced by the incubation of slices in high glucose could lead to an increase in the number of neurons unresponsive to a low-glucose challenge, since our findings demonstrated a voltage-dependent depolarization by low glucose (i.e., the more negative the RMP, the greater the membrane potential response amplitude). This effect was indeed observed. Neurons unresponsive to low glucose accounted for 35% when incubated in normal aCSF, but ~70% when incubated in high-glucose aCSF. Interestingly, Balfour *et al*. (2006) reported that ~80% of NTS/DMX neurons of rats were unresponsive to glucose removal, however these authors performed the electrophysiological recordings using a control aCSF with 10 mM glucose, supporting that K_{ATP} channels are probably already closed in NTS neurons incubated in high glucose, what cause depolarization of the RMP, and could contribute to increase the number of cells that do not respond to a low glucose challenge, as we demonstrated in the current work. These authors also showed the expression of the K_{ATP} channel subunit SUR1 in cells that did not respond to low glucose, what suggest that in these neurons K_{ATP} channels are present but could be saturated by high glucose-derived ATP levels. On the other hand, K_{ATP} channels are inwardly rectifying, conducting less potassium current at potentials positive to equilibrium potential of potassium (Hibino *et al.*, 2010). It means that the insensitivity to tolbutamide of neurons incubated in high glucose could be caused by the more positive RMP indeed, which could be the result of another factor related to the higher basal glucose concentration.

Low extracellular glucose induce K_{ATP} channels to open following a decrease in intracellular ATP levels (Hibino *et al.*, 2010), but we observed a hyperpolarization response to low glucose in only two of 60 NTS neurons incubated in 5 mM glucose aCSF. Other groups have reported that the sensitivity to glucose is not observed in all NTS neurons expressing K_{ATP} channel subunits (Dallaporta *et al.*, 2000; Balfour *et al.*, 2006). Our findings demonstrate that most NTS neurons exhibit a voltage-dependent depolarization and decreased input resistance in response to a low-glucose solution, but these effects were not correlated. These findings together suggest that the depolarizing effect might be generated by more than one conductance. Balfour *et al*. (2007) showed the opening of an inwardly rectifying current in some neurons of the NTS in response to glucose removal, what could contribute to the depolarizing response seen in our recordings. In the ventromedial hypothalamus, low glucose lead to the closure of chloride channels and membrane depolarization in some neurons (Routh *et al.*, 2014), although in a subset of GLUT2-expressing NTS neurons this mechanism was not involved (Lamy *et al.*, 2014). Other ionic mechanisms than ion channels can contribute to depolarize neurons under low-glucose availability. The Na^+/K^+-ATPase pumps are important generators of electrochemical gradients in cells. High-glucose levels stimulate the Na^+/K^+-ATPase pump and triggers membrane hyperpolarization in glucose-excited neurons in the lateral hypothalamus (Oomura *et al.*, 1974), and brain hypoglycemia reduces the activity of the Na^+/K^+-ATPase pump (Lees, 1991) and may lead to depolarization of neurons (Balfour & Trapp, 2007). Therefore, Na^+/K^+-ATPase pump constitute an additional component altering the RMP of NTS neurons in response to glucose levels changes. However, this mechanism is not compatible to the voltage-dependency we observed.

Lamy *et al*. (2014) reported an increase in membrane input resistance by the closure of leak potassium channels in GLUT2-expressing GABAergic neurons activated by low glucose in mice. This could account for the response we observed, but again, the low glucose-sensitive current observed in this report was a linear non-rectifying current, which is not compatible to the voltage-dependency we observed. Our results regarding the effect triggered by low glucose suggest an action on a voltage-dependent current, like a block of a G-protein-activated inwardly rectifying potassium (GIRK) current, or an activation of the h current (I_h).

Because the effect on input resistance was seemingly unrelated to the depolarization induced by low glucose, we believe that other conductances could be activated by low glucose, but not significantly affecting the RMP. Thus, in contrast to glucose-excited neurons that rely on a K_{ATP} blockage for increasing firing activity in response to high-glucose availability (Thorens, 2012), neurons responsive to low glucose seem to present more heterogeneous mechanisms. This scenario might not be surprising, since distinct signaling pathways may be activated under conditions of energy deprivation (i.e., low-glucose levels). Unlike hyperglycemia, which can develop neuropathies over the years, brain hypoglycemia may short-term compromise neuronal activity and survival (Cryer, 2007; Mergenthaler *et al.*, 2013).

The cooperation of astrocytes for maintaining neuronal energy requirements via an astrocyte-neuron lactate shuttle is a matter of intense debate (Dienel, 2012; Bak & Schousboe, 2017). However, some studies have showed that astrocytes in the NTS are sensitive to extracellular glucose levels, and thus could boost lactate into neurons to generate ATP under conditions of low-glucose availability. In rats, acute hypoglycemia reduces glycogen contents in NTS astrocytes (Suh *et al.*, 2007), and low-glucose solution or the presence of 2-DG increases intracellular calcium in NTS astrocytes (McDougal *et al.*, 2013). Therefore, it is reasonable to hypothesized that the net effect of low extracellular glucose levels on K_{ATP} conductance in neurons would be set by an increase in ATP levels (i.e., inhibiting K_{ATP} currents) produced by astrocyte-derived lactate (possibly by glucogenolysis) opposing a reduced amount of ATP caused by low glucose (i.e., activating K_{ATP} currents). However, we found that even after the blockage of K_{ATP} channels the neurons could depolarize in response to low glucose if the membrane potential was hyperpolarized. This suggests that even if astrocytes are shunting lactate into neurons, the low glucose-induced depolarization is not triggered by the closure of K_{ATP} channels.

Hypoglycemia is a common occurrence in patients with type 1 diabetes (T1D) mainly due to pharmacological interventions driven to control glycemia (Mergenthaler *et al.*, 2013). Several evidences have suggested that subsequent episodes of hypoglycemia may lead to a habituation of the brain to increase glucose supply and/or use alternative substrates of energy (e.g., lactate and ketone bodies) (McNay & Cotero, 2010; Litvin *et al.*, 2013). Episodes of recurrent hypoglycemia facilitates neuronal uptake of lactate under hypoglycemia in rats (Herzog *et al.*, 2013). Accordingly, the glucose metabolism seems to be better preserved during hypoglycemia in patients with insulin-dependent diabetes mellitus (Boyle *et al.*, 1995) and in T1D patients (van de Ven *et al.*, 2013) compared to healthy subjects. However, if on the one hand the neuroprotective adaptations sustain neural activity during a subsequent episode of brain hypoglycemia, on the other hand they are suggested to be the cause of blunted counterregulatory responses to hypoglycemia and decreased ability of patients to detect it, namely hypoglycemia-associated autonomic failure (Cryer, 2005; Litvin *et al.*, 2013). In other words, the brain adapts itself to preferentially use other energy substrates than glucose to meet its energy demand and ends up not detecting low-glucose levels. For instance, recurrent hypoglycemia increases the threshold for low-glucose detection in ventromedial hypothalamic neurons in comparison to control rats (Song & Routh, 2006). Our results clear show a mechanism of membrane depolarization driven by a rapid reduction in external glucose that could be potentially relevant for neurometabolic responses to hypoglycemia. Interestingly, when neurons are maintained in a hyperglycemic solution (similar to high post-prandial circulating glucose levels), the number of NTS neurons responsive to low glucose decreases, and the latency for peak response increases, indicating that the establishment of more depolarized RMP by the closure of K_{ATP} channels due to increased intracelullar ATP levels could be an additional permissive mechanism contributing to reduced brain sensitivity to a low-glucose challenge. Thus, the energetics adaptation of the brain to support a subsequent hypoglycemia episode could be enhancing basal intracellular ATP levels, leading neurons to a more positive RMP, and contributing for the less sensitivity of neurons in response to low glucose.

Interestingly, since both K_{ATP} blockage and low glucose either produce membrane depolarization, but low glucose is ineffective to depolarize neurons when K_{ATP} are fully blocked, we believe that the depolarization induced by low external glucose might represent a form of homeostatic regulation of the RMP in order to avoid an excessive hyperpolarization by unblocking K_{ATP} channels under a hypoglycemia episode, which could be essential for the proper functioning of the vital autonomic functions controlled by the NTS.

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Figures

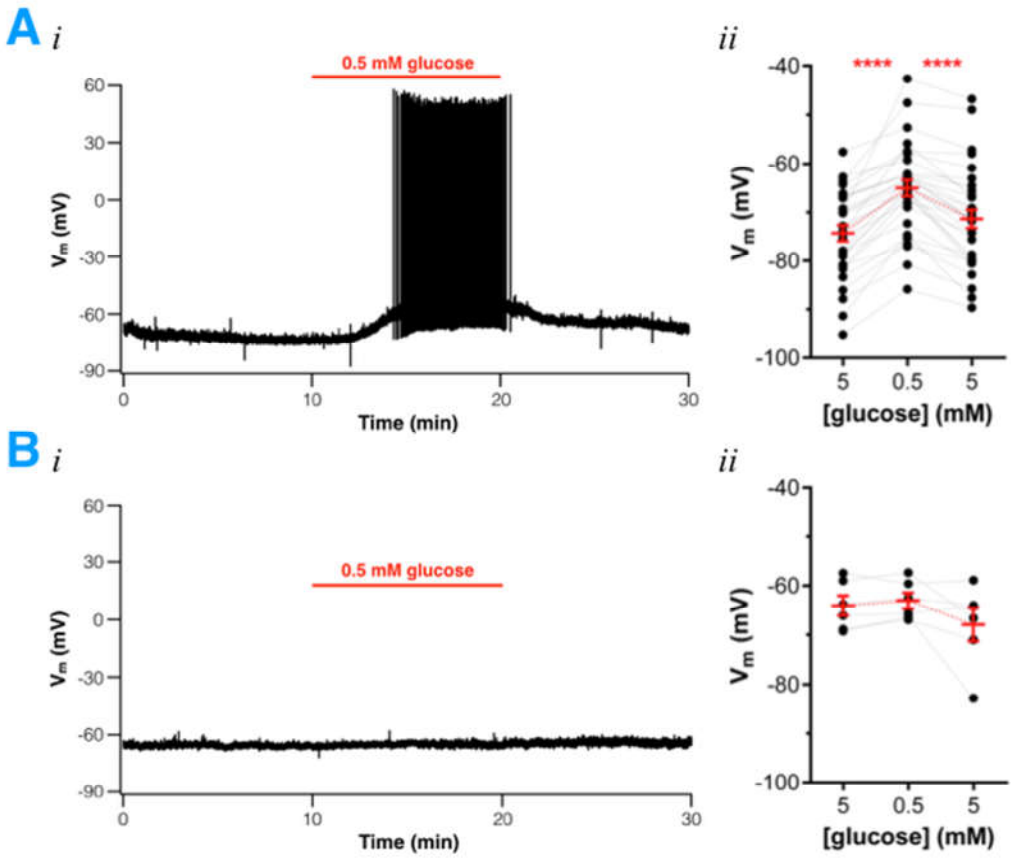

Figure 1. Most NTS neurons depolarize in response to low glucose. **A,** subset of neurons depolarized by low glucose. A*i*, representative recording of a responsive neuron showing a reversible depolarization and increased firing activity induced by low glucose. A*ii*, Summary of the effect of low glucose on resting membrane potential. **B,** subset of neurons unresponsive to low glucose. B*i*, representative recording of a non-responsive neuron to low glucose. B*ii*, Summary of the effect of low glucose on membrane potential of neurons. V_m, membrane potential. $^{****}p < 0.0001$.

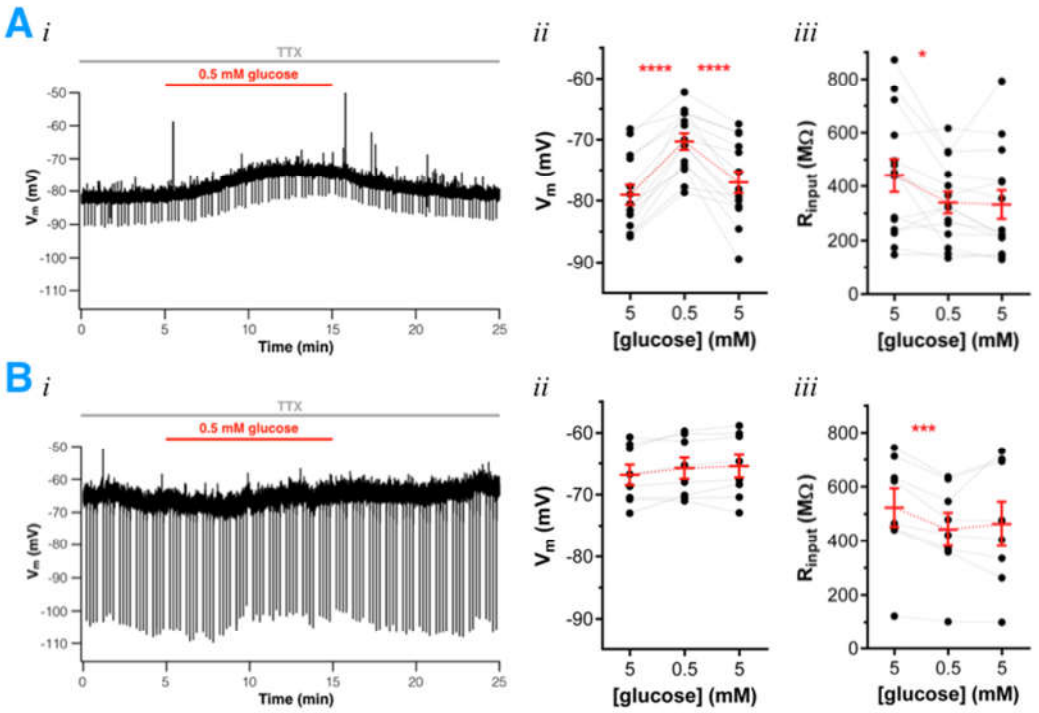

Figure 2. NTS neurons sense low glucose is indenpenent of voltage-dependent sodium channels. **A,** subset of neurons depolarized by low glucose in the presence of tetrodotoxin (TTX). A*i*, representative recording of a responsive neuron showing a reversible depolarization and decreased input resistance (R_{input}) induced by low glucose. The graphs show the summary of the effect of low glucose on (*i*) membrane potential (V_m) and (*ii*) input resistance (R_{input}) of neurons. **B,** subset of neurons unresponsive to low glucose in the presence of TTX. B*i*, representative recording of a non-responsive neuron to low glucose. The graphs show the summary of the effect of low glucose on (*i*) V_m and (*ii*) R_{input} of neurons. R_{input}, input resistance; TTX, tetrodotoxin; V_m, membrane potential. $^{*}p < 0.05$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

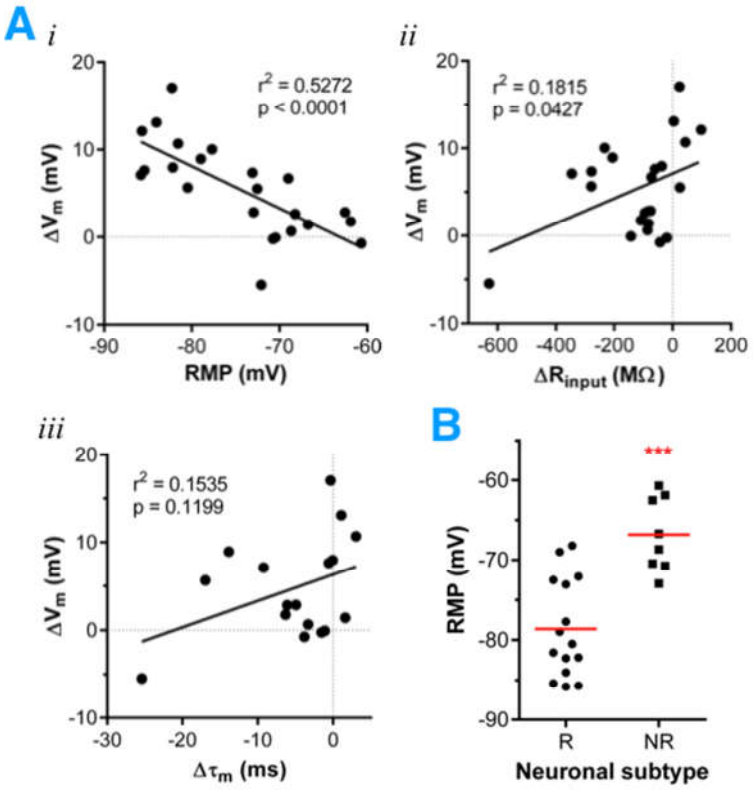

Figure 3. Depolarization triggered by low glucose is dependent on membrane potential in NTS neurons. **A,** Linear correlation between the low-glucose response amplitude and (*i*) resting membrane potential, (*ii*) input resistance, and (*iii*) membrane time constant of neurons. **B,** comparison of the RMP between neurons responsive and unresponsive to low glucose. NR, non-resposive; R, responsive; R_{input}, input resistance; RMP, resting membrane potential; τ_m, membrane time constant. $^{***}p < 0.001$.

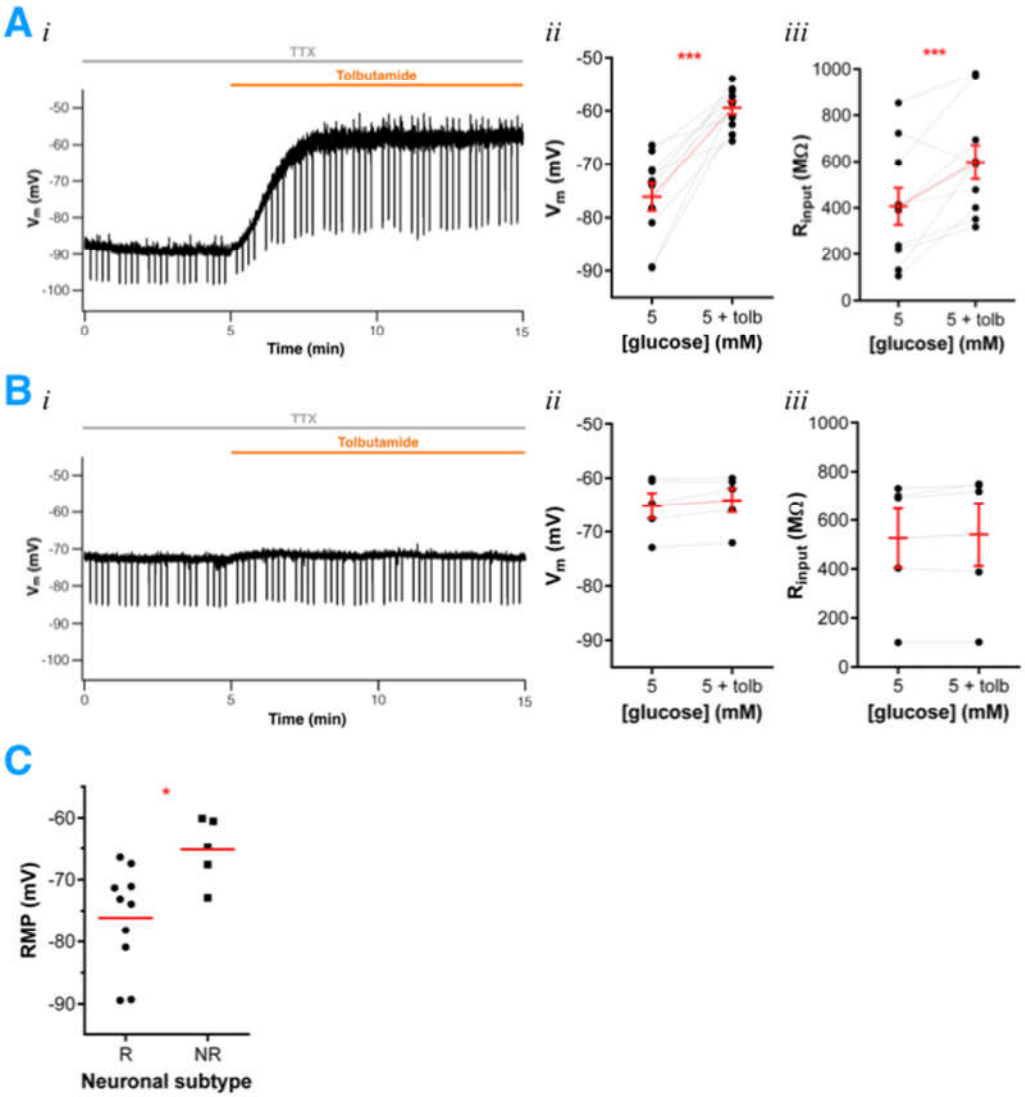

Figure 4. Presence of ATP-sensitive potassium (K_{ATP}) channels in NTS neurons. **A,** subset of neurons responsive to tolbutamide. The graphs show the summary of the effect of tolbutamide on (*i*) membrane potential (V_m) and (*ii*) input resistance (R_{input}) of neurons. **B,** subset of neurons unresponsive to tolbutamide. The graphs show the summary of the effect of tolbutamide application on (*i*) V_m and (*ii*) R_{input}. **C,** comparison of the RMP between neurons responsive and unresponsive to tolbutamide. NR, non-resposive; R, responsive; R_{input}, input resistance; RMP, resting membrane potential; Tolb, tolbutamide. $^{*}p < 0.05$; $^{*}p < 0.001$.

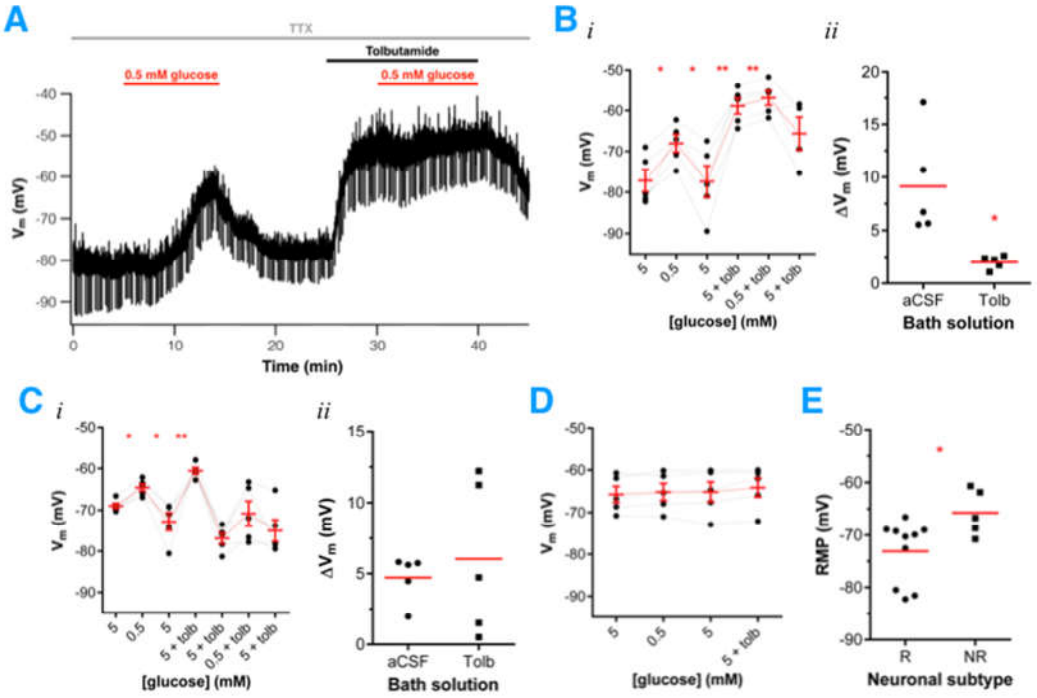

Figure 5. The occlusion of K_{ATP} channels suppresses the depolarization induced by low glucose. **A,** representative recording of a neuron responsive to low glucose showing that tolbutamide blunts the depolarizing effect to a subsequent low-glucose challenge. **B,** neurons responsive to low glucose are depolarized by tolbutamide (*i*), and this effect occludes low-glucose sensing (*ii*). **D,** neurons unreponsive to low glucose does not also respond to tolbutamide application. **E,** comparison of the RMP between neurons responsive and unresponsive to both low glucose and tolbutamide. NR, non-resposive; R, responsive; RMP, resting membrane potential; Tolb, tolbutamide; TTX, tetrodotoxin; V_m, membrane potential. $^{*}p < 0.05$; $^{**}p < 0.01$.

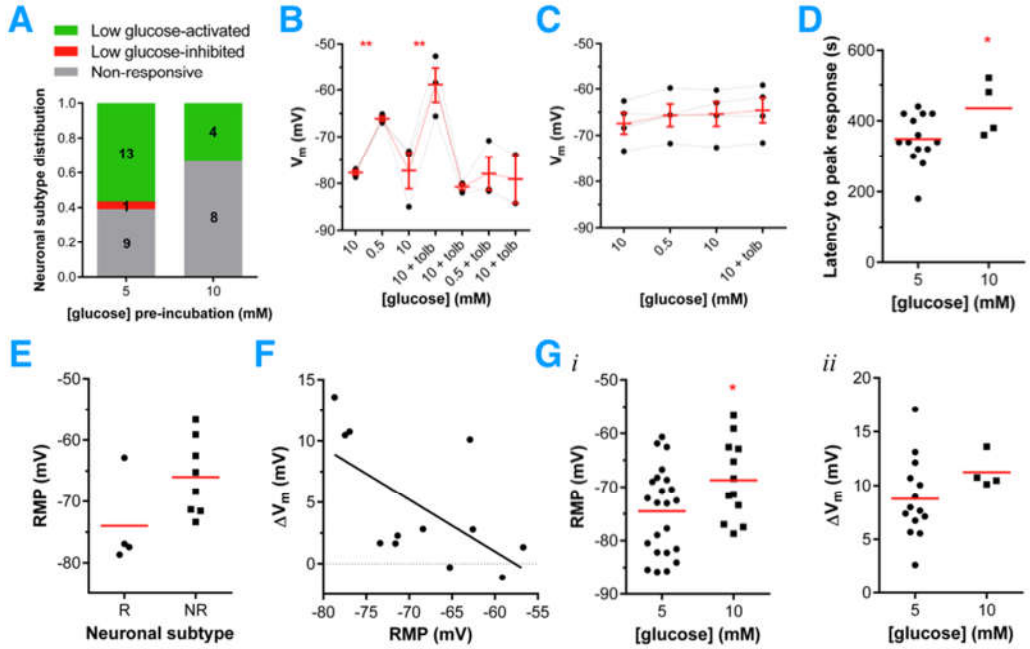

Figure 6. Incubation in high glucose (10 mM) decreases the number of NTS neurons sensitive to low glucose. **A,** Comparison of the distribution of the neurons according their response to low glucose between neurons incubated in different glucose solutions. **B,** neurons responsive to low glucose are depolarized by tolbutamide application. **C,** neurons unresponsive to low glucose do not also respond to tolbutamide. **D and E,** comparison of the (D) resting membrane potential and (E) latency to peak response between neurons responsive and non-responsive to low glucose. **F,** linear correlation between the low glucose amplitude response and the RMP. **G.** Comparison of the (*i*) RMP and (*ii*) low-glucose response amplitude between neurons incubated in high glucose and normal aCSF (5 mM glucose). NR, non-responsive; R, responsive; RMP, resting membrane potential; Tolb, tolbutamide; V_m, membrane potential. $^{*}p < 0.05$; $^{**}p < 0.01$.