

**MOLECULAR CHANGES IN THE DORSAL VAGAL COMPLEX OF DIABETIC MICE**

**PhD Thesis**

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This dissertation is dedicated to my family, Bret, Miklos, Viktor, Justin, Maddie, Morgan, Roxy-ka, and my extended family in Hungary for their unlimited emotional support.

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**PUBLICATIONS RELATED TO THE THESIS:**

1. K.C. **Halmos**, P. Gyarmati, H. Xu, S. Maimaiti, G. Jancsó, G. Benedek, B.N. Smith, Molecular and functional changes in glucokinase expression in the brainstem dorsal vagal complex in a murine model of type 1 diabetes. *Neuroscience* 2015 In Press. doi:10.1016/j.neuroscience.2015.08.023

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2. Bach EC, **Halmos KC**, Smith BN Enhanced NMDA receptor-mediated modulation of excitatory neurotransmission in the dorsal vagal complex of streptozotocin-treated, chronically hyperglycemic mice. *PLOS ONE* 2015 Mar 23;10(3):e0121022.

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3. Boychuk CR, **Halmos, K**, Smith BN. Diabetes induces GABA receptor plasticity in murine vagal motor neurons. *J Neurophysiol.* 2015 May 20;jn.00209.2015. doi: 10.1152/jn.00209.2015 In Press.

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## OTHER PUBLICATIONS:

1. Luther JA, **Halmos KC**, Tasker JG: A slow transient potassium current expressed in a subset of neurosecretory neurons of the hypothalamic paraventricular nucleus. *J Neurophysiology* 2000, 84:1814-1825
2. Luther JA, Daftary SS, Boudaba C, Gould GC, **Halmos KC**, Tasker JG: Neurosecretory and non-neurosecretory parvocellular neurones of the hypothalamic paraventricular nucleus express distinct electrophysiological properties. *J Neuroendocrinol.* 2002 Dec;14(12):929-32.
3. Boudaba C, Linn DM, **Halmos KC**, Tasker JG: Increased tonic activation of presynaptic metabotropic glutamate receptors in the rat supraoptic nucleus following chronic dehydration. *J Physiol.* 2003 Sept 15 551(3):815-23.
4. Di S, Malcher-Lopes R, Halmos KC, Tasker JG: Nongenomic glucocorticoid inhibition via endocannabinoid release in the hypothalamus: a fast feedback mechanism. *J Neurosci.* 2003 Jun 15;23(12):4850-7.
5. Haam J, Popescu IR, Morton LA, Halmos KC, Teruyama R, Ueta Y, Tasker JG. GABA is excitatory in adult vasopressinergic neuroendocrine cells. *J Neurosci.* 2012 Jan 11;32(2):572- 82.
6. Haam J, Halmos KC, Di S, Tasker JG. Nutritional state-dependent ghrelin activation of vasopressin neurons via retrograde trans-neuronal-glia stimulation of excitatory GABA circuits. *J. Neurosci* 2014 Apr 30;34(18):6201-13.

## **Chapter 1: Introduction**

### **The role of the CNS in the diabetes**

Diabetes mellitus is a major health concern, afflicting over 29 million people in the United States. The International Diabetes Federation estimates that around 52 million people are living with diabetes in the European Region, or about 8.4% of men and 7.8% of women aged 20–79 years (World Health Organization, WHO). Serious complications resulting from diabetes include heart disease, stroke, hypertension, blindness, nervous system damage, and autonomic dysfunction. A major impediment to developing successful diabetes treatments (versus treating symptoms) is relative knowledge gap regarding the multifaceted and redundant systems that contribute to control of metabolic homeostasis. Due to its effects on peripheral organs that modulate hormone homeostasis, diabetes has classically been treated as a peripheral disease. However, the pathological involvement of the CNS has earned increased attention more recently, as various studies have linked changes neuronal function and synaptic reorganization of central nervous system (CNS) neurons in response to glucose dysregulation and diabetes (Kalsbeek et al. 2010; Levin et al. 1999; Obici et al. 2002; Yi et al. 2010; Zsombok and Smith 2009).

Much recent research on central control of whole-body metabolism and altered neuronal function in diabetes has focused on functions of neurons in preautonomic hypothalamic nuclei (Ashford et al. 1990; Levin et al. 1999; Ritter et al. 2000). Despite this hypothalamic focus, abundant evidence indicates that brainstem dorsal vagal complex (DVC) plays a primary and critical role in glucose-sensitive modulation of plasma glucose and insulin levels, feeding and energy balance (Ritter et al. 1981; Ritter et al. 2000; Zsombok and Smith 2009). Subsets of DVC neurons and synaptic terminals are glucose-sensitive (Balfour et al. 2006; Boychuk et al. 2015a; Browning 2013; Ferreira et al. 2001; Lamy et al. 2014; Oomura et al. 1974; Wan et al. 2008), consistent with longstanding evidence that glucose-sensing neurons in this region regulates both feeding and blood glucose concentrations (Ritter et al. 1981; Ritter et al. 2000). Injection of a glucoprivic glucose analogue into the vagal complex, but not hypothalamic areas, increases both feeding and hyperglycemia in rats (Ritter et al. 2000). Neurons in the DVC clearly respond to altered glucose concentrations and also influence blood glucose content, yet little is known about the mechanisms underlying these effects in the diabetic state.

## **The Dorsal Vagal Complex**

### **Circuit organization and neurotransmission in the Dorsal Vagal Complex**

The dorsal vagal complex (DVC) of the caudal brainstem regulates central parasympathetic reflex and integrative visceral functions, including those of the digestive system. Viscerosensory inputs mediated by cranial nerves VII, IX, and X in conjunction with centrally-originating afferents, synapse in the nucleus of the solitary tract (NTS). The NTS consists of a heterogeneous population of cells, which incorporate mechanical and chemical stimuli from peripheral organs with centrally derived neuronal information as well as signals mediated by circulating hormones and metabolites. The NTS integrates this information and transmits to other autonomic centers, including the dorsal motor nucleus of the vagus nerve (DMV) (Browning 2013; Travagli et al. 2006). The DMV contains preganglionic motor neurons that project to all thoracic and most subdiaphragmatic peripheral organs. Vagal motor activity is tightly regulated by synaptic inputs arising from GABA and glutamate neurons of the adjacent nucleus NTS (Davis et al. 2004; Travagli et al. 1991), and NTS neurons also project to other central autonomic regulatory centers. As a unit, primary viscerosensory afferents, second-order sensory neurons in the NTS, and preganglionic parasympathetic vagal motor neurons in the DMV mediate vago-vagal reflexes—includes cardiovascular and gastrointestinal reflexes (Browning 2013; Travagli et al. 2006). These reflexes can often be modulated by synaptic input from other brain areas and, at least for the NTS, by substances in the blood (e.g., glucose). As an example, glucose infusion into the proximal duodenum induces a decrease in hepatic gluconeogenesis, and this “reflex” is prevented by blocking glutamatergic neurotransmission in the NTS or by cutting the hepatic branch of the vagus nerve, indicating a vago-vagal “gut-brainstem-liver” glucose-regulatory circuit (Breen et al. 2013). Neurons in the NTS are therefore considered to serve both reflex and integrative functions in regulating vagal output.

DMV neurons maintain tonic pacemaker activity (Travagli et al. 2006). This activity, however, is tightly regulated by predominantly GABAergic and glutamatergic presynaptic neurons of the NTS (Davis et al. 2004; Glatzer and Smith 2005; Travagli et al. 2006). GABAergic inputs maintain a tonic inhibitory drive that prominently regulates vagal outflow, with glutamatergic inputs serving a phasic excitatory role (Browning and Travagli 2011; Gao

and Smith 2010). Increased vagal outflow lowers hepatic gluconeogenesis, enhances pancreatic exocrine function (i.e. release of insulin), stimulates gastric emptying (Pocai et al. 2005b; Travagli et al. 2006), and also stimulates glucagon release under some conditions (Lamy et al. 2014). Local neuronal connections within the DVC are therefore important regulators of peripheral glucose metabolism.

### **Energy homeostasis in the DVC and pathological consequences**

Diabetes mellitus is a metabolic disease of glucose characterized by the body's inability to maintain glucose homeostasis. This can arise either from pancreatic  $\beta$ -cell islet destruction and the resultant inability of the body to synthesize insulin (Type 1 diabetes), or abnormal insulin secretion and/or insulin resistance (Type 2 diabetes)(Zimmet et al. 2001). In addition, hepatic gluconeogenesis/glyconeogenesis is dysregulated, resulting in continuously dysregulated glucose production (Pocai et al. 2005a; Pocai et al. 2005b; Schwartz et al. 2013). The DVC can be modulated by a complex array of peripheral as well as CNS signals (Travagli et al. 2006). Gastric reflexes are in part triggered by mechano- and chemoreceptors in the gut. Therefore, when the stomach becomes full and consequently experiences mechanical distension or chemoreceptors are activated by various chemicals, including glucose or peptides, vagal afferents that synapse in the NTS become activated to trigger the cessation of feeding (Travagli et al. 2006). NTS activity is also transmitted to hypothalamic nuclei, which in turn provide additional signals to the DVC that are related to the overall metabolic status (Pocai et al. 2005b). This represents a balance where the information from the gut transmitted to DVC which eventually influences hepatic glucose regulation. Thus, gastric vagal afferent signaling initiates digestion-related reflexes in the gut as well as metabolic activity that controls glucose metabolism (Breen et al. 2013). This "gut-brain-liver" circuit exemplifies a physiological response system whose homeostatic imbalance can lead to pathological complications (Saltzman and McCallum 1983).

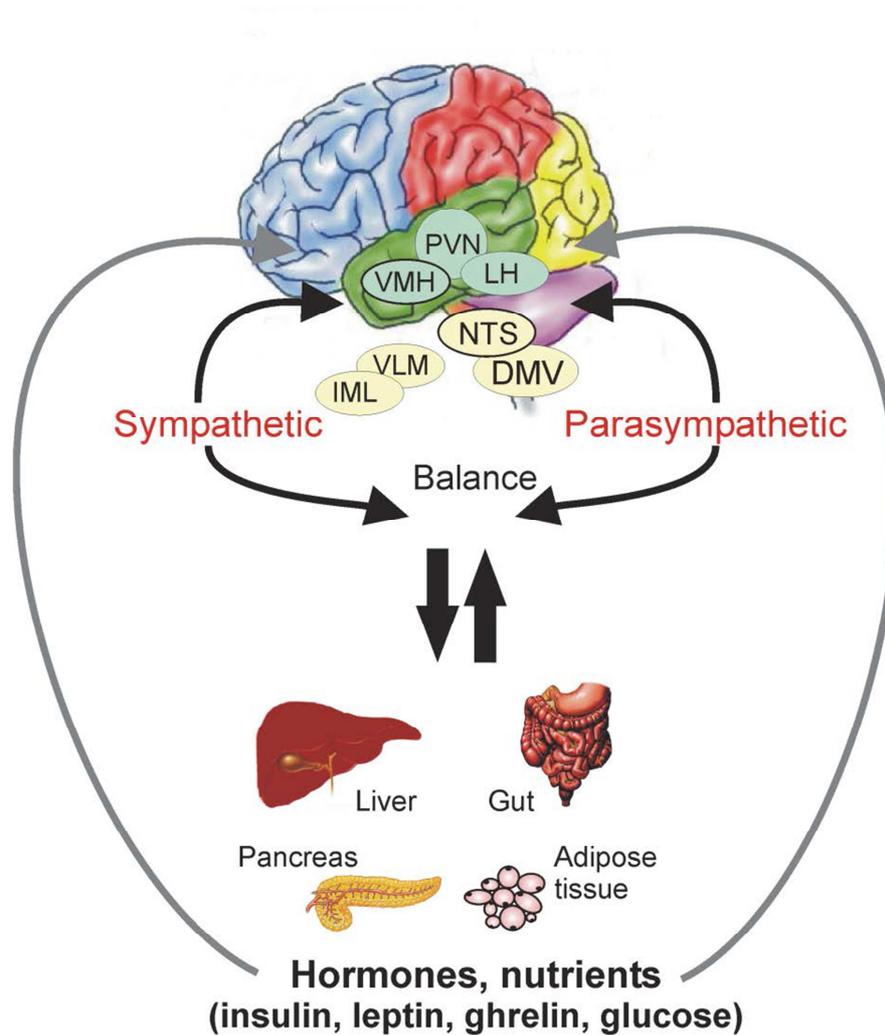


Figure 1. Schematic of hypothesized parallel brain-digestive system-brain circuits in hypothalamus and brainstem. Hypothalamic nuclei involved in autonomic regulation and feeding make reciprocal connections with neurons in the dorsal vagal complex that regulate visceral function. Within the vagal complex, the NTS receives visceral afferent neural input and projects heavily to vagal motor neurons of the DMV. The NTS is also reciprocally connected to hypothalamic nuclei involved in autonomic regulation, especially the PVN. Both PVN and NTS also project to central sympathetic nervous system regulatory centers. Likewise, both brainstem and hypothalamic areas receive information about peptide and glucose content in the blood. Abbreviations: DMV, dorsal motor nucleus of the vagus; IML, intermediolateral cell column; LH, lateral hypothalamus; NTS, nucleus tractus solitarius; PVN, paraventricular nucleus; VLM, ventrolateral medulla; VMH, ventromedial hypothalamus. From Zsombok and Smith, 2009.

Central detection of systemic energy status is provided by fenestrated capillaries in the NTS, which allow a direct indication of circulating hormones and metabolites (Gross et al. 1990; Merchenthaler 1991). Particular interest has recently developed the NTS in the ability of this nucleus to directly respond to hormones and metabolites that mediate glucose homeostasis. Glucose modulates efferent activity of the DVC through subpopulation of cells in the NTS and the DMV that respond to changing glucose concentrations by altering their firing properties. These glucose-sensitive neurons respond to either hypoglycemic or hyperglycemic conditions to elicit vagal efferent responses important to maintaining and reestablishing glucose homeostasis (Balfour et al. 2006; Balfour and Trapp 2007; Boychuk et al. 2015a; Ferreira et al. 2001; Lamy et al. 2014). Thus, NTS neuron activity and responses to visceral and other synaptic input occurs in the context of local glucose concentration.

In conjunction with glucose, other metabolites that have been well established for their peripheral role in glucose homeostasis also have direct effects on neurons within the DVC. These hormonal players include: insulin, leptin, ghrelin, and glucagon-like peptide-1 (GLP-1) to name a few. Importantly insulin and leptin modulate synaptic input to the DMV and on NTS neurons through the activation of insulin and leptin receptors, respectively, that results in a decrease of glutamate release on gastric related neurons. Both were shown to act in a PI3K-dependent mechanism, suggesting their activation of signaling cascades similar to those observed in peripheral organs and by hypothalamic nuclei (Blake and Smith 2012; Williams and Smith 2006; Williams et al. 2007). GLP-1 activity has been shown to enhance both the excitatory and inhibitory activity to pancreatic-projecting neurons in the DMV (Wan et al. 2007). Presumably, the direct effects of circulating metabolic signals like glucose and peptides at the level of the DVC suggests that they are important players in moment to moment efferent output modulation.

To begin to understand the effects diabetes has on synaptic connections of the DVC, the streptozotocin (STZ) model of Type 1 diabetes was used in electrophysiological and molecular studies in the DMV. This model involves injecting animals systematically with STZ, which kills the insulin-producing pancreatic  $\beta$ -cells, resulting in chronic hyperglycemia in conjunction with hypoinsulinemia (Craighead 1980). These studies revealed that chronically increased blood glucose concentrations in the STZ model of type 1 diabetes is accompanied by altered neurotransmitter function in the DVC (Bach et al. 2015; Blake and Smith 2014; Boychuk et al. 2015b; Zsombok et al. 2011). These effects persisted for hours (at least) after glucose was

standardized to control levels *in vitro*. In addition to insulin-dependent receptor trafficking (Zsombok et al. 2011), postsynaptic and action potential responses to glucose of specific sets of vagal complex neurons are suppressed in this model after several days of hyperglycemia (Browning 2013). Thus, chronic glycemc dysregulation in diabetes is accompanied by altered neuronal function in the DVC.

The complexity of the synaptic rules governing homeostatic gastrointestinal reflex responses elicited by the DVC emphasizes the need to better understand these principles and how they can be modulated under physiological as well pathological circumstances. A more comprehensive understanding of their mechanisms may serve as a potential tool to manipulate glucose homeostasis and prevent the occurrence of diabetes. As a result, studying the DVC in the context of diabetes has moved the spotlight from the hypothalamus to the brainstem and increased attention in the global epidemic of the metabolic disorders (Zsombok and Smith 2009).

### **Glucokinase function**

Glucokinase is part of the hexokinase (HK) family (often called HK type IV), an enzyme that catalyzes the phosphorylation of glucose to glucose 6-phosphate (G6P), which then undergoes glycolysis in mitochondria to increase the ATP/ADP ratio. Most cells in the body, including pancreatic  $\beta$  cells, express KATP channels in the membrane, which are open when ATP levels are low and are closed by increased intracellular ATP. Increased production of ATP due to GCK-dependent glucose metabolism results in the ATP-dependent closure of KATP channels and, consequently, membrane depolarization. Influx of  $\text{Ca}^{2+}$  through voltage-dependent  $\text{Ca}^{2+}$  channels stimulates the  $\text{Ca}^{2+}$ -dependent release of insulin from  $\beta$ -cells. Glucokinase is also present in cells of the liver, gut, and brain, and the enzyme plays an important role in the regulation of glucose metabolism in all these areas by acting as a sensitive glucose sensor (Balfour et al. 2006; Lam et al. 2007; Matschinsky et al. 1968; Oomura et al. 1974). Rising or falling glucose concentrations triggers shifts in cellular function and metabolism, for example after a meal or when fasting. Hepatic glucose homeostasis is largely maintained by G6PC and GCK, which act in opposition to regulate the intracellular levels of free glucose (Haeusler et al. 2015). Thus, the coordinated regulation of these two enzymes ultimately dominates the way of the flow of glucose into or out of the hepatocyte. During fasting, an increased ratio of G6PC/GCK causes glucose efflux. If the ratio decreases, then it creates increased influx of

glucose. (Haeusler et al. 2015). Paired with hepatic gluconeogenesis, the process by which amino acids are metabolized to generate glucose in hepatocytes, this process is important in both hepatic and pancreatic control of blood glucose levels. GCK is similarly important for glucose sensing in hypothalamic and other glucose sensing neurons, including in the DVC (Boychuk et al. 2015b; Dunn-Meynell et al. 2002), where mRNA is localized to neurons that exhibit electrophysiological sensitivity to glucose (Lamy et al. 2014). In the NTS, these glucose-sensing neurons comprise a subset of inhibitory, GABAergic cells, which are usually either excited or inhibited by increasing glucose concentration in a GCK-dependent manner (Boychuk et al. 2015a; Lamy et al. 2014). Under conditions of normal glucose concentration, intracellular ATP is relatively low in these neurons, allowing KATP channels to remain open. In the NTS, GCK regulates responses to hyperglycemia by increasing intracellular ATP levels, which closes KATP channels and depolarizes the neuronal membrane. The response to hypoglycemia typically involves decreased ATP levels, which allows KATP channels to open and results in a hyperpolarization of the membrane (Balfour et al. 2006). Although less typical, other mechanisms, which trigger opening or closing of BK or “leak” K channels have also been hypothesized to mediate GCK-dependent responses in glucose-inhibited neurons (Lamy et al. 2014; Shanley et al. 2002).

### **KATP channels and their role in diabetes**

The importance of KATP channels in insulin secretion was established over 30 years ago (Ashcroft et al. 1984), and these channels have more recently been shown to mediate most responses to glucose in neurons (Balfour et al. 2006; Boychuk et al. 2015a; Dunn-Meynell et al. 2002). The KATP channel can be pharmacologically regulated by sulfonylurea drugs, which are widely used to treat type 2 diabetes; they work by binding to and closing the KATP channel, consequently depolarizing the cell membrane. Conversely, drugs such as diazoxide can be utilized to open the channel (K<sup>+</sup> channel openers).

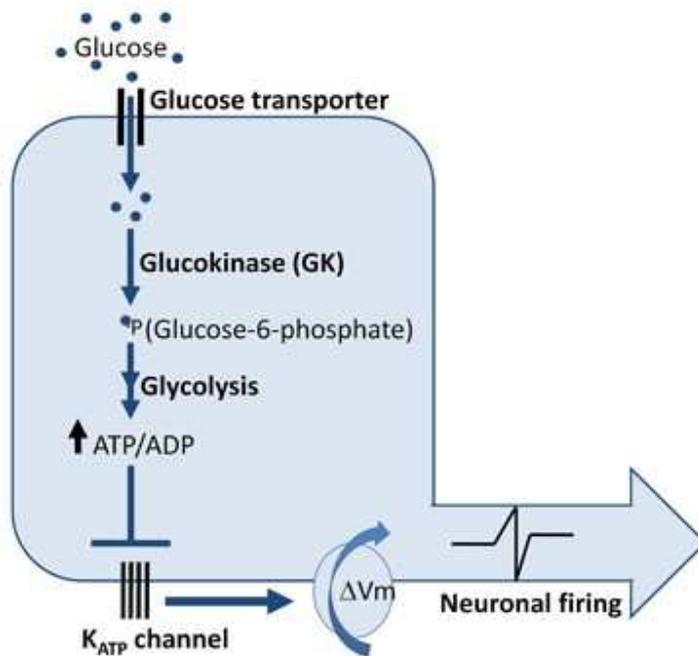


Figure 2. Coupling of glucose to ATP-sensitive K<sup>+</sup> (KATP) channel function by glucokinase (GCK). Glucose is transported across the cell membrane by a glucose transporter, where it is converted via a GCK-catalyzed mechanism to glucose-6-phosphate (G-6-P). Through glycolysis, G-6-P results in an increase in the intracellular ATP/ADP ratio. Increased ATP blocks KATP channels in the membrane, resulting in membrane depolarization and increased action potential firing. Adapted from Kelly A. Diggs-Andrews, B.S., Julie M. Silverstein, M.D., and Simon J. Fisher, M.D., Ph.D. Cyberrounds.com

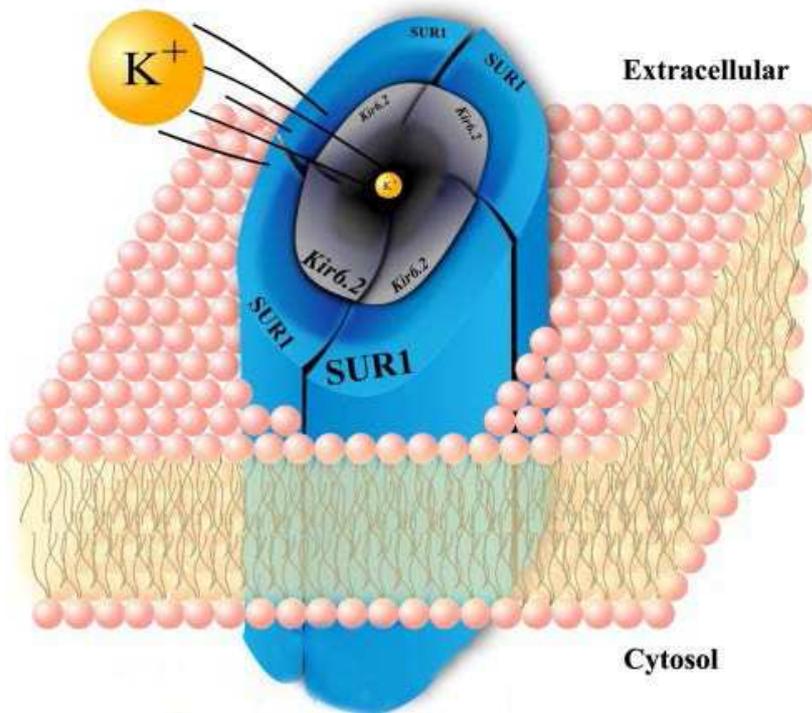


Figure 3. Schematic diagram of the ATP-dependent  $K^+$  (KATP) channel. Four Kir6.2 subunits, which form the selective potassium pore, and four SUR1 subunits, which bind sulfonylurea drugs like tolbutamide, are indicated. Adapted from Bonfanti et al., 2015.

The KATP channel consists of two essential subunits: Kir6.2, which is the pore-forming unit and belongs to the inwardly rectifying K<sup>+</sup> channel family, and sulfonylurea receptor 1 (SUR1). The channel is an octameric complex of four Kir6.2 and four SUR1 subunits. The binding of ATP to Kir6.2 results in closure of KATP channels, while sulfonylurea drugs (e.g., tolbutamide) or KATP channel openers (e.g., diazoxide) bind to SUR1 via two cytosolic binding domains (Bonfanti et al. 2015). Given the central role of the KATP channel in insulin secretion it is not surprising that mutations in the genes encoding the subunits of this channel can result in both hypo- and hyperglycemia (Babenko et al. 2006; Gloyn et al. 2006; Gloyn et al. 2003; Proks et al. 2006).

In NTS GABA neurons, blocking KATP channels prevented the excitatory effects of increased glucose concentration (Boychuk et al. 2015a) but inhibitory glucose effects were maintained. This suggests a similar mechanism of depolarization for NTS GABA neurons and pancreatic  $\beta$  cells. In either case responses of NTS GABA cells require GCK, implicating intracellular metabolic processing of glucose for both responses. The channel mediating the glucose-induced hyperpolarization in these neurons is not known. Whereas glucose-inhibited neurons in other brain areas can utilize KATP channel opening to hyperpolarize the membrane, still other neurons use BK or leak K<sup>+</sup> channels (Lamy et al. 2014; Shanley et al. 2002). In either case responses of NTS GABA cells require GCK, implicating intracellular metabolic processing of glucose for both responses.

Because of the involvement of GCK and KATP channel modulation in the neuronal response to glucose, and altered responsiveness of NTS neurons in models of type 1 diabetes, we tested the hypothesis that GCK or KATP channel expression is altered after several days of chronic hyperglycemia/hypoinsulemia in the streptozotocin (STZ) treated mouse. Understanding how glucose sensitivity in the dorsal brainstem is altered in diabetes may offer hypothesis to guide development of alternative therapies for the disease.

## **Chapter 2: Materials and Methods**

### **Animals**

Mice were treated and cared for in accordance with National Institutes of Health guidelines, and all procedures were approved by the University of Kentucky Animal Care and Use Committee (Animal Welfare Assurance Number A3336-01). Euthanasia was accomplished by anesthesia with isoflurane to effect, followed by decapitation while anesthetized. Juvenile and young adult (28–42 days) male CD-1 (Harlan Laboratories, Indianapolis, IN) or transgenic ‘GIN’ mice (FVB-Tg (GadGFP) 4570Swn/J; The Jackson Laboratory, Bar Harbor, ME) were used for all experiments and housed under a standard 14-h light-10-h dark cycle, with food and water provided without restriction. The GIN mice express EGFP in the somatostatinergic subset of GABA neurons in the NTS, which comprise a large proportion of NTS neurons (Glatzer et al. 2007; Williams and Smith 2006).

### **Streptozotocin Injections**

Intra-peritoneal injections of streptozotocin (STZ; 200mg/kg in 0.9% NaCl), which kills insulin-secreting pancreatic  $\beta$  cells, was used to induce chronic hyperglycemia in mice. For single STZ injections, mice were fasted (4–6 h) prior to injection. Prior to fasting and STZ injections, blood glucose concentration (tail puncture) was measured using a Nova Max PLUS glucometer. Control mice were either injected with saline (0.9% NaCl) or untreated. No differences in electrophysiological parameters were observed between normoglycemic saline-injected and untreated mice; they were therefore pooled and considered as a single control group. Systemic glucose levels were measured daily. Onset of hyperglycemia (i.e., blood glucose level of above 300mg/dl) varied between animals, but occurred between 1 and 3 days post-STZ injection and remained elevated until the day of the experiment. Animals were used for electrophysiological recordings and molecular analyses after 3-4 days of continuous hyperglycemia.

### **Brain slice preparation**

On-cell and whole-cell voltage-clamp recordings were made using brain stem slices prepared from male and female GIN mice, 4-5 wks. of age. Animals were deeply anesthetized by

isoflurane inhalation (IsoThesia; Henry Schein, Melville, NY) and decapitated while anesthetized. The brain was rapidly removed and blocked to isolate the brainstem and then glued to a sectioning stage. Transverse (coronal) brain stem slices (300  $\mu\text{m}$ ) containing the caudal NTS (i.e.,  $\pm 600 \mu\text{m}$  rostral and caudal to area postrema) were made in ice cold, oxygenated (95% O<sub>2</sub>-5% CO<sub>2</sub>) artificial cerebrospinal fluid (ACSF) using a vibrating microtome (Vibratome Series 1000; Technical Products, St. Louis, MO). The ACSF contained (in mM): 124 NaCl, 3 KCl, 2 CaCl<sub>2</sub>, 1.3 MgCl<sub>2</sub>, 1.4 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, and 11 or 2.5 glucose (pH 7.15–7.3); osmolality was adjusted to 290–310 mOsm/kg with sucrose; equimolar ACSF was made with by substitution sucrose for glucose substitution experiments. Slices were incubated for an equilibration period for  $\geq 1$  h in warmed (30–33°C), oxygenated ACSF prior to recording.

### **Electrophysiology**

A single brain slice was transferred to a recording chamber mounted on a fixed stage under an upright microscope (BX51WI; Olympus, Melville, NY), where they were continually perfused by warmed (30–33°C), oxygenated ACSF. EGFP-labeled NTS neurons were targeted for recording under a 40x water-immersion objective with fluorescence and infrared-differential interference contrast (IR-DIC) optics, as previously described (Gao et al. 2009; Glatzer et al. 2007; Williams et al. 2007). For recordings from EGFP-labeled NTS neurons, initial visualization was made briefly under epifluorescence using a fluorescein isothiocyanate (FITC) filter set (excitation filter wavelengths: 450-490 nm).

On-cell and whole-cell patch-clamp recordings were obtained in the NTS using pipettes pulled from borosilicate glass (Garner Glass, Claremont, CA; open tip resistance 4-6 M $\Omega$ ) using a Sutter P-87 horizontal puller (Sutter Instrument Co., Novato, CA). Pipettes were filled with a solution containing (in mM): 130 K<sup>+</sup>-gluconate (or Cs<sup>+</sup>-gluconate), 1 NaCl, 5 EGTA, 10 HEPES, 1 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>, 3 KOH (or CsOH), 2-4 ATP (pH 7.15 – 7.3). Electrophysiological signals were recorded using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA), low-pass filtered at 3 kHz, and recorded onto a PC-style computer (Digidata 1440A, Molecular Devices) using pClamp 10.2 software (Molecular Devices). A 2-4 G $\Omega$  seal was maintained for on-cell recordings. For whole-cell voltage-clamp recordings, series resistance was monitored throughout the recordings and data were used for analysis if the series resistance remained  $< 25$

MΩ and changed by  $\leq 20\%$  during the recording. Once a recording was obtained, cells were allowed to equilibrate for ~10 min before beginning data collection.

In whole-cell voltage-clamp recordings, resting potential was determined by monitoring the voltage at which no current was injected. Spontaneous EPSCs (sEPSC) and TTX-resistant (i.e., miniature) EPSCs (mEPSCs) were examined at a holding potential of -65 mV and IPSCs (sIPSCs and mIPSCs) were examined at 0 mV using pipettes containing Cs-gluconate to block K<sup>+</sup> currents, thereby improving voltage control and reducing noise. All drugs were bath-applied until a steady-state was reached. Drugs used included: the GCK inhibitor glucosamine (GA; 5 μM); the NMDA receptor antagonist AP5 (50 μM); the AMPA/KA receptor antagonist CNQX (10 μM); the GABAA receptor blocker picrotoxin (100 μM). AP5, CNQX, and picrotoxin were received from Sigma-Aldrich (St. Louis, MO). GA was received from MP Biomedical (Santa Ana, CA). For effects of elevated glucose concentration, slices were transferred to ACSF containing 2.5 mM glucose for at least 1 hr prior to on-cell recording. Spontaneous action potential activity was recorded for 10 min prior to changing to ACSF containing 15 mM glucose for 10 min before returning to 2.5 mM glucose solution (washout). Recordings of 2-3 min epochs were made prior to and after 10 min of drug- or 15 mM glucose-containing ACSF application. Washout was at least 15 min.

### **RNA isolation**

Brainstem slices (300 μm) were isolated as described for electrophysiological recordings. The dorsal vagal complex, including most of both the DMV and NTS, was visualized under a dissecting microscope and excised from the rest of the brainstem with a 1 mm diameter biopsy punch (Miltex, Inc. York, PA). Resulting punched-tissue were then suspended in 500 μL of TRIzol (Sigma-Aldrich) and gently shaken periodically for 5 min. Chloroform (100–250 μl) was added and tubes were vortexed for 15 s and then maintained at 4°C for 20 min and subsequently centrifuged at 12,000 rpm for 15 min at 4°C. The pellet was discarded and the supernatant containing RNA was transferred into fresh 1.5 ml centrifuge tubes, mixed with 500 μL of ice-cold propanol, incubated at room temperature for 10 min, and centrifuged at 12,000 rpm for 10 min at 4°C. Propanol was decanted and RNA was washed by re-suspension in 500 μL 75% ethanol followed by centrifugation at 7500–12,000 rpm for 10 min at 4°C. The wash step was repeated, the ethanol decanted, and RNA samples were air-dried for 10–20 min. RNA samples

were re-suspended in 8–10  $\mu$ L RNase-free water and stored at  $-80^{\circ}\text{C}$  or immediately reverse transcribed into cDNA.

### **TaqMan QPCR**

RNA samples were reverse transcribed in reverse-transcription master mix containing: 1  $\mu$ l random nonamers (50  $\mu$ M; Sigma-Aldrich), 5  $\mu$ l MMLV RT buffer (5x) (Fisher Scientific, Pittsburgh, PA), 5  $\mu$ l dNTPs (10 mM; Fisher), 2  $\mu$ l DEPC-treated H<sub>2</sub>O (Fisher), 1  $\mu$ l reverse transcriptase (Fisher), and RNase inhibitor (1  $\mu$ l; Fisher) in a thermocycler (Mastercycler, Eppendorf) at  $42^{\circ}\text{C}$  for 90 min followed by 5 min at  $95^{\circ}\text{C}$ . Negative controls included no template and RNase free sterile water instead of template. No RNA was detected in these controls. Primers and probes are shown in Table 1.

Master mix included 10  $\mu$ l of TaqMan Universal master mix II (Applied Biosystems), 1  $\mu$ l of primer/probe mix and 9  $\mu$ l Of cDNA+RNase-free sterile H<sub>2</sub>O. Samples were loaded into a 96-well plate in triplicate (Bio-Rad, Hercules, CA). Samples were centrifuged for 2 min at 1000 RPM and placed in an Applied Biosystems thermocycler (ABI 7500; Life Technologies, Grand Island, NY) for PCR analysis. Samples were held at  $95^{\circ}\text{C}$  2 min and cycled 50 times at  $95^{\circ}\text{C}$  for 30 s,  $60^{\circ}\text{C}$  for 15 s and at  $72^{\circ}\text{C}$  15 s. Gene transcript level from each mouse was individually measured; target transcript CT value was normalized to the CT value for  $\beta$ -actin. Results were analyzed by the 2<sup>-ddct</sup> method (Livak and Schmittgen 2001) and statistical data was evaluated using an unpaired student t-test. Differences of  $p < 0.05$  were considered significant.

Table 1. Primers and probes used in these experiments (all from Integrated DNA Technologies, Coral Gables, FL).

<b><math>\beta</math>-actin, Bactin, NM_007393 Actb;</b>
Probe 5'-/56-FAM/CTGGCCTCA/ZEN/CTGTCCACCTTCC/3IABkFQ/-3'
Primer 1 5'-GACTCATCGTACTCCTGCTTG-3'
Primer 2 5'-GATTACTGCTCTGGCTCCTAG-3'
<b>Glucokinase,GCK, NM_010292.4,</b>
Probe 5'-/56-FAM/CCTGAAGAA/ZEN/GGTGATGAGCCGG/3IABkFQ/-3'
Primer 1 5'-CGTAGGTGGGCAACATCTTT-3'
Primer 2 5'-AGAGCAGATCCTGGCAGAG-3'
<b>Glucose 6 Phosphatase G6pdx,NM_008062,</b>
Probe 5'-/56-FAM/CATGAGTCA/ZEN/GACAGGCTGGAACCG/3IABkFQ/-3'
Primer 1 5'-TGGTTCGACAGTTGATTGGAG-3'
Primer 2 5'-GAAGCAGTCACCAAGAACATTC-3'
<b>SUR1, Abcc8 NM_011510;</b>
Probe 5'-/56-FAM/ATCTGAGCG/ZEN/ACCACCTGATGCAG/3LabKFQ/-3'
Primer 1 5'-CACTGTCTCTTTGTCATCCC-3'
Primer 2 5'-CCTCTACCAGCACACCAATG-3'
<b>Kir6.2, Kcnj11, NM_001204411</b>
Probe 5'-/56-FAM/ACTCCAACA/ZEN/GCCCGCTCTACG/3IABkFQ/-3'
Primer 1 5'-GTTTCTACCACGCCTTCCAA-3'
Primer 2 5'-CCACTCATCATCTACCACGTC-3'
<b>Glucose Transporter 2 Glut2) Slc2a2 NM_031197,</b>
Probe 5'-/56-FAM/TCTTCACCA/ZEN/ACTGCCCTTGTC/3IABkFQ/-3'
Primer 1 5'-GATTGCCCAGAATAAAGCTGAG-3'
Primer 2 5'-GATCGCTCCAACCACACTC-3'

## Western Blots

Brainstem slices (300  $\mu\text{m}$ ) were cut as described above, the dorsal vagal complex was isolated from the slice, and tissue punches containing the DMV and NTS were immediately transferred to 40–60  $\mu\text{l}$  of lysis buffer consisting of 0.15M NaCl, 5mM EDTA (pH 8), 1% Triton X-100, 10mM Tris-HCl (pH 7.4), 10 $\mu\text{l}/\text{ml}$  of 100mM PMSF (174.2 mg/10ml in methanol), and 100 $\mu\text{l}/\text{ml}$  of 0.5M NaF (pH 10). Each sample was sonicated and centrifuged immediately at 12,000 RPM for 3 min. Supernatant was aspirated, aliquoted, and stored at  $-80^{\circ}\text{C}$  until further use. Protein concentration was measured using a Bradford Protein Assay and 20  $\mu\text{g}$  of protein was loaded per lane for Western blot analysis. The appropriate volume of sample together with equal amounts of loading buffer was boiled in water for 2 min. Samples and ladder were loaded into precast SDS polyacrylamide gels and electrophoresed at 50 mA for 45–80 min. Proteins were then transferred at 200 mA for 2 hours onto polyvinylidene difluoride membranes for Western blot analysis. Membranes were blocked in 1:1 Odyssey blocking buffer/TBS/0.1% Tween 20 for 1 hr at room temperature. Due to well-separated molecular weights of the GCK (band at 65 kD) and  $\beta$ -actin (band at 40–45 kD) protein membranes were cut in half and incubated over-night at  $4^{\circ}\text{C}$ , with a rabbit monoclonal anti-GCK (1:1000; Abcam, Cambridge, MA) and a rabbit monoclonal anti- $\beta$ -actin (1:10000; Abcam) antibody in Odyssey blocking buffer/TBS/0.1% Tween 20. Membranes were washed 4 times (5 min) with TBS on a shaker and treated for 1 hr with fluorescence-conjugated anti-rabbit IgG (IRDye 680RD; Li-Cor Biosciences, Lincoln, NE). Membranes were then washed (4 x 5 min) and scanned on a densitometer (Odyssey model 9120, Li-Cor Biosciences) to quantify band density. Background density was subtracted from the GCK band density and normalized to  $\beta$ -actin, which was used as a loading control.

## Data analysis

Recordings were analyzed using Clampfit 10.2 (Molecular Devices) and MiniAnalysis 6.0.7 software (Synaptosoft, Decatur, GA). At least two minutes of continuous recording under each condition was used to assess mean action potential frequency or EPSC and IPSC frequency and amplitude. The Kolmogorov-Smirnov (K-S) intra-assay test was used to determine statistical significance of drug- or glucose-induced changes in the frequency of action potentials, EPSCs, or IPSCs within a recording. For all electrophysiological experiments, a paired, two-tailed

Student's t-test was used to determine statistical significance between STZ-treated and saline-treated mice (Graphpad Prism, San Diego, CA). For quantitative RT-PCR and Western blots, an unpaired Student t-test was used to detect differences between samples from each group. Statistical significance for all measures was set at  $p < 0.05$ .

### **Chapter 3: Molecular and Functional changes in glucokinase expression in the brainstem dorsal vagal complex in a murine model of type 1 diabetes**

#### **Introduction**

Neurons in the brainstem nucleus of the solitary tract (NTS) receive glutamatergic, primary vagal afferent synaptic input from the gut and other thoracic and abdominal viscera. Vagal afferents rapidly convey information about gastrointestinal distention and nutrient content to the NTS, where that information is processed, integrated with neuronal and humoral signals, and transmitted to other brain areas, including to vagal motor neurons of the dorsal motor nucleus of the vagus (DMV). Neurons in the NTS respond to acutely altered glucose concentration with either increases or decreases in neural excitability and altered synaptic input (Balfour et al. 2006; Boychuk et al. 2015a; Lamy et al. 2014; Oomura et al. 1974; Wan et al. 2008), which are glucokinase (GCK)-dependent. The depolarizing response is mediated by inactivation of ATP-sensitive K<sup>+</sup> (KATP) channels (Balfour et al. 2006; Boychuk et al. 2015a) and KATP channel modulation prevents the glucose-induced, GABA mediated inhibition of vagal motor neurons (Ferreira et al. 2001). Type I diabetes is characterized by uncontrolled hyperglycemia due to reduced insulin secretion from pancreatic beta cells. Synaptic and other cellular responses in the dorsal vagal complex are altered in models of type 1 diabetes, even after normalizing glucose concentration (Bach et al. 2015; Blake and Smith 2014; Boychuk et al. 2015b; Browning 2013; Zsombok et al. 2011). Vagal reflexes are often blunted during chronic hyperglycemia, and altered vagal function may contribute to diabetes-associated visceral dysfunction (Saltzman and McCallum 1983; Undeland et al. 1998), suggesting that chronically-elevated glucose alters responsiveness of neurons in the dorsal vagal complex.

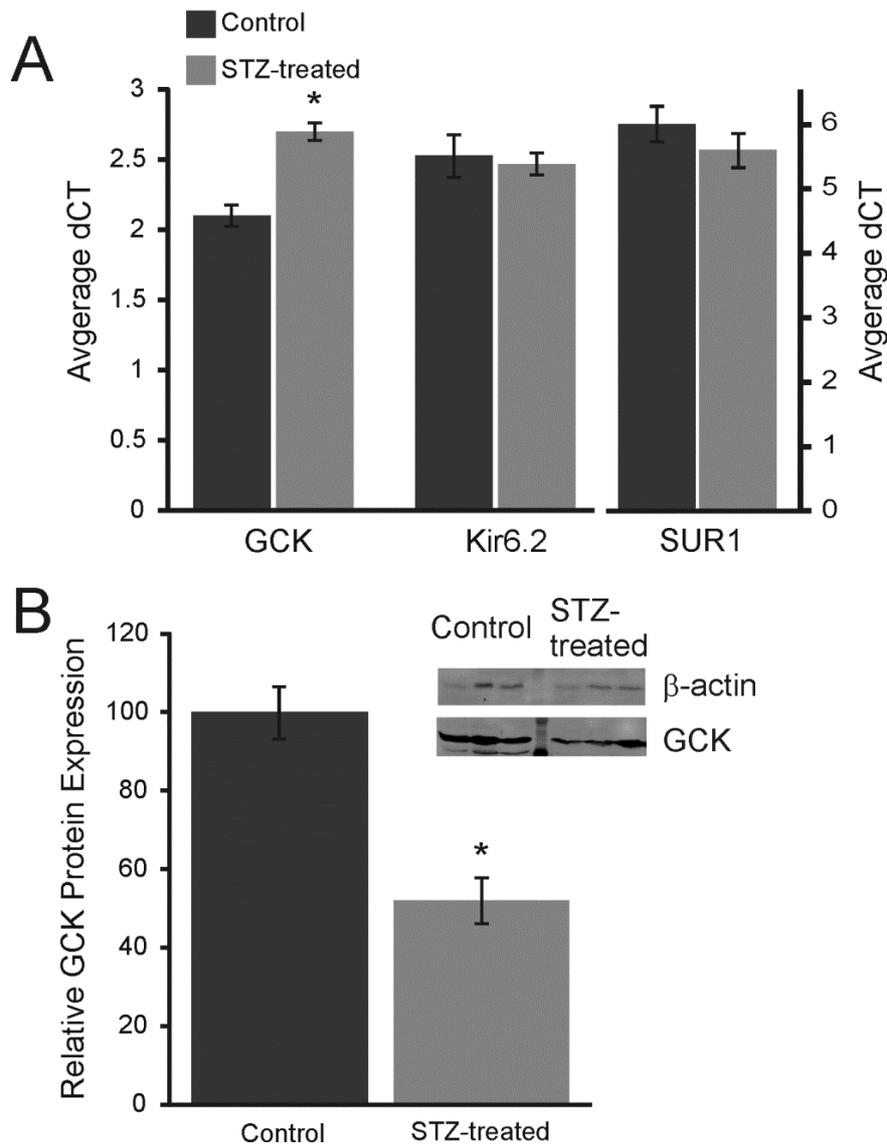
Because of the involvement of GCK and KATP channel modulation in the neuronal response to glucose, and the altered responsiveness of NTS neurons in animal models of type 1 diabetes, we tested the hypothesis that GCK or KATP channel expression is altered after several days of chronic hyperglycemia/hypoinsulemia in the streptozotocin (STZ)-treated mouse. Understanding how glucose sensitivity in the dorsal brainstem is altered in diabetes may offer hypotheses to guide development of alternative therapies for the disease.

## Results

*Molecular expression of GCK and KATP channels.* Punches of tissue containing the dorsal brainstem were collected from normoglycemic (n=8; glucose index  $180 \pm 4$  mg/dl) and STZ-treated CD-1 mice that were hyperglycemic for 3-4 days (n=8;  $469 \pm 5$  mg/dl). All target transcript measurements were normalized to  $\beta$ -actin expression. Quantitative RT-PCR revealed a significant decrease in GCK expression in the dorsal vagal complex from STZ-treated hyperglycemic CD1 mice versus controls ( $p < 0.05$ ; Fig. 3.1). GCK expression was similarly reduced in the vagal complex of GIN mice (n=5 control, n=7 STZ-treated;  $p < 0.05$ ). No significant expression differences were detected for Kir6.2 or SUR1 transcripts between normoglycemic and hyperglycemic CD-1 mice ( $p > 0.05$ ; Fig. 3.1). Molecular expression of GCK, but not components of the KATP channel, was reduced in STZ-treated, hyperglycemic mice relative to control mice.

To determine if the decrease in mRNA transcription correlated with decreased protein expression, Western blots were performed on punches from an additional 8 control and 8 STZ-treated hyperglycemic CD-1 mice. Western blot analysis indicated that GCK protein expression was significantly reduced in the dorsal vagal complex of STZ-treated hyperglycemic mice ( $p < 0.05$ ; Fig. 3.1).

*Effect of GCK inhibition on synaptic input to NTS neurons.* Glucokinase inhibition prevents responses to acute hypoglycemia in a subset of GABAergic NTS neurons (Lamy et al. 2014). Since GCK expression was reduced in the NTS of STZ-treated mice after several days of hyperglycemia, responses to the GCK inhibitor, GA (5  $\mu$ M) were determined in GABAergic medial NTS (mNTS) neurons, identified by expression of EGFP in acute slices from normoglycemic control (n=5) and STZ-treated GIN mice (n=5). In GABAergic mNTS neurons from normal mice, GA application decreased the frequency of sEPSCs by  $\geq 20\%$  in 80% of neurons (12 of 15 neurons;  $2.83 \pm 0.34$  Hz control ACSF;  $1.72 \pm 0.22$  Hz in GA; n=15;  $p < 0.05$ ; Fig. 3.2), with no change in sEPSC frequency in the remaining three neurons. No effect on synaptic current amplitude was detected ( $p > 0.05$ ). In the presence of TTX (2  $\mu$ M) to prevent action potential firing, there was no significant effect of GA on miniature EPSC (mEPSC) frequency in control mice ( $p > 0.05$ ); mEPSC frequency was decreased slightly in only one of five



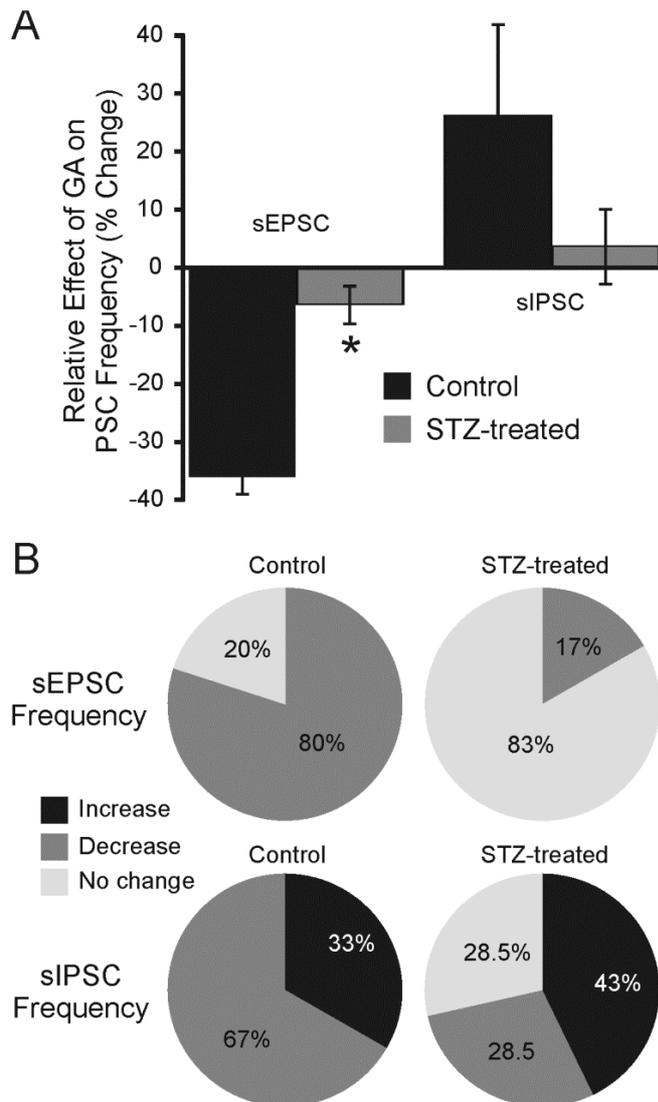
**Figure 3.1.** Expression of glucokinase (GCK) and ATP-sensitive  $K^+$  ( $K_{ATP}$ ) channels in the dorsal vagal complex of normoglycemic control and hyperglycemic, streptozotocin (STZ)-treated mice. **A.** Molecular expression of GCK mRNA was significantly reduced in the dorsal vagal complex of STZ-treated mice after 3-4 days of chronic hyperglycemia versus control mice ( $p < 0.05$ ;  $n = 8$  for each group). Expression of Kir6.2 and SUR1, components of the  $K_{ATP}$  channel, were not significantly altered ( $p > 0.05$ ). **B.** Western blot analysis indicated that GCK protein expression was lower in the dorsal vagal complex of STZ-treated mice ( $n = 6$ ) than controls ( $p < 0.05$ ;  $n = 8$ ). Inset: Sample blot of  $\beta$ -actin and GCK protein expression from control and STZ-treated mice; center lane is protein ladder. Asterisks indicate  $p < 0.05$ .

neurons from three mice, so effects in STZ-treated mice were not assessed. These findings are consistent with an effect of GCK in mediating spontaneous excitatory synaptic responses to ambient glucose levels in NTS neurons (Wan and Browning, 2008) and indicated that GCK blockade inhibited excitatory, glutamatergic synaptic input to most GABA neurons.

In GABAergic mNTS neurons from STZ-treated mice after 3-5 days of hyperglycemia (n=5), effects of GA on sEPSC frequency were significantly less robust than in controls ( $p < 0.05$ ; Chi-square), being reduced (-22%) in only one of six neurons and unchanged in the remaining five cells (Fig. 3.2). Overall, mean sEPSC frequency was unchanged in the presence of GA ( $7.63 \pm 1.04$  Hz in control ACSF;  $7.04 \pm 0.83$  Hz in GA; n=6;  $p > 0.05$ ). Consistent with the decreased GCK expression in the vagal complex, modulation of excitatory synaptic activity by GA was reduced in GABAergic mNTS neurons from STZ-treated, hyperglycemic mice.

Effects of GA application on sIPSC frequency and amplitude were also determined. In GABAergic mNTS neurons from control mice (n=5), GA application was without effect on the overall population ( $1.22 \pm 0.2$  Hz control, ACSF;  $1.25 \pm 0.30$  Hz, GA; n=9;  $p > 0.05$ ), but was either increased (n=3) or decreased (n=6) in individual neurons (Fig. 3.2). The amplitude of sIPSCs was unchanged by GA ( $p > 0.05$ ). Similar to results in control mice, there was no overall effect on sIPSC frequency in neurons from STZ-treated mice ( $1.04 \pm 0.30$  Hz control ACSF;  $1.01 \pm 0.31$  Hz GA; n=7;  $p > 0.05$ ). sIPSC amplitude was also unchanged ( $p > 0.05$ ). In neurons from STZ-treated mice, GA application either increased (n=3), decreased (n=2) or was without effect (n=2) on sIPSC frequency (Fig. 3.2). There was no significant effect of GA on mIPSC frequency in control mice; mIPSC frequency was increased slightly in only two of five neurons from four mice, so effects in STZ-treated mice were not assessed. Although sIPSC frequency in GABAergic mNTS neurons was usually altered by GA application, robust differences between responses in neurons from control and STZ-treated mice were not observed ( $p > 0.05$ ; Chi-square).

*Glucose effect on Action Potentials.* Neurons in the NTS are glucose sensors, and this sensitivity may be especially prominent in GABAergic NTS neurons (Boychuk et al. 2015a; Ferreira et al. 2001; Lamy et al. 2014). Since GCK expression was reduced in the NTS of STZ-treated mice after several days of hyperglycemia, we examined action potential frequency of



**Figure 3.2.** Modulation of synaptic responses in NTS neurons by glucosamine (GA). **A.** Graph showing the relative overall effect of GA on sEPSCs and sIPSCs in GABAergic mNTS neurons from control and STZ-treated mice (n=6-15). sEPSC frequency was suppressed in the presence of GA in 80% of neurons from control mice, but GA was generally without effect on sEPSC frequency in most neurons (83%) from STZ-treated mice. Asterisk indicates significant difference from control group for sEPSC frequency. Effects of GA on sIPSCs were variable in both control and STZ-treated mice. **B.** Pie graphs indicating the percentage of neurons in which GA application evoked a  $\geq 20\%$  change in frequency of either sEPSCs or sIPSCs in mice from each treatment group.

GABAergic NTS neurons in response to elevating glucose from 2.5 to 15 mM using on-cell recordings. Increasing glucose concentration resulted in a >20% change in action potential frequency in 77% of neurons (10 of 13 cells) in neurons from normoglycemic control mice (n=6). Increasing glucose concentration resulted in an increase in action potential frequency in seven of 13 neurons ( $122 \pm 57\%$  increase; Fig. 3.3), a decrease in three neurons ( $-27 \pm 2\%$  decrease), and no change in three neurons. In neurons from STZ-treated, hyperglycemic mice (n=6), elevating glucose resulted in a change in action potential frequency in only five of 15 neurons (33%), a significantly lower percentage of responses than in neurons from control mice ( $p < 0.05$ , two-tailed Chi-square). The frequency of action potentials was increased in three neurons ( $111 \pm 22\%$ ), decreased in two cells ( $-55 \pm 10\%$ ), and was unaffected in the remaining 10 cells. Whereas increasing glucose concentration resulted in a significant and large change in action potential frequency in the majority of neurons in normoglycemic mice, action potential frequency in most neurons from hyperglycemic mice was unaffected by increasing glucose concentration.

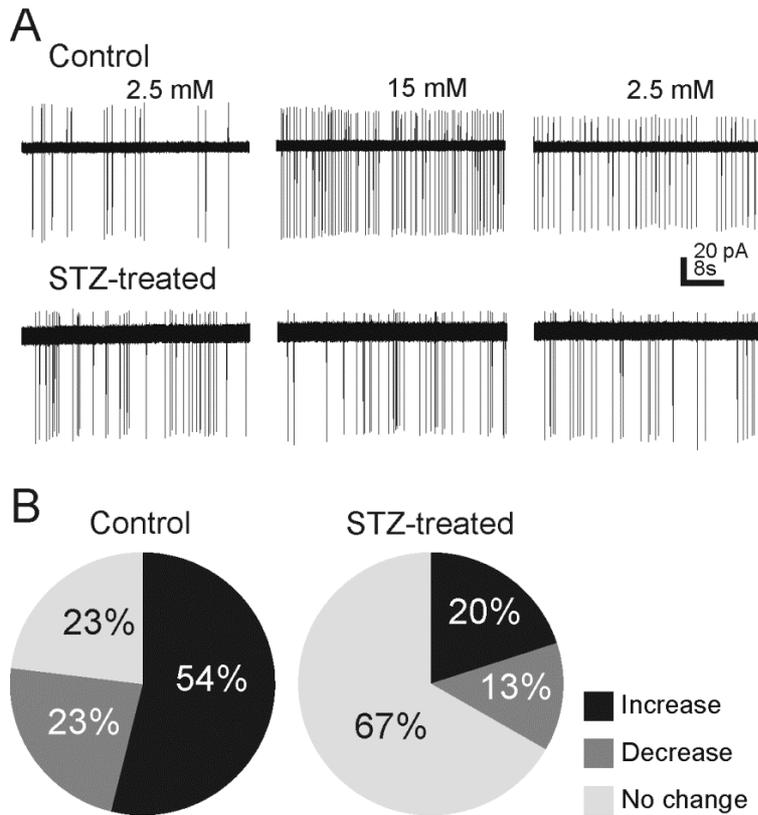


Figure 3.3. Effects of elevated glucose on sodium currents (action potentials) in NTS neurons from control and STZ-treated mice. **A.** Examples of on-cell recordings in GABAergic mNTS neurons from control (upper) and STZ-treated (lower) mice showing spontaneous action potential (sodium current) activity in ACSF containing 2.5 mM glucose, 15 mM glucose, and after 15 min wash to 2.5 mM glucose. **B.** Pie graphs indicating the proportion of neurons (n=13 control; n=15 STZ-treated mice) that responded with an increase, decrease, or no change in action potentials when glucose concentration was elevated. Glucose elevation most often increased action potential firing in neurons from control mice, whereas firing rate was unchanged in most neurons from STZ-treated mice.

## Discussion

Neurons in the NTS receive direct input from the primary viscerosensory vagal afferents and comprise the initial response component of central parasympathetic regulatory circuits. Subsets of these neurons, and GABA neurons in particular, are known to be glucose-sensitive (Balfour et al. 2006; Boychuk et al. 2015a; Browning 2013; Lamy et al. 2014; Oomura et al. 1974; Wan and Browning 2008). Several physiological aspects of central vagal circuitry are altered functionally in diabetes (Bach et al. 2015; Blake and Smith 2014; Boychuk et al. 2015b; Browning 2013; Zsombok et al. 2011), consistent with modified parasympathetic regulation of the viscera concurrent with the disease (Saltzman and McCallum 1983). Glucose sensing in NTS neurons involves GCK, which catalyzes the conversion of glucose to glucose-6 phosphate in neurons and other cells (Balfour et al. 2006; Briski et al. 2009), resulting in increased ATP/ADP ratio. In several neural systems, membrane responses after increased glucose concentration are caused by ATP binding to KATP channels to affect changes in membrane potential. Diabetes induces changes in GCK or KATP channel expression in specific hypothalamic nuclei (Levin and Dunn-Meynell 1998; Nishio et al. 2006), which are consistent with altered or compensatory neuronal responses to chronically-elevated glucose or hypoinsulemia in STZ-treated rats. Altered electrophysiological responsiveness of NTS neurons in type 1 diabetes has been demonstrated (Bach et al. 2015; Browning 2013), which could contribute to visceral dysregulation in diabetes, but the mechanisms of this plasticity are unknown. Here, we found that molecular and functional expression of GCK—but not components of the KATP channel—were diminished in the vagal complex of mice with type 1 diabetes. Consistent with decreased mRNA transcription, GCK protein levels were reduced, neuronal and synaptic responses to GCK blockade were attenuated, and neuronal activity responses to increased glucose concentration were diminished. A similar decrease in GCK expression and activity was reported in the arcuate nucleus, but not in other glucose-responsive hypothalamic nuclei, of STZ-treated rats (Nishio et al. 2006). The present findings suggest that responses of NTS neurons to increased glucose concentration may be altered as a consequence of chronic hyperglycemia or hypoinsulinemia in a GCK-dependent manner, similar to glucose-sensitive neurons of the arcuate nucleus.

Neurons in the NTS normally respond to elevated or reduced glucose concentration with either increases or decreases in excitability (Balfour et al. 2006; Boychuk et al. 2015a; Browning

2013; Lamy et al. 2014; Oomura et al. 1974; Wan et al. 2008), which are often GCK-dependent. Glucose-induced increases in excitation are mediated by inactivation of KATP channels in NTS neurons (Balfour et al. 2006; Boychuk et al. 2015a) and KATP channel modulation prevents the glucose-induced, GABA mediated inhibition of vagal motor neurons (Ferreira et al. 2001). We tested the hypothesis that expression of molecular components of the KATP channel was altered after several days of hyperglycemia. KATP channels in central neurons are mainly composed of SUR1 and Kir6.2 subunits and SUR1 is expressed by glucose-sensing NTS cells (Balfour et al. 2006). In STZ-treated rats, SUR binding is increased in neurons of the dorsomedial, ventromedial, and lateral—but not paraventricular—hypothalamic nuclei after seven days of hyperglycemia (Levin and Dunn-Meynell 1998), suggesting increased KATP channel expression that may serve a compensatory function in specific neurons. We found that molecular expression of SUR1 and Kir6.2 was unchanged in the vagal complex of mice with type 1 diabetes, which is inconsistent with the hypothesis that SUR1 activity increases in the NTS after several days of chronic hyperglycemia.

Since KATP channel-mediated responses to glucose in the NTS require GCK, the decrease in GCK expression suggests a mechanism for blunted responsiveness to glucose that has been proposed to occur in chronically hyperglycemic mice (Browning 2013). The decreased molecular and protein expression of GCK we observed was consistent with an attenuation of the electrophysiological response to GCK inhibition. Blockade of GCK activity resulted in altered synaptic excitability of most GABAergic NTS neurons from normoglycemic mice, and this effect was mainly action potential-dependent, consistent with tonic GCK-mediated effects on neuronal activity in the slice. Notably, it is conceivable that blockade of GCK activity might deplete energy supplies. This potential confound is mitigated, however, by the fact that application was limited to 10 min in this study. Further, postsynaptic current amplitude was not altered by GA, suggesting that neural responsiveness was not overtly affected. The effects of blocking GCK were reduced in neurons from mice with type 1 diabetes, especially on action potential-dependent glutamate release, suggesting that GABAergic NTS neurons may be less responsive to excitatory synaptic input in mice with type 1 diabetes. Moreover, responsiveness of GABAergic NTS neurons to increased glucose concentration was attenuated in STZ-treated, hyperglycemic mice. This further implies that synaptic activity in the NTS normally occurs in

the context of glucose concentration, since GCK mediates glucose-responsiveness in these neurons.

Previous studies indicated that responses of NTS neurons to acute hypoglycemia required GCK activity (Balfour et al. 2006; Lamy et al. 2014). Responses to transient hypoglycemia were previously reported in NTS neurons that expressed glucose transporter 2 (GLUT2) and the expression of the transporter was colocalized in a subset of GABA neurons (Lamy et al. 2014). We recently showed that GABA neurons were either depolarized or hyperpolarized by glucose, and the depolarization in particular was sensitive to blockade of GCK activity or by blocking KATP channels (Boychuk et al. 2015a). Here, we found that attenuated neuronal responses to transient hyperglycemia in mice with type 1 diabetes coincided with reduced molecular, protein, and functional GCK expression in the vagal complex, suggesting that prolonged hyperglycemia affects glucose responsiveness in the vagal complex.

Elevated glucose concentration increases glutamatergic synaptic transmission from vagal afferents in rats and mice, and these effects were reported to be attenuated in a model of type 1 diabetes (Browning 2013). Our results are consistent with this report, and offer a mechanistic explanation for the loss of response. The glucoregulatory response to nutritive substances applied in the intestine is prevented by blocking ionotropic glutamate receptors in the NTS (Cheung et al. 2009; Wang et al. 2008), suggesting that glutamatergic, vagal afferent activation of NTS neurons is required for this response. Blockade of NMDA receptors in the vagal complex prevents the positive effect of bariatric surgery on systemic blood glucose levels, an effect hypothesized to occur by inhibiting a “gut-brainstem-liver” circuit in the vagal complex (Verberne et al. 2014).

Our results support the hypothesis that diminished GCK expression in the vagal complex of mice with type 1 diabetes results in reduced responsiveness of NTS neurons to glucose, including the response to synaptic glutamate release. It is likely that glutamatergic, visceral afferent synaptic input to the NTS, including input mediating mechano- and chemoreceptor activity in the gut (Barber and Burks 1987), occurs in the context of glucose concentration. Restoring GCK expression may help restore physiological responsiveness of NTS neurons to glucose, thereby helping to normalize parasympathetic regulation of visceral function, including glucose homeostasis, in diabetes.

## Chapter 4 : General Discussion

Our findings suggest a new direction to guide research on how diabetes is governed by the brain, and will hopefully will prompt development of new therapies to treat how diabetes, based on regulation of vagal circuitry. Chronic hyperglycemia modifies vagally-mediated visceral function. Parasympathetic visceral tone is decreased and that leads to a collapse of metabolic homeostasis, which often occurs with chronically elevated gluconeogenesis. GABAergic input from the NTS to the DMV neurons is less effective in regulating vagal output after several days of hyperglycemia. Hyperglycemia creates fundamental changes in the balance of synaptic input to vagal motor neurons, altering the function of glutamatergic NTS neurons that projects to the DMV (Bach et al. 2015; Zsombok and Smith 2009). The sustained upregulation of glutamate release in the DMV in the hyperglycemic state likely contributes to continued visceral dysregulation, even after glucose levels are normalized. In addition, sustained changes in GABAergic signaling in the DMV may also contribute to vagal dysregulation in diabetes (Boychuk et al. 2015b). Here, I found that GCK expression in the NTS was diminished, and this was accompanied by decreased glucose responsiveness in most NTS GABA neurons. Together, these data implicate sustained changes in regulation of central vagal neurons as a possible contributor to autonomic dysregulation in diabetes. Chronic dysregulation of visceral autonomic control may be a contributing factor in the development of insulin resistance in diabetes (Carnethon et al. 2003).

GABAergic NTS neurons sense changes in glucose concentration by  $K^+$  channel- and GCK-dependent alterations in membrane potential and responses to primary afferent input. We identified the GABA neurons responses in the NTS were altered when blood glucose concentration was elevated in the DVC of intact mice. Increased glucose concentration resulted in an action potential frequency change in the majority of GABAergic NTS neurons from normoglycemic, but not in diabetic mice as we mentioned (Chapter 3). NTS GABA neurons are principal mediators of direct glucose effects in central parasympathetic circuits, and these neurons are also critical modulators of ongoing vagal motor activity. The inability of these neurons to appropriately integrate synaptic and other electrical activity in the context of glucose concentration seems likely to have profound effects on parasympathetic visceral regulation in diabetes. A reduction in molecular and functional GCK expression was detected in the DVC of

diabetic mice, consistent with the reduced glucose-sensitivity of NTS GABA neurons. This supports our hypothesis is that long-term changes in gene expression underlies altered GCK function is altered glucose sensing in these neurons.

Hyperglycemia significantly decreases GCK expression in other regions of the brain that have been implicated in regulating metabolic homeostasis. In the dorsomedial and ventromedial nuclei of the hypothalamus, expression of elements of the KATP channel are also altered in diabetes, but no such changes in Kir6.2 or SUR1 expression were observed in the present study. This could be due to the relatively short time of the diabetes symptoms in this study (i.e., 3-4 days) relative to the study in the hypothalamus (Dunn-Meynell et al. 2002). Longer periods of hyperglycemia may eventually result in compensatory increases in KATP expression (Levin et al. 1999). In addition, AMPK-dependent protein trafficking can also be affected by GCK activity. Experiments to assess KATP channel function may help determine if channel internalization occurs in diabetes, as occurs for other proteins in this model (Zsombok et al. 2011). Alternatively, other channels (i.e., BK or leak K) may mediate the glucose-induced hyperpolarization in NTS GABA neurons, since blocking KATP channels with tolbutamide did not always affect the glucose-induced hyperpolarization in these cells (Boychuk et al. 2015a). Investigation of potential molecular changes in AMPK or other K channels may provide more comprehensive insight to the effects of diabetes development in the DVC.

The triggers for the diabetes-induced response plasticity in NTS GABA neurons are not known, however several possibilities exist. Chronic exposure to glucose might induce a compensatory reduction in GCK. In addition, insulin is known to traffic proteins to cell membranes of diabetic mice, including the TRPV1 receptor on GABAergic terminals contacting DMV neurons (Zsombok et al. 2011). Since hypoglycemia is a hallmark of the model used in these studies, it is possible that reduced insulin levels may also contribute to the changes observed here. Alternatively, glutamate is the primary neurotransmitter released by viscerosensory afferents, and elevated glucose concentration enhances glutamate release from these terminals (Boychuk et al. 2015a; Wan and Browning 2008). In addition, glutamate release is chronically increased in the DMV of diabetic mice (Bach et al. 2015; Zsombok et al. 2011). It is possible that continually increased glutamate in the vagal complex of mice with type 1 diabetes induces activity-dependent changes in protein expression, phosphorylation, or

trafficking that underlie the diminished GCK expression and response to glucose in the NTS. Further studies are necessary to resolve these questions.

Pressing questions prompted by these studies include whether the GCK expression decrease and loss of glucose-responsiveness in diabetic mice can be resolved readily or by using means other than glycemic control. Further, understanding the effect on systemic glucose concentration of normalizing GCK expression in the DVC would also help determine the necessity of appropriate neuronal responses in the DVC for regulating metabolic homeostasis. Notably, activation of vagal afferent terminals by a number of substances (e.g., GLP-1, CCK, PYY, fatty acids, glucose) at the level of the gut results in altered blood glucose concentration in a manner that involves a brain-centered glucose regulatory system (Breen et al. 2013; Wang et al. 2008). Several studies have suggested the blood [glucose] regulating output is mediated by vagal inhibition of hepatic gluconeogenesis (Breen et al. 2013; Breen et al. 2012), but others have suggested that the hepatic vagus nerve is not necessary for effects of Roux-en-Y bypass surgery on body weight (Shin et al. 2012). Regardless of output, a non-insulin-dependent, brain-initiated glucose control system has been proposed, in which disruption of both brain and insulin glucoregulatory systems contribute to diabetes (Schwartz et al. 2013). There is growing evidence that gastric bypass surgery rapidly resolves type 2 diabetes (i.e., independent of weight loss) in patients and animal models (Chambers et al. 2011; Ryan et al. 2014; Schauer et al. 2012), and one report indicates similar results in the STZ-treated, type 1 diabetes model (Breen et al. 2013). It is interesting to postulate that reinstatement of normal glucose-responsiveness in the NTS, possibly due to effects of the surgery on vagal afferent signaling, may provide a mechanism accounting for the positive and rapid effects of bariatric surgery on blood glucose regulation.

The results of this study suggest a novel perspective on how neurons in the brainstem respond to metabolic changes associated with diabetes. Because of their unique involvement in visceral regulation, this perspective also includes insight into the role of brainstem circuitry in the etiology of the disease itself. The eventual goal of this line of research is to discover new potential therapies aimed at modulating NTS neuron responses to systemic hyperglycemia in an effort to reinstate autonomic control over glucose homeostasis. The issues identified here provide the basis for further investigation into development of therapies to treat diabetes, based on modulation of neural function in the DVC.

## Appendices

### Appendix 1: List of Abbreviations

ACSF-Artificial cerebrospinal fluid

ADP-Adenosine Diphosphate

ATP-Adenosine Triphosphate

Ct-Cycle threshold

DMV-Dorsal motor nucleus of the vagus nerve

DVC-Dorsal Vagal complex

EPSC-Excitatory postsynaptic current

IML, intermediolateral cell column

IPSC-Inhibitory postsynaptic current

GA-Glucosamine

GCK-Glucokinase

Glut2-Glucose transporter 2

LH, lateral hypothalamus

NTS, nucleus tractus solitarius

PVN, paraventricular nucleus

TTX-Tetrodotoxin

sEPSC-spontaneous EPSC

sIPSC-spontaneous IPSC

STZ-Streptozotocin

SUR1-Sulfonylurea receptor 1

VLM, ventrolateral medulla

VMH, ventromedial hypothalamus

**Appendix 2:** Equipment used for Electrophysiological data acquisition and brainstem slice preparation

List of Equipment

Vibrating Microtome (Vibratome 1000 Plus)

Pipette Puller (Sutter P-87)

Vibration Isolation Table (TMC)

Microscope (Olympus BX51WI)

Digitizer (Digidata 1320A and 1440A)

Patch-Clamp Amplifier (Axopatch 200B or Multiclamp 700B)

Signal timer for electrical stimulation (Master-8)

PCR Machine- ABI 7500 Fast Time (Applied Biosciences)

NanoDrop (Fischer)

ABI 7500 Real Time PCR (ABI)

### Appendix 3. Glucose concentrations of type 1 diabetic animals used for experiments

Bodyweight, day1	Blood glucose, day 1	Bodyweight, day2	Blood glucose, day2	Bodyweight, day3	Blood glucose, day3	Bodyweight, day4	Blood glucose, day4	Bodyweight, day5	Blood glucose, day 5
26.7 gr	187mg/dl	28.2 gr	195mg/dl	27.2 gr	188mg/dl	29.4 gr	550mg/dl	25.2 gr	554mg/dl
28.9 gr	179mg/dl	30.0 gr	378mg/dl	26.7 gr	547mg/dl	27.3 gr	599mg/dl	23.8 gr	553mg/dl
31.5 gr	186mg/dl	30.1 gr	362mg/dl	27.8 gr	599mg/dl	27.3 gr	599mg/dl	27.8 gr	575mg/dl
27.4 gr	107mg/dl	27.6 gr	126mg/dl	28.0 gr	186mg/dl	29.0 gr	191mg/dl	29.1 gr	178mg/dl
29.0 gr	184mg/dl	29.4 gr	181mg/dl	31.2 gr	175mg/dl	31.6 gr	189mg/dl	31.5 gr	174mg/dl
28.4 gr	177mg/dl	28.6 gr	200mg/dl	30.0 gr	192mg/dl	31.0 gr	190mg/dl		
23.5 gr	178mg/dl	23.6 gr	172mg/dl	23.9 gr	167mg/dl	24.2 gr	181mg/dl		
29.0 gr	199mg/dl	28.4 gr	140mg/dl	26.5 gr	599mg/dl	26.8 gr	599mg/dl	26.2 gr	399mg/dl
30.0 gr	166mg/dl	30.3 gr	171mg/dl	30.0 gr	195mg/dl	30.3 gr	599mg/dl	22.7 gr	599mg/dl
30.1 gr	194mg/dl	30.2 gr	190mg/dl	28.6 gr	412mg/dl	27.0 gr	599mg/dl	26.4 gr	392mg/dl
27.5 gr	183mg/dl	29.6 gr	197mg/dl	31.0 gr	170mg/dl	31.1 gr	181mg/dl		
27.3 gr	152mg/dl	29.8 gr	203mg/dl	30.7 gr	179mg/dl	31.0 gr	180mg/dl		
26.2 gr	172mg/dl	27.2 gr	186mg/dl	27.0 gr	201mg/dl	27.1 gr	184mg/dl		
26.1 gr	164mg/dl	28.0 gr	178mg/dl	28.2 gr	174mg/dl	28.3 gr	175mg/dl		
27.5 gr	157mg/dl	28.4 gr	167mg/dl	29.0 gr	202mg/dl	29.6 gr	194mg/dl		
27.6 gr	135mg/dl	29.3 gr	184mg/dl	30.0 gr	226mg/dl				
26.8 gr	180mg/dl	29.4 gr	191mg/dl	29.4 gr	197mg/dl				
25.1 gr	156mg/dl	26.7 gr	170mg/dl	26.5 gr	172mg/dl				
24.5 gr	119mg/dl	20.0 gr	529mg/dl	19.0 gr	555mg/dl	17.5 gr	599mg/dl		
23.9 gr	100mg/dl	20.6 gr	510mg/dl	19.3 gr	599mg/dl	18.2 gr	483mg/dl		
27.8 gr	131mg/dl	25.6 gr	599mg/dl	24.9 gr	599mg/dl	20.4 gr	514mg/dl		
26.8 gr	142mg/dl	25.8 gr	553mg/dl	25.0 gr	530mg/dl	23.4 gr	569mg/dl		
28.4 gr	161mg/dl	26.4 gr	509mg/dl	25.3 gr	490mg/dl	24.6 gr	590mg/dl		
26.2 gr	162mg/dl	26.0 gr	493mg/dl	26.0 gr	492mg/dl	25.6 gr	486mg/dl		
29.2 gr	147mg/dl	28.7 gr	507mg/dl	28.4 gr	599mg/dl	28.1 gr	523mg/dl		
26.1 gr	167mg/dl	25.8 gr	510mg/dl	24.8 gr	516mg/dl	23.4 gr	580mg/dl		
22.6 gr	225mg/dl	22.7 gr	368mg/dl	21.5 gr	415mg/dl	21.5 gr	440mg/dl	21.0 gr	454mg/dl
20.2 gr	242mg/dl	20.2 gr	380mg/dl	19.2 gr	465mg/dl	17.3 gr	471mg/dl	18.8 gr	600mg/dl
23.4 gr	200mg/dl	22.3 gr	390mg/dl	22.1 gr	450mg/dl	22.0 gr	480mg/dl	21.9 gr	550mg/dl
21.6 gr	187mg/dl	21.6 gr	440mg/dl	21.0 gr	465mg/dl	20.9 gr	520mg/dl	20.2 gr	590mg/dl

## References

- Ashcroft FM, Harrison DE, and Ashcroft SJ.** Glucose induces closure of single potassium channels in isolated rat pancreatic beta-cells. *Nature* 312: 446-448, 1984.
- Ashford ML, Boden PR, and Treherne JM.** Glucose-induced excitation of hypothalamic neurones is mediated by ATP-sensitive K<sup>+</sup> channels. *Pflugers Arch* 415: 479-483, 1990.
- Babenko AP, Polak M, Cave H, Busiah K, Czernichow P, Scharfmann R, Bryan J, Aguilar-Bryan L, Vaxillaire M, and Froguel P.** Activating mutations in the ABCC8 gene in neonatal diabetes mellitus. *The New England journal of medicine* 355: 456-466, 2006.
- Bach EC, Halmos KC, and Smith BN.** Enhanced NMDA Receptor-Mediated Modulation of Excitatory Neurotransmission in the Dorsal Vagal Complex of Streptozotocin-Treated, Chronically Hyperglycemic Mice. *PloS one* 10: e0121022, 2015.
- Balfour RH, Hansen AM, and Trapp S.** Neuronal responses to transient hypoglycaemia in the dorsal vagal complex of the rat brainstem. *The J Physiol* 570: 469-484, 2006.
- Balfour RH, and Trapp S.** Ionic currents underlying the response of rat dorsal vagal neurones to hypoglycaemia and chemical anoxia. *J Physiol* 579: 691-702, 2007.
- Barber WD, and Burks TF.** The response of brainstem neurons to chemical activation of gastric sensory receptors. *Gastroenterology Clinics of North America* 16: 521-524, 1987.
- Blake CB, and Smith BN.** cAMP-dependent insulin modulation of synaptic inhibition in neurons of the dorsal motor nucleus of the vagus is altered in diabetic mice. *Am J Physiol Regul Integr Comp Physiol* 307: R711-720, 2014.
- Blake CB, and Smith BN.** Insulin reduces excitation in gastric-related neurons of the dorsal motor nucleus of the vagus. *Am J Physiol Regul Integr Comp Physiol* 303: R807-814, 2012.
- Boychuk CR, Gyarmati P, Xu H, and Smith BN.** Glucose sensing by GABAergic neurons in the mouse nucleus tractus solitarius. *J Neurophysiol* jn 00310 02015, 2015a.
- Boychuk CR, Halmos K, and Smith BN.** Diabetes induces GABA receptor plasticity in murine vagal motor neurons. *J Neurophysiol* jn 00209 02015, 2015b.
- Breen DM, Rasmussen BA, Cote CD, Jackson VM, and Lam TK.** Nutrient-sensing mechanisms in the gut as therapeutic targets for diabetes. *Diabetes* 62: 3005-3013, 2013.
- Breen DM, Rasmussen BA, Kokorovic A, Wang R, Cheung GW, and Lam TK.** Jejunal nutrient sensing is required for duodenal-jejunal bypass surgery to rapidly lower glucose concentrations in uncontrolled diabetes. *Nature Medicine* 18: 950-955, 2012.
- Briski KP, Cherian AK, Genabai NK, and Vavaiya KV.** In situ coexpression of glucose and monocarboxylate transporter mRNAs in metabolic-sensitive caudal dorsal vagal complex catecholaminergic neurons: transcriptional reactivity to insulin-induced hypoglycemia and caudal hindbrain glucose or lactate repletion during insulin-induced hypoglycemia. *Neuroscience* 164: 1152-1160, 2009.
- Browning KN.** Modulation of gastrointestinal vagal neurocircuits by hyperglycemia. *Front Neurosci* 7:217.: 10.3389/fnins.2013.00217. eCollection 02013., 2013.
- Browning KN, and Travagli RA.** Plasticity of vagal brainstem circuits in the control of gastrointestinal function. *Auton Neurosci* 161: 6-13, 2011.
- Carnethon MR, Golden SH, Folsom AR, Haskell W, and Liao D.** Prospective investigation of autonomic nervous system function and the development of type 2 diabetes: the Atherosclerosis Risk In Communities study, 1987-1998. *Circulation* 107: 2190-2195, 2003.
- Chambers AP, Stefater MA, Wilson-Perez HE, Jessen L, Sisley S, Ryan KK, Gaitonde S, Sorrell JE, Toure M, Berger J, D'Alessio DA, Sandoval DA, Seeley RJ, and Woods SC.**

Similar effects of roux-en-Y gastric bypass and vertical sleeve gastrectomy on glucose regulation in rats. *Physiol Behav* 105: 120-123, 2011.

**Cheung GW, Kokorovic A, Lam CK, Chari M, and Lam TK.** Intestinal cholecystokinin controls glucose production through a neuronal network. *Cell Metab* 10: 99-109, 2009.

**Craighead JE.** Experimental models of juvenile onset (insulin-dependent) diabetes mellitus. *Monogr Pathol* 21: 166-176, 1980.

**Davis SF, Derbenev AV, Williams KW, Glatzer NR, and Smith BN.** Excitatory and inhibitory local circuit input to the rat dorsal motor nucleus of the vagus originating from the nucleus tractus solitarius. *Brain Res* 1017: 208-217, 2004.

**Dunn-Meynell AA, Routh VH, Kang L, Gaspers L, and Levin BE.** Glucokinase is the likely mediator of glucosensing in both glucose-excited and glucose-inhibited central neurons. *Diabetes* 51: 2056-2065, 2002.

**Ferreira M, Jr., Browning KN, Sahibzada N, Verbalis JG, Gillis RA, and Travagli RA.** Glucose effects on gastric motility and tone evoked from the rat dorsal vagal complex. *J Physiol* 536: 141-152, 2001.

**Gao H, Glatzer NR, Williams KW, Derbenev AV, Liu D, and Smith BN.** Morphological and electrophysiological features of motor neurons and putative interneurons in the dorsal vagal complex of rats and mice. *Brain Res* 1291: 40-52, 2009.

**Gao H, and Smith BN.** Tonic GABAA receptor-mediated inhibition in the rat dorsal motor nucleus of the vagus. *J Neurophysiol* 103: 904-914, 2010.

**Glatzer NR, Derbenev AV, Banfield BW, and Smith BN.** Endomorphin-1 modulates intrinsic inhibition in the dorsal vagal complex. *J Neurophysiol* 98: 1591-1599, 2007.

**Glatzer NR, and Smith BN.** Modulation of synaptic transmission in the rat nucleus of the solitary tract by endomorphin-1. *J Neurophysiol* 93: 2530-2540, 2005.

**Gloyn AL, Siddiqui J, and Ellard S.** Mutations in the genes encoding the pancreatic beta-cell KATP channel subunits Kir6.2 (KCNJ11) and SUR1 (ABCC8) in diabetes mellitus and hyperinsulinism. *Human Mutation* 27: 220-231, 2006.

**Gloyn AL, Weedon MN, Owen KR, Turner MJ, Knight BA, Hitman G, Walker M, Levy JC, Sampson M, Halford S, McCarthy MI, Hattersley AT, and Frayling TM.** Large-scale association studies of variants in genes encoding the pancreatic beta-cell KATP channel subunits Kir6.2 (KCNJ11) and SUR1 (ABCC8) confirm that the KCNJ11 E23K variant is associated with type 2 diabetes. *Diabetes* 52: 568-572, 2003.

**Gross PM, Wall KM, Pang JJ, Shaver SW, and Wainman DS.** Microvascular specializations promoting rapid interstitial solute dispersion in nucleus tractus solitarius. *Am J Physiol* 259: R1131-1138, 1990.

**Haeusler RA, Camastra S, Astiarraga B, Nannipieri M, Anselmino M, and Ferrannini E.** Decreased expression of hepatic glucokinase in type 2 diabetes. *Mol Metab* 4: 222-226, 2015.

**Kalsbeek A, Bruinstroop E, Yi CX, Klieverik LP, La Fleur SE, and Fliers E.** Hypothalamic control of energy metabolism via the autonomic nervous system. *Ann N Y Acad Sci* 1212: 114-129, 2010.

**Lam TK, Gutierrez-Juarez R, Poci A, Bhanot S, Tso P, Schwartz GJ, and Rossetti L.** Brain glucose metabolism controls the hepatic secretion of triglyceride-rich lipoproteins. *Nature Medicine* 13: 171-180, 2007.

**Lamy CM, Sanno H, Labouebe G, Picard A, Magnan C, Chatton JY, and Thorens B.** Hypoglycemia-activated GLUT2 neurons of the nucleus tractus solitarius stimulate vagal activity and glucagon secretion. *Cell Metab* 19: 527-538. doi: 10.1016/j.cmet.2014.1002.1003., 2014.

- Levin BE, and Dunn-Meynell AA.** Effect of streptozotocin-induced diabetes on rat brain sulfonylurea binding sites. *Brain Res Bull* 46: 513-518, 1998.
- Levin BE, Dunn-Meynell AA, and Routh VH.** Brain glucose sensing and body energy homeostasis: role in obesity and diabetes. *Am J Physiol* 276: R1223-1231, 1999.
- Livak KJ, and Schmittgen TD.** Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, Calif)* 25: 402-408, 2001.
- Matschinsky FM, Kauffman FC, and Ellerman JE.** Effect of hyperglycemia on the hexose monophosphate shunt in islets of Langerhans. *Diabetes* 17: 475-480, 1968.
- Merchenthaler I.** Neurons with access to the general circulation in the central nervous system of the rat: a retrograde tracing study with fluoro-gold. *Neuroscience* 44: 655-662, 1991.
- Nishio T, Toyoda Y, Hiramatsu M, Chiba T, and Miwa I.** Decline in glucokinase activity in the arcuate nucleus of streptozotocin-induced diabetic rats. *Biol & Pharm Bull* 29: 216-219, 2006.
- Obici S, Zhang BB, Karkanias G, and Rossetti L.** Hypothalamic insulin signaling is required for inhibition of glucose production. *Nat Med* 8: 1376-1382, 2002.
- Oomura Y, Ooyama H, Sugimori M, Nakamura T, and Yamada Y.** Glucose inhibition of the glucose-sensitive neurone in the rat lateral hypothalamus. *Nature* 247: 284-286, 1974.
- Pocai A, Lam TK, Gutierrez-Juarez R, Obici S, Schwartz GJ, Bryan J, Aguilar-Bryan L, and Rossetti L.** Hypothalamic K(ATP) channels control hepatic glucose production. *Nature* 434: 1026-1031, 2005a.
- Pocai A, Obici S, Schwartz GJ, and Rossetti L.** A brain-liver circuit regulates glucose homeostasis. *Cell Metab* 1: 53-61, 2005b.
- Proks P, Arnold AL, Bruining J, Girard C, Flanagan SE, Larkin B, Colclough K, Hattersley AT, Ashcroft FM, and Ellard S.** A heterozygous activating mutation in the sulphonylurea receptor SUR1 (ABCC8) causes neonatal diabetes. *Human Molecular Genetics* 15: 1793-1800, 2006.
- Ritter RC, Slusser PG, and Stone S.** Glucoreceptors controlling feeding and blood glucose: location in the hindbrain. *Science* 213: 451-452, 1981.
- Ritter S, Dinh TT, and Zhang Y.** Localization of hindbrain glucoreceptive sites controlling food intake and blood glucose. *Brain Res* 856: 37-47, 2000.
- Ryan KK, Tremaroli V, Clemmensen C, Kovatcheva-Datchary P, Myronovych A, Karns R, Wilson-Perez HE, Sandoval DA, Kohli R, Backhed F, and Seeley RJ.** FXR is a molecular target for the effects of vertical sleeve gastrectomy. *Nature* 509: 183-188, 2014.
- Saltzman MB, and McCallum RW.** Diabetes and the stomach. *The Yale Journal of Biology and Medicine* 56: 179-187, 1983.
- Schauer PR, Kashyap SR, Wolski K, Brethauer SA, Kirwan JP, Pothier CE, Thomas S, Abood B, Nissen SE, and Bhatt DL.** Bariatric surgery versus intensive medical therapy in obese patients with diabetes. *New Eng J Med* 366: 1567-1576, 2012.
- Schwartz MW, Seeley RJ, Tschop MH, Woods SC, Morton GJ, Myers MG, and D'Alessio D.** Cooperation between brain and islet in glucose homeostasis and diabetes. *Nature* 503: 59-66, 2013.
- Shanley LJ, Irving AJ, Rae MG, Ashford ML, and Harvey J.** Leptin inhibits rat hippocampal neurons via activation of large conductance calcium-activated K<sup>+</sup> channels. *Nat Neurosci* 5: 299-300, 2002.

- Shin AC, Zheng H, and Berthoud HR.** Vagal innervation of the hepatic portal vein and liver is not necessary for Roux-en-Y gastric bypass surgery-induced hypophagia, weight loss, and hypermetabolism. *Ann Surg* 255: 294-301, 2012.
- Travagli RA, Gillis RA, Rossiter CD, and Vicini S.** Glutamate and GABA-mediated synaptic currents in neurons of the rat dorsal motor nucleus of the vagus. *Am J Physiol* 260: G531-536, 1991.
- Travagli RA, Hermann GE, Browning KN, and Rogers RC.** Brainstem circuits regulating gastric function. *Annu Rev Physiol* 68: 279-305, 2006.
- Undeland KA, Hausken T, Gilja OH, Aanderud S, and Berstad A.** Gastric meal accommodation studied by ultrasound in diabetes. Relation to vagal tone. *Scandinavian Journal of Gastroenterology* 33: 236-241, 1998.
- Verberne AJ, Sabetghadam A, and Korim WS.** Neural pathways that control the glucose counterregulatory response. *Front Neurosci* 8: 38, 2014.
- Wan S, and Browning KN.** D-glucose modulates synaptic transmission from the central terminals of vagal afferent fibers. *Am J Physiol Gastrointest Liver Physiol* 294: G757-763, 2008.
- Wan S, Browning KN, Coleman FH, Sutton G, Zheng H, Butler A, Berthoud HR, and Travagli RA.** Presynaptic melanocortin-4 receptors on vagal afferent fibers modulate the excitability of rat nucleus tractus solitarius neurons. *J Neurosci* 28: 4957-4966, 2008.
- Wan S, Browning KN, and Travagli RA.** Glucagon-like peptide-1 modulates synaptic transmission to identified pancreas-projecting vagal motoneurons. *Peptides* 28: 2184-2191, 2007.
- Wang PY, Caspi L, Lam CK, Chari M, Li X, Light PE, Gutierrez-Juarez R, Ang M, Schwartz GJ, and Lam TK.** Upper intestinal lipids trigger a gut-brain-liver axis to regulate glucose production. *Nature* 452: 1012-1016, 2008.
- Williams KW, and Smith BN.** Rapid inhibition of neural excitability in the nucleus tractus solitarius by leptin: implications for ingestive behaviour. *J Physiol* 573: 395-412, 2006.
- Williams KW, Zsombok A, and Smith BN.** Rapid inhibition of neurons in the dorsal motor nucleus of the vagus by leptin. *Endocrinology* 148: 1868-1881, 2007.
- Yi CX, La Fleur SE, Flier JS, and Kalsbeek A.** The role of the autonomic nervous liver innervation in the control of energy metabolism. *Biochim Biophys Acta* 1802: 416-431, 2010.
- Zimmet P, Alberti KG, and Shaw J.** Global and societal implications of the diabetes epidemic. *Nature* 414: 782-787, 2001.
- Zsombok A, Bhaskaran MD, Gao H, Derbenev AV, and Smith BN.** Functional plasticity of central TRPV1 receptors in brainstem dorsal vagal complex circuits of streptozotocin-treated hyperglycemic mice. *J Neurosci* 31: 14024-14031, 2011.
- Zsombok A, and Smith BN.** Plasticity of central autonomic neural circuits in diabetes. *Biochim Biophys Acta* 1792: 423-431, 2009.

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## **APPENDIX**

**I.**

Molecular and Functional Changes in Glucokinase Expression in the Brainstem Dorsal  
Vagal Complex in a Murine Model of Type 1 Diabetes

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## **ABSTRACT**

Glucose concentration changes in the nucleus tractus solitarius (NTS) affect visceral function and metabolism by influencing central vagal circuits, especially inhibitory, GABAergic NTS neurons. Acutely elevated glucose can alter NTS neuron activity, and prolonged hyperglycemia and hypoinsulemia in animal models of type 1 diabetes results in plasticity of neural responses in the NTS. NTS neurons contributing to metabolic regulation therefore act as central glucose sensors and are functionally altered in type 1 diabetes. Glucokinase (GCK) mediates cellular utilization of glucose, linking increased glucose concentration to excitability changes mediated by ATP-sensitive K<sup>+</sup> channels (K<sub>ATP</sub>). Using quantitative RT-PCR, Western blot, and in vitro electrophysiology, we tested the hypothesis that changes in GCK expression in the NTS accompanied development of diabetes symptoms in the streptozotocin (STZ)-treated mouse model of type 1 diabetes. After several days of hyperglycemia in STZ-treated mice, RNA expression of GCK, but not Kir6.2 or SUR1, was decreased versus controls in the dorsal vagal complex. Electrophysiological recordings in vitro indicated that neural responses to acute hyperglycemia, and synaptic responsiveness to blockade of GCK with glucosamine, were attenuated in GABAergic NTS neurons from STZ-treated mice, consistent with reduced molecular and functional expression of GCK in the vagal complex of hyperglycemic, STZ-treated mice. Altered autonomic responses to glucose in type 1 diabetes may therefore involve reduced functional GCK expression in the dorsal vagal complex.

**Keywords:** GABA neuron, hyperglycemia, K<sub>ATP</sub> channel, nucleus tractus solitarius, postsynaptic current, vagus

## INTRODUCTION

Diabetes mellitus, defined by unequivocally elevated blood glucose levels, affects over 29 million people in the United States (Centers for Disease Control and Prevention, 2014). Some of the serious complications of diabetes include heart disease, stroke, hypertension, blindness, nervous system damage, and gastrointestinal dysfunction. Treatments for the disease remain inadequate, despite substantial investment to reduce symptoms and complications of the disease. Multiple 'preautonomic' areas of the brain contribute to systemic glucose homeostasis (Zsombok and Smith, 2009, Kalsbeek et al., 2010, Yi et al., 2010) and are also affected by elevated blood glucose levels. In particular, neural circuits in the hindbrain play a critical role in regulating plasma glucose and insulin levels. More specifically, vagally-mediated parasympathetic output critically regulates visceral functions related to metabolic homeostasis, and abundant evidence indicates that the brainstem dorsal vagal complex plays a primary and critical role in glucose-sensitive modulation of plasma glucose and insulin levels, feeding, and energy balance (Ritter et al., 1981, Laughton and Powley, 1987, Ritter et al., 2000, Zsombok and Smith, 2009).

Neurons in the brainstem nucleus of the solitary tract (NTS) receive glutamatergic, primary vagal afferent synaptic input from the gut and other thoracic and abdominal viscera. Vagal afferents rapidly convey information about gastrointestinal distention and nutrient content to the NTS, where that information is processed, integrated with neuronal and humoral signals, and transmitted to other brain areas, including to vagal motor neurons of the dorsal motor nucleus of the vagus (DMV). Neurons in the NTS respond to acutely altered glucose concentration with either increases or decreases in

neural excitability and altered synaptic input (Oomura et al., 1974, Balfour et al., 2006, Wan and Browning, 2008, Lamy et al., 2014, Boychuk et al., 2015a), which are glucokinase (GCK)-dependent. The depolarizing response is mediated by inactivation of ATP-sensitive K<sup>+</sup> (K<sub>ATP</sub>) channels (Balfour et al., 2006, Boychuk et al., 2015a) and K<sub>ATP</sub> channel modulation prevents the glucose-induced, GABA mediated inhibition of vagal motor neurons (Ferreira et al., 2001). Type I diabetes is characterized by uncontrolled hyperglycemia due to reduced insulin secretion from pancreatic beta cells. Synaptic and other cellular responses in the dorsal vagal complex are altered in models of type 1 diabetes, even after normalizing glucose concentration (Zsombok et al., 2011, Browning, 2013, Blake and Smith, 2014, Bach et al., 2015, Boychuk et al., 2015b). Vagal reflexes are often blunted during chronic hyperglycemia, and altered vagal function may contribute to diabetes-associated visceral dysfunction (Saltzman and McCallum, 1983, Undeland et al., 1998), suggesting that chronically-elevated glucose alters responsiveness of neurons in the dorsal vagal complex.

Because of the involvement of GCK and K<sub>ATP</sub> channel modulation in the neuronal response to glucose, and the altered responsiveness of NTS neurons in animal models of type 1 diabetes, we tested the hypothesis that GCK or K<sub>ATP</sub> channel expression is altered after several days of chronic hyperglycemia/hypoinsulemia in the streptozotocin (STZ)-treated mouse. Understanding how glucose sensitivity in the dorsal brainstem is altered in diabetes may offer hypotheses to guide development of alternative therapies for the disease.

## **EXPERIMENTAL PROCEDURES**

### **Animals**

Mice were treated and cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all procedures were approved by the University of Kentucky Animal Care and Use Committee (Animal Welfare Assurance Number A3336–01). Euthanasia was accomplished by anesthesia with isoflurane to effect (IsoThesia; Henry Schein, Melville, NY), followed by decapitation while anesthetized. Juvenile and young adult (28–42 days) male CD-1 (Harlan Laboratories, Indianapolis, IN) or transgenic ‘GIN’ mice (FVB-Tg (GadGFP)4570Swn/J; The Jackson Laboratory, Bar Harbor, ME) were used for all experiments and housed under a standard 14-h light-10-h dark cycle, with food and water provided without restriction. The GIN mice express EGFP in a subset of GABA neurons in the NTS, which comprise a large proportion of NTS neurons (Oliva et al., 2000, Williams and Smith, 2006, Glatzer et al., 2007, Boychuk et al., 2015a).

### **Streptozotocin Injection**

Intra-peritoneal injection of STZ (200mg/kg in 0.9% NaCl), which kills insulin-secreting pancreatic  $\beta$  cells, was used to induce chronic hyperglycemia in mice. Blood glucose concentration (non-fasted) was measured by tail puncture using a Nova Max PLUS glucometer from normal mice, which were then fasted for 4–6 h prior to STZ or vehicle injection. Control mice were either injected with saline (0.9% NaCl) or untreated. No differences in electrophysiological parameters were observed between normoglycemic saline-injected and untreated mice; they were therefore pooled and considered as a single control group. Blood glucose levels (non-fasted) were measured

daily. Onset of hyperglycemia (i.e., blood glucose concentration above 300mg/dl) varied between animals, but occurred between 1 and 3 days post-STZ injection and remained elevated until the day of the experiment. Animals were used for electrophysiological recordings and molecular analyses after 3-4 days of continuous hyperglycemia.

### **Brain slice preparation**

On-cell and whole-cell voltage-clamp recordings were made using brain stem slices prepared from male GIN mice, 4-5 weeks of age. Animals were deeply anesthetized by isoflurane inhalation (IsoThesia; Henry Schein) and decapitated while anesthetized. The brain was rapidly removed and blocked to isolate the brainstem and then glued to a sectioning stage. Transverse (coronal) brain stem slices (300  $\mu\text{m}$ ) containing the caudal NTS (i.e.,  $\pm 600$   $\mu\text{m}$  rostral and caudal to area postrema) were made in ice cold, oxygenated (95%  $\text{O}_2$ -5%  $\text{CO}_2$ ) artificial cerebrospinal fluid (ACSF) using a vibrating microtome (Vibratome Series 1000; Technical Products, St. Louis, MO). The ACSF contained (in mM): 124 NaCl, 3 KCl, 2  $\text{CaCl}_2$ , 1.3  $\text{MgCl}_2$ , 1.4  $\text{NaH}_2\text{PO}_4$ , 26  $\text{NaHCO}_3$ , and 11 or 2.5 glucose (pH 7.15–7.3); osmolality was adjusted to 290–310 mOsm/kg with sucrose; equimolar ACSF was made with by substitution sucrose for glucose substitution experiments. Slices were incubated for an equilibration period for  $\geq 1$  h in warmed (30–33°C), oxygenated ACSF prior to recording.

### **Electrophysiology**

A single brain slice was transferred to a recording chamber mounted on a fixed stage under an upright microscope (BX51WI; Olympus, Melville, NY), where it was continually perfused by warmed (30–33°C), oxygenated ACSF. EGFP-labeled NTS neurons were targeted for recording under a 40x water-immersion objective with fluorescence and infrared-differential interference contrast (IR-DIC) optics, as previously described (Williams and Smith, 2006, Glatzer et al., 2007, Gao et al., 2009, Boychuk et al., 2015a). For recordings from EGFP-labeled NTS neurons, initial visualization was made briefly under epifluorescence using a fluorescein isothiocyanate (FITC) filter set (excitation filter wavelengths: 450-490 nm).

On-cell and whole-cell patch-clamp recordings were obtained in the NTS using pipettes pulled from borosilicate glass (Garner Glass, Claremont, CA; open tip resistance 4-6 M $\Omega$ ) using a Sutter P-87 horizontal puller (Sutter Instrument Co., Novato, CA). Pipettes were filled with a solution containing (in mM): 130 K<sup>+</sup>-gluconate (or Cs<sup>+</sup>-gluconate), 1 NaCl, 5 EGTA, 10 HEPES, 1 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>, 3 KOH (or CsOH), 2-4 ATP (pH 7.15 – 7.3). Electrophysiological signals were recorded using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA), low-pass filtered at 3 kHz, and recorded onto a PC-style computer (Digidata 1440A, Molecular Devices) using pClamp 10.2 software (Molecular Devices). A 2-4 G $\Omega$  seal was maintained for on-cell recordings. For whole-cell voltage-clamp recordings, series resistance was monitored throughout the recordings and data were used for analysis if the series resistance remained <25 M $\Omega$  and changed by  $\leq$ 20% during the recording. Once a recording was obtained, cells were allowed to equilibrate for ~10 min before beginning data collection.

In whole-cell voltage-clamp recordings, resting potential was determined by monitoring the voltage at which no current was injected. Spontaneous EPSCs (sEPSC) were examined at a holding potential of -65 mV and IPSCs (sIPSCs) were examined at 0 mV using pipettes containing Cs-gluconate to block K<sup>+</sup> currents, thereby improving voltage control and reducing noise. Miniature EPSCs and IPSCs (mEPSCs and mIPSCs) were recorded in the presence of tetrodotoxin (2 μM). The GCK inhibitor glucosamine (GA; 5μM; MP Biomedical, Santa Ana, CA) was applied with the ACSF for 10 min. For effects of elevated glucose concentration, slices were incubated in ACSF containing 2.5 mM glucose for at least 1 hr prior to on-cell recording. Spontaneous action potential activity was recorded for 10 min prior to changing to ACSF containing 15 mM glucose for 10 min before returning to 2.5 mM glucose solution (washout). Recordings of 2-3 min epochs were made prior to and after 10 min of GA- or 15 mM glucose-containing ACSF application. Washout was at least 15 min.

### **RNA isolation**

Brainstem slices (300μm) from male CD-1 or GIN mice were isolated in cold ACSF, as described for electrophysiological recordings. The dorsal vagal complex, including most of both the DMV and NTS, was visualized under a dissecting microscope and excised from the rest of the brainstem with a 1 mm diameter biopsy punch (Miltex, Inc. York, PA). Resulting punched-tissue was then suspended in 500 μL of TRIzol (Sigma-Aldrich) and gently shaken periodically for 5min. Chloroform (100–250 μl) was added and tubes were vortexed for 15 s, maintained at 4°C for 20 min, and subsequently centrifuged at 12,000 rpm for 15 min at 4°C. The pellet was discarded and the supernatant containing RNA was transferred into fresh 1.5 ml centrifuge tubes,

mixed with 500  $\mu$ L of ice-cold propanol, incubated at room temperature for 10 min, and centrifuged at 12,000 rpm for 10 min at 4°C. Propanol was decanted and RNA was washed by re-suspension in 500  $\mu$ L 75% ethanol followed by centrifugation at 7500–12,000 rpm for 10 min at 4°C. The wash step was repeated, the ethanol decanted, and RNA samples were air-dried for 10–20 min. RNA samples were re-suspended in 8–10  $\mu$ L RNase-free water and stored at -80°C or immediately reverse-transcribed into cDNA.

### **TaqMan QPCR**

RNA samples were reverse transcribed in reverse-transcription master mix containing: 1  $\mu$ l random nonamers (50  $\mu$ M; Sigma-Aldrich), 5  $\mu$ l MMLV RT buffer (5x) (Fisher Scientific, Pittsburgh, PA), 5  $\mu$ l dNTPs (10 mM; Fisher), 2  $\mu$ l DEPC-treated H<sub>2</sub>O (Fisher), 1  $\mu$ l reverse transcriptase (Fisher), and RNase inhibitor (1  $\mu$ l; Fisher) in a thermocycler (Mastercycler, Eppendorf) at 42°C for 90 min followed by 5 min at 95°C. Negative controls included no template and RNase-free sterile water instead of template. No RNA was detected in these controls. Primers and probes were:  $\beta$ -actin, Bactin, NM\_007393 Actb; SUR1, Abcc8 NM\_011510; and Kir6.2, Kcnj11, NM\_001204411 (Integrated DNA Technologies, Coralville, IA) and GCK, NM\_010292.4 (Sigma). Master mix included 10  $\mu$ l of TaqMan Universal master mix II (Applied Biosystems, Life Technologies, Grand Island, NY), 1  $\mu$ l of primer/probe mix and 9  $\mu$ l Of cDNA+RNase-free sterile H<sub>2</sub>O. Samples were loaded into a 96-well plate (Bio-Rad, Hercules, CA). Samples were centrifuged for 2 min at 1000 RPM and placed in an Applied Biosystems thermocycler (ABI 7500) for PCR analysis. Samples were held at 95°C 2 min and cycled 50 times at 95°C for 30 s, 60°C for 15 s and at 72°C 15 s. Gene

transcript level from each mouse was measured in triplicate; target transcript cycle threshold (CT) value was normalized to the CT value for  $\beta$ -actin for each sample; resulting difference CT (dCT) values were compared statistically. Results were also analyzed by the 2ddct method (Livak and Schmittgen, 2001). Differences of  $p < 0.05$  were considered significant.

### **Western Blots**

Brainstem slices (300  $\mu$ m) were cut as described above, the dorsal vagal complex was isolated from the slice, and tissue punches containing the DMV and NTS were immediately transferred to 40–60  $\mu$ l of lysis buffer consisting of 0.15M NaCl, 5mM EDTA (pH 8), 1% Triton X-100, 10mM Tris-HCl (pH 7.4), 10 $\mu$ l/ml of 100mM PMSF (174.2 mg/10ml in methanol), and 100 $\mu$ l/ml of 0.5M NaF (pH 10). Each sample was sonicated and centrifuged immediately at 12,000 RPM for 3 min. Supernatant was aspirated, aliquoted, and stored at -80°C until further use. Protein concentration was measured using a Bradford Protein Assay and 20  $\mu$ g of protein was loaded per lane for Western blot analysis. The appropriate volume of sample together with equal amounts of loading buffer was boiled in water for 2 min. Samples and ladder were loaded into precast SDS polyacrylamide gels and electrophoresed at 50 mA for 45–80 min. Proteins were then transferred at 200 mA for 2 hours onto polyvinylidene difluoride membranes for Western blot analysis. Membranes were blocked in 1:1 Odyssey (Li-Cor Biosciences, Lincoln, NE ) blocking buffer/TBS/0.1% Tween 20 for 1 hr at room temperature. Due to well-separated molecular weights of the GCK (band at 65 kD) and  $\beta$ -actin (band at 40–45 kD) protein membranes were cut in half and incubated over-

night at 4°C, with a rabbit monoclonal anti-GCK (1:1000; Abcam, Cambridge, MA) and a rabbit monoclonal anti-β-actin (1:10000; Abcam) antibody in Odyssey blocking buffer/TBS/0.1% Tween 20. Membranes were washed 4 times (5 min) with TBS on a shaker and treated for 1 hr with fluorescence-conjugated anti-rabbit IgG (IRDye 680RD; Li-Cor Biosciences). Membranes were then washed (4 x 5 min) and scanned on a densitometer (Odyssey model 9120, Li-Cor Biosciences) to quantify band density. Background density was subtracted from the GCK band density and normalized to β-actin, which was used as a loading control.

### **Data analysis**

Recordings were analyzed using Clampfit 10.2 (Molecular Devices) and MiniAnalysis 6.0.7 software (Synaptosoft, Decatur, GA). At least two minutes of continuous recording under each condition was used to assess mean action potential frequency or EPSC and IPSC frequency and amplitude. The Kolmogorov-Smirnov (K-S) intra-assay test was used to determine statistical significance of drug- or glucose-induced changes in the frequency of action potentials, EPSCs, or IPSCs within a recording. For all electrophysiological experiments, a two-tailed Student's *t*-test was used to identify effects within neurons; a two-tailed Chi-square test was used to compare proportions of responding cells between STZ-treated and saline-treated mice (Graphpad Prism, San Diego, CA). For quantitative RT-PCR and Western blots, an unpaired Student *t*-test was used to detect differences between samples from each group. Values are presented as mean ± SEM; statistical significance for all measures was set at  $p < 0.05$ .

## RESULTS

**Molecular expression of GCK and  $K_{ATP}$  channels.** Punches of tissue containing the dorsal brainstem were collected from normoglycemic (n=8; glucose index  $180 \pm 4$  mg/dl) and STZ-treated CD-1 mice that were hyperglycemic for 3-4 days (n=8;  $469 \pm 5$  mg/dl). All target transcript measurements were normalized to  $\beta$ -actin expression. Quantitative RT-PCR revealed a significant decrease in GCK expression in the dorsal vagal complex from STZ-treated hyperglycemic CD1 mice versus controls ( $p < 0.05$ ; Fig. 1). GCK expression was similarly reduced in the vagal complex of GIN mice (n=5 control, n=7 STZ-treated;  $p < 0.05$ ). No significant expression differences were detected for Kir6.2 or SUR1 transcripts between normoglycemic and hyperglycemic CD-1 mice ( $p > 0.05$ ; Fig. 1). Molecular expression of GCK, but not components of the  $K_{ATP}$  channel, was reduced in STZ-treated, hyperglycemic mice relative to control mice.

To determine if the decrease in mRNA transcription correlated with decreased protein expression, Western blots were performed on punches from an additional 8 control and 8 STZ-treated hyperglycemic CD-1 mice. Western blot analysis indicated that GCK protein expression was significantly reduced in the dorsal vagal complex of STZ-treated hyperglycemic mice ( $p < 0.05$ ; Fig. 1).

**Effect of GCK inhibition on synaptic input to NTS neurons.** Glucokinase inhibition prevents responses to acute hypoglycemia in a subset of GABAergic NTS neurons (Lamy et al., 2014). Since GCK expression was reduced in the NTS of STZ-treated mice after several days of hyperglycemia, responses to the GCK inhibitor, GA (5  $\mu$ M) were determined in GABAergic medial NTS (mNTS) neurons, identified by expression of EGFP in acute slices from normoglycemic control (n=5) and STZ-treated

GIN mice (n=5). In GABAergic mNTS neurons from normal mice, GA application decreased the frequency of sEPSCs by  $\geq 20\%$  in 80% of neurons (12 of 15 neurons;  $2.83 \pm 0.34$  Hz control ACSF;  $1.72 \pm 0.22$  Hz in GA; n=15;  $p < 0.05$ ; Fig. 2), with no change in sEPSC frequency in the remaining three neurons. No effect on synaptic current amplitude was detected ( $p > 0.05$ ). In the presence of TTX ( $2 \mu\text{M}$ ) to prevent action potential firing, there was no significant effect of GA on miniature EPSC (mEPSC) frequency in control mice ( $p > 0.05$ ); mEPSC frequency was decreased slightly in only one of five neurons from three mice, so effects in STZ-treated mice were not assessed. These findings are consistent with an effect of GCK in mediating spontaneous excitatory synaptic responses to ambient glucose levels in NTS neurons (Wan and Browning, 2008) and indicated that GCK blockade inhibited excitatory, glutamatergic synaptic input to most GABA neurons.

In GABAergic mNTS neurons from STZ-treated mice after 3-5 days of hyperglycemia (n=5), effects of GA on sEPSC frequency were significantly less robust than in controls ( $p < 0.05$ ; Chi-square), being reduced (-22%) in only one of six neurons and unchanged in the remaining five cells (Fig. 2). Overall, mean sEPSC frequency was unchanged in the presence of GA ( $7.63 \pm 1.04$  Hz in control ACSF;  $7.04 \pm 0.83$  Hz in GA; n=6;  $p > 0.05$ ). Consistent with the decreased GCK expression in the vagal complex, modulation of excitatory synaptic activity by GA was reduced in GABAergic mNTS neurons from STZ-treated, hyperglycemic mice.

Effects of GA application on sIPSC frequency and amplitude were also determined. In GABAergic mNTS neurons from control mice (n=5), GA application was without effect on the overall population ( $1.22 \pm 0.2$  Hz control, ACSF;  $1.25 \pm 0.30$  Hz, GA; n=9;

$p > 0.05$ ), but was either increased ( $n=3$ ) or decreased ( $n=6$ ) in individual neurons (Fig. 2). The amplitude of sIPSCs was unchanged by GA ( $p > 0.05$ ). Similar to results in control mice, there was no overall effect on sIPSC frequency in neurons from STZ-treated mice ( $1.04 \pm 0.30$  Hz control ACSF;  $1.01 \pm 0.31$  Hz GA;  $n=7$ ;  $p > 0.05$ ). sIPSC amplitude was also unchanged ( $p > 0.05$ ). In neurons from STZ-treated mice, GA application either increased ( $n=3$ ), decreased ( $n=2$ ) or was without effect ( $n=2$ ) on sIPSC frequency (Fig. 2). There was no significant effect of GA on mIPSC frequency in control mice; mIPSC frequency was increased slightly in only two of five neurons from four mice, so effects in STZ-treated mice were not assessed. Although sIPSC frequency in GABAergic mNTS neurons was usually altered by GA application, robust differences between responses in neurons from control and STZ-treated mice were not observed ( $p > 0.05$ ; Chi-square).

**Glucose effect on Action Potentials.** Neurons in the NTS are glucose sensors, and this sensitivity may be especially prominent in GABAergic NTS neurons (Ferreira et al., 2001, Lamy et al., 2014, Boychuk et al., 2015a). Since GCK expression was reduced in the NTS of STZ-treated mice after several days of hyperglycemia, we examined action potential frequency of GABAergic NTS neurons in response to elevating glucose from 2.5 to 15 mM using on-cell recordings. Increasing glucose concentration resulted in a  $>20\%$  change in action potential frequency in 77% of neurons (10 of 13 cells) in neurons from normoglycemic control mice ( $n=6$ ). Increasing glucose concentration resulted in an increase in action potential frequency in seven of 13 neurons ( $122 \pm 57\%$  increase; Fig. 3), a decrease in three neurons ( $-27 \pm 2\%$  decrease), and no change in three neurons. In neurons from STZ-treated,

hyperglycemic mice (n=6), elevating glucose resulted in a change in action potential frequency in only five of 15 neurons (33%), a significantly lower percentage of responses than in neurons from control mice ( $p < 0.05$ , two-tailed Chi-square). The frequency of action potentials was increased in three neurons ( $111 \pm 22\%$ ), decreased in two cells ( $-55 \pm 10\%$ ), and was unaffected in the remaining 10 cells. Whereas increasing glucose concentration resulted in a significant and large change in action potential frequency in the majority of neurons in normoglycemic mice, action potential frequency in most neurons from hyperglycemic mice was unaffected by increasing glucose concentration.

## **DISCUSSION**

Neurons in the NTS receive direct input from the primary viscerosensory vagal afferents and comprise the initial response component of central parasympathetic regulatory circuits. Subsets of these neurons, and GABA neurons in particular, are known to be glucose-sensitive (Oomura et al., 1974, Balfour et al., 2006, Wan and Browning, 2008, Browning, 2013, Lamy et al., 2014, Boychuk et al., 2015a). Several physiological aspects of central vagal circuitry are altered functionally in diabetes (Zsombok et al., 2011, Browning, 2013, Blake and Smith, 2014, Bach et al., 2015, Boychuk et al., 2015b), consistent with modified parasympathetic regulation of the viscera concurrent with the disease (Saltzman and McCallum, 1983). Glucose sensing in NTS neurons involves GCK, which catalyzes the conversion of glucose to glucose-6 phosphate in neurons and other cells (Balfour et al., 2006, Briski et al., 2009), resulting in increased ATP/ADP ratio. In several neural systems, membrane responses after

increased glucose concentration are caused by ATP binding to  $K_{ATP}$  channels to affect changes in membrane potential. Diabetes induces changes in GCK or  $K_{ATP}$  channel expression in specific hypothalamic nuclei (Levin and Dunn-Meynell, 1998, Nishio et al., 2006), which are consistent with altered or compensatory neuronal responses to chronically-elevated glucose or hypoinsulinemia in STZ-treated rats. Altered electrophysiological responsiveness of NTS neurons in type 1 diabetes has been demonstrated (Browning, 2013, Bach et al., 2015), which could contribute to visceral dysregulation in diabetes, but the mechanisms of this plasticity are unknown. Here, we found that molecular and functional expression of GCK—but not components of the  $K_{ATP}$  channel—were diminished in the vagal complex of mice with type 1 diabetes. Consistent with decreased mRNA transcription, GCK protein levels were reduced, neuronal and synaptic responses to GCK blockade were attenuated, and neuronal activity responses to increased glucose concentration were diminished. A similar decrease in GCK expression and activity has been reported in the arcuate nucleus, but not in other glucose-responsive hypothalamic nuclei, of STZ-treated rats (Nishio et al., 2006). The present findings suggest that responses of NTS neurons to increased glucose concentration may be altered as a consequence of chronic hyperglycemia or hypoinsulinemia in a GCK-dependent manner, similar to glucose-sensitive neurons of the arcuate nucleus.

Neurons in the NTS normally respond to elevated or reduced glucose concentration with either increases or decreases in excitability (Oomura et al., 1974, Balfour et al., 2006, Wan and Browning, 2008, Browning, 2013, Lamy et al., 2014, Boychuk et al., 2015a), which are often GCK-dependent. Glucose-induced increases in excitation are

mediated by inactivation of  $K_{ATP}$  channels in NTS neurons (Balfour et al., 2006, Boychuk et al., 2015a) and  $K_{ATP}$  channel modulation prevents the glucose-induced, GABA mediated inhibition of vagal motor neurons (Ferreira et al., 2001). We tested the hypothesis that expression of molecular components of the  $K_{ATP}$  channel was altered after several days of hyperglycemia.  $K_{ATP}$  channels in central neurons are mainly composed of SUR1 and Kir6.2 subunits and SUR1 is expressed by glucose-sensing NTS cells (Balfour et al., 2006). In STZ-treated rats, SUR binding is increased in neurons of the dorsomedial, ventromedial, and lateral—but not paraventricular—hypothalamic nuclei after seven days of hyperglycemia (Levin and Dunn-Meynell, 1998), suggesting increased  $K_{ATP}$  channel expression that may serve a compensatory function in specific neurons. We found that molecular expression of SUR1 and Kir6.2 was unchanged in the vagal complex of mice with type 1 diabetes, which is inconsistent with the hypothesis that SUR1 activity increases in the NTS after several days of chronic hyperglycemia.

Since  $K_{ATP}$  channel-mediated responses to glucose in the NTS require GCK, the decrease in GCK expression suggests a mechanism for blunted responsiveness to glucose that has been proposed to occur in chronically hyperglycemic mice (Browning, 2013). The decreased molecular and protein expression of GCK we observed was consistent with an attenuation of the electrophysiological response to GCK inhibition. Blockade of GCK activity resulted in altered synaptic excitability of most GABAergic NTS neurons from normoglycemic mice, and this effect was mainly action potential-dependent, consistent with tonic GCK-mediated effects on neuronal activity in the slice. Notably, it is conceivable that blockade of GCK activity might deplete energy supplies.

This potential confound is mitigated, however, by the fact that application was limited to 10 min in this study. Further, postsynaptic current amplitude was not altered by GA, suggesting that neural responsiveness was not overtly affected. The effects of blocking GCK were reduced in neurons from mice with type 1 diabetes, especially on action potential-dependent glutamate release, suggesting that GABAergic NTS neurons may be less responsive to excitatory synaptic input in mice with type 1 diabetes. Moreover, responsiveness of GABAergic NTS neurons to increased glucose concentration was attenuated in STZ-treated, hyperglycemic mice. This further implies that synaptic activity in the NTS normally occurs in the context of glucose concentration, since GCK mediates glucose-responsiveness in these neurons.

Previous studies indicated that responses of NTS neurons to acute hypoglycemia required GCK activity (Balfour et al., 2006, Lamy et al., 2014). Responses to transient hypoglycemia were previously reported in NTS neurons that expressed glucose transporter 2 (GLUT2) and the expression of the transporter was colocalized in a subset of GABA neurons (Lamy et al., 2014). We recently showed that GABA neurons were either depolarized or hyperpolarized by glucose, and the depolarization in particular was sensitive to blockade of GCK activity or by blocking  $K_{ATP}$  channels (Boychuk et al., 2015a). Here, we found that attenuated neuronal responses to transient hyperglycemia in mice with type 1 diabetes coincided with reduced molecular, protein, and functional GCK expression in the vagal complex, suggesting that prolonged hyperglycemia affects glucose responsiveness in the vagal complex.

Elevated glucose concentration increases glutamatergic synaptic transmission from vagal afferents in rats and mice, and these effects were reported to be attenuated in a

model of type 1 diabetes (Browning, 2013). Our results are consistent with this report, and offer a mechanistic explanation for the loss of response. The glucoregulatory response to nutritive substances applied in the intestine is prevented by blocking ionotropic glutamate receptors in the NTS (Wang et al., 2008, Cheung et al., 2009), suggesting that glutamatergic, vagal afferent activation of NTS neurons is required for this response. Blockade of NMDA receptors in the vagal complex prevents the positive effect of bariatric surgery on systemic blood glucose levels, an effect hypothesized to occur by inhibiting a “gut-brainstem-liver” circuit in the vagal complex (Verberne et al., 2014).

Our results support the hypothesis that diminished GCK expression in the vagal complex of mice with type 1 diabetes results in reduced responsiveness of NTS neurons to glucose, including the response to synaptic glutamate release. It is likely that glutamatergic, visceral afferent synaptic input to the NTS, including input mediating mechano- and chemoreceptor activity in the gut (Barber and Burks, 1987), occurs in the context of glucose concentration. Restoring GCK expression may help restore physiological responsiveness of NTS neurons to glucose, thereby helping to normalize parasympathetic regulation of visceral function, including glucose homeostasis, in diabetes.

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## REFERENCES

- Bach EC, Halmos KC, Smith BN (2015) Enhanced NMDA receptor-mediated modulation of excitatory neurotransmission in the dorsal vagal complex of streptozotocin-treated, chronically hyperglycemic mice. *PloS one* 10:e0121022.
- Balfour RH, Hansen AM, Trapp S (2006) Neuronal responses to transient hypoglycaemia in the dorsal vagal complex of the rat brainstem. *The Journal of physiology* 570:469-484.
- Barber WD, Burks TF (1987) The response of brainstem neurons to chemical activation of gastric sensory receptors. *Gastroenterology clinics of North America* 16:521-524.
- Blake CB, Smith BN (2014) cAMP-dependent insulin modulation of synaptic inhibition in neurons of the dorsal motor nucleus of the vagus is altered in diabetic mice. *American journal of physiology Regulatory, integrative and comparative physiology* 307:R711-720.
- Boychuk CR, Gyarmati P, Xu H, Smith BN (2015a) Glucose sensing by GABAergic neurons in the mouse nucleus tractus solitarius. *Journal of neurophysiology* jn 0031002015.
- Boychuk CR, Halmos K, Smith BN (2015b) Diabetes induces GABA receptor plasticity in murine vagal motor neurons. *Journal of neurophysiology* jn 0020902015.
- Briski KP, Cherian AK, Genabai NK, Vavaiya KV (2009) In situ coexpression of glucose and monocarboxylate transporter mRNAs in metabolic-sensitive caudal dorsal vagal complex catecholaminergic neurons: transcriptional reactivity to insulin-induced hypoglycemia and caudal hindbrain glucose or lactate repletion during insulin-induced hypoglycemia. *Neuroscience* 164:1152-1160.
- Browning KN (2013) Modulation of gastrointestinal vagal neurocircuits by hyperglycemia. *Front Neurosci* 7:217.:10.3389/fnins.2013.00217. eCollection 02013.
- Centers for Disease Control and Prevention A, GA (2014) National Diabetes Statistics Report: Estimates of Diabetes and Its Burden in the United States, 2014. US Department of Health and Human Services.
- Cheung GW, Kokorovic A, Lam CK, Chari M, Lam TK (2009) Intestinal cholecystokinin controls glucose production through a neuronal network. *Cell Metab* 10:99-109.
- Ferreira M, Jr., Browning KN, Sahibzada N, Verbalis JG, Gillis RA, Travagli RA (2001) Glucose effects on gastric motility and tone evoked from the rat dorsal vagal complex. *The Journal of physiology* 536:141-152.
- Gao H, Glatzer NR, Williams KW, Derbenev AV, Liu D, Smith BN (2009) Morphological and electrophysiological features of motor neurons and putative interneurons in the dorsal vagal complex of rats and mice. *Brain Res* 1291:40-52.
- Glatzer NR, Derbenev AV, Banfield BW, Smith BN (2007) Endomorphin-1 modulates intrinsic inhibition in the dorsal vagal complex. *Journal of neurophysiology* 98:1591-1599.
- Kalsbeek A, Bruinstroop E, Yi CX, Klieverik LP, La Fleur SE, Fliers E (2010) Hypothalamic control of energy metabolism via the autonomic nervous system. *Ann N Y Acad Sci* 1212:114-129.
- Lamy CM, Sanno H, Labouebe G, Picard A, Magnan C, Chatton JY, Thorens B (2014) Hypoglycemia-activated GLUT2 neurons of the nucleus tractus solitarius stimulate vagal activity and glucagon secretion. *Cell Metab* 19:527-538. doi: 510.1016/j.cmet.2014.1002.1003.
- Laughton WB, Powley TL (1987) Localization of efferent function in the dorsal motor nucleus of the vagus. *Am J Physiol* 252:R13-R25.
- Levin BE, Dunn-Meynell AA (1998) Effect of streptozotocin-induced diabetes on rat brain sulfonylurea binding sites. *Brain research bulletin* 46:513-518.

- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* (San Diego, Calif) 25:402-408.
- Nishio T, Toyoda Y, Hiramatsu M, Chiba T, Miwa I (2006) Decline in glucokinase activity in the arcuate nucleus of streptozotocin-induced diabetic rats. *Biological & pharmaceutical bulletin* 29:216-219.
- Oliva AA, Jr., Jiang M, Lam T, Smith KL, Swann JW (2000) Novel hippocampal interneuronal subtypes identified using transgenic mice that express green fluorescent protein in GABAergic interneurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 20:3354-3368.
- Oomura Y, Ooyama H, Sugimori M, Nakamura T, Yamada Y (1974) Glucose inhibition of the glucose-sensitive neurone in the rat lateral hypothalamus. *Nature* 247:284-286.
- Ritter RC, Slusser PG, Stone S (1981) Glucoreceptors controlling feeding and blood glucose: location in the hindbrain. *Science* 213:451-452.
- Ritter S, Dinh TT, Zhang Y (2000) Localization of hindbrain glucoreceptive sites controlling food intake and blood glucose. *Brain Res* 856:37-47.
- Saltzman MB, McCallum RW (1983) Diabetes and the stomach. *The Yale journal of biology and medicine* 56:179-187.
- Undeland KA, Hausken T, Gilja OH, Aanderud S, Berstad A (1998) Gastric meal accommodation studied by ultrasound in diabetes. Relation to vagal tone. *Scandinavian journal of gastroenterology* 33:236-241.
- Verberne AJ, Sabetghadam A, Korim WS (2014) Neural pathways that control the glucose counterregulatory response. *Front Neurosci* 8:38.
- Wan S, Browning KN (2008) D-glucose modulates synaptic transmission from the central terminals of vagal afferent fibers. *Am J Physiol Gastrointest Liver Physiol* 294:G757-763.
- Wang PY, Caspi L, Lam CK, Chari M, Li X, Light PE, Gutierrez-Juarez R, Ang M, Schwartz GJ, Lam TK (2008) Upper intestinal lipids trigger a gut-brain-liver axis to regulate glucose production. *Nature* 452:1012-1016.
- Williams KW, Smith BN (2006) Rapid inhibition of neural excitability in the nucleus tractus solitarius by leptin: implications for ingestive behaviour. *The Journal of physiology* 573:395-412.
- Yi CX, La Fleur SE, Flier JS, Kalsbeek A (2010) The role of the autonomic nervous liver innervation in the control of energy metabolism. *Biochim Biophys Acta* 1802:416-431.
- Zsombok A, Bhaskaran MD, Gao H, Derbenev AV, Smith BN (2011) Functional plasticity of central TRPV1 receptors in brainstem dorsal vagal complex circuits of streptozotocin-treated hyperglycemic mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 31:14024-14031.
- Zsombok A, Smith BN (2009) Plasticity of central autonomic neural circuits in diabetes. *Biochim Biophys Acta* 1792:423-431.

## Figures

**Figure 1.** Expression of glucokinase (GCK) and ATP-sensitive K<sup>+</sup> (K<sub>ATP</sub>) channels in the dorsal vagal complex of normoglycemic control and hyperglycemic, streptozotocin (STZ)-treated mice. **A.** Molecular expression of GCK mRNA was significantly reduced in the dorsal vagal complex of STZ-treated mice after 3-4 days of chronic hyperglycemia versus control mice ( $p < 0.05$ ;  $n = 8$  for each group). Expression of Kir6.2 and SUR1, components of the K<sub>ATP</sub> channel, were not significantly altered ( $p > 0.05$ ). **B.** Western blot analysis indicated that GCK protein expression was lower in the dorsal vagal complex of STZ-treated mice ( $n = 6$ ) than controls ( $p < 0.05$ ;  $n = 8$ ). Inset: Sample blot of  $\beta$ -actin and GCK protein expression from control and STZ-treated mice; center lane is protein ladder. Asterisks indicate  $p < 0.05$ .

**Figure 2.** Modulation of synaptic responses in NTS neurons by glucosamine (GA). **A.** Graph showing the relative overall effect of GA on sEPSCs and sIPSCs in GABAergic mNTS neurons from control and STZ-treated mice ( $n = 6-15$ ). sEPSC frequency was suppressed in the presence of GA in 80% of neurons from control mice, but GA was generally without effect on sEPSC frequency in most neurons (83%) from STZ-treated mice. Asterisk indicates significant difference from control group for sEPSC frequency. Effects of GA on sIPSCs were variable in both control and STZ-treated mice. **B.** Pie graphs indicating the percentage of neurons in which GA application evoked a  $\geq 20\%$  change in frequency of either sEPSCs or sIPSCs in mice from each treatment group.

**Figure 3.** Effects of elevated glucose on sodium currents (action potentials) in NTS neurons from control and STZ-treated mice. **A.** Examples of on-cell recordings in GABAergic mNTS neurons from control (upper) and STZ-treated (lower) mice showing spontaneous action potential (sodium current) activity in ACSF containing 2.5 mM glucose, 15 mM glucose, and after 15 min wash to 2.5 mM glucose. **B.** Pie graphs indicating the proportion of neurons (n=13 control; n=15 STZ-treated mice) that responded with an increase, decrease, or no change in action potentials when glucose concentration was elevated. Glucose elevation most often increased action potential firing in neurons from control mice, whereas firing rate was unchanged in most neurons from STZ-treated mice.

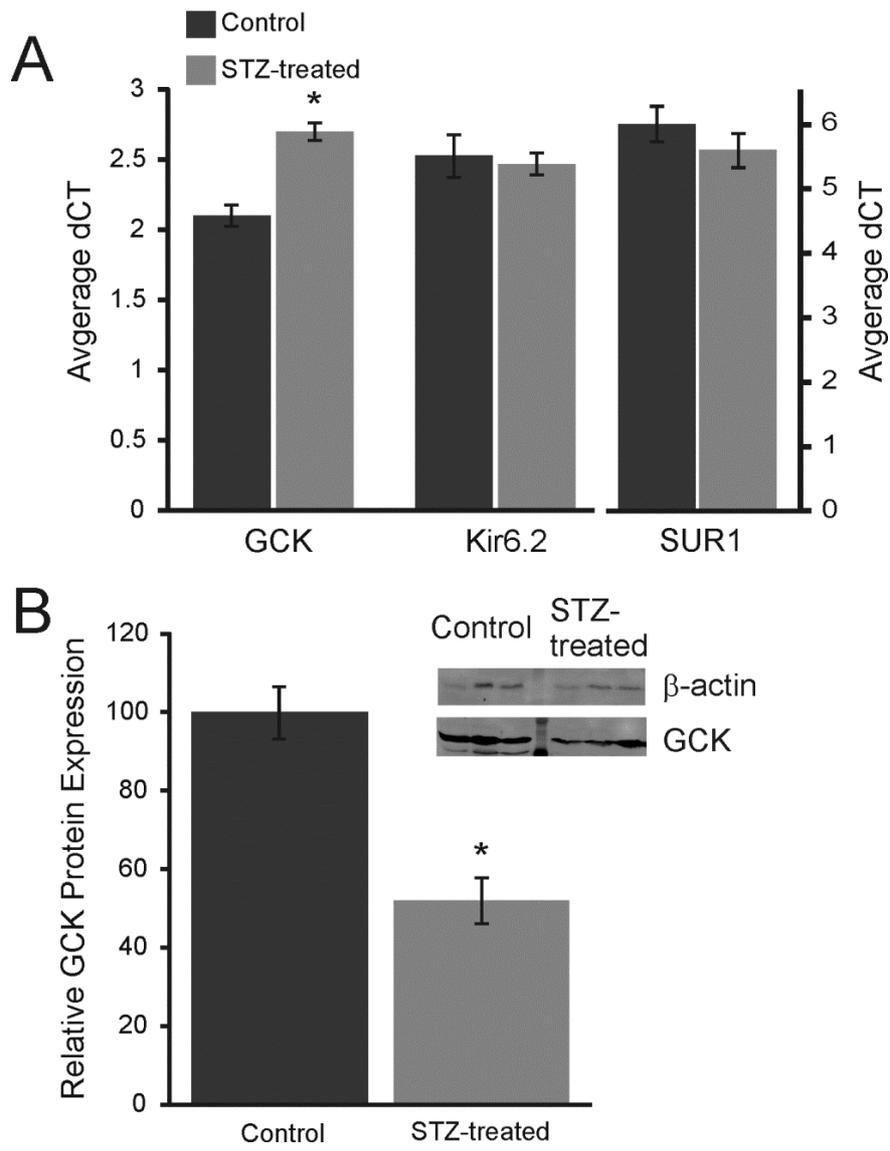


Figure 1

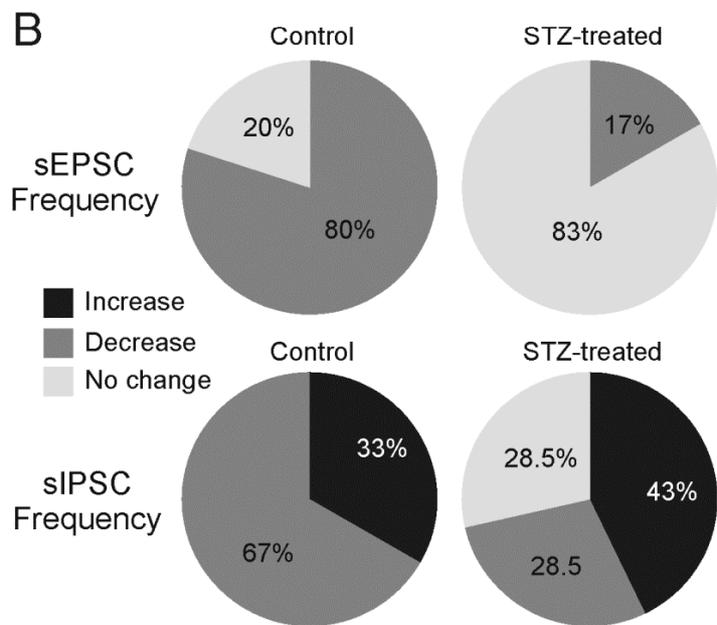
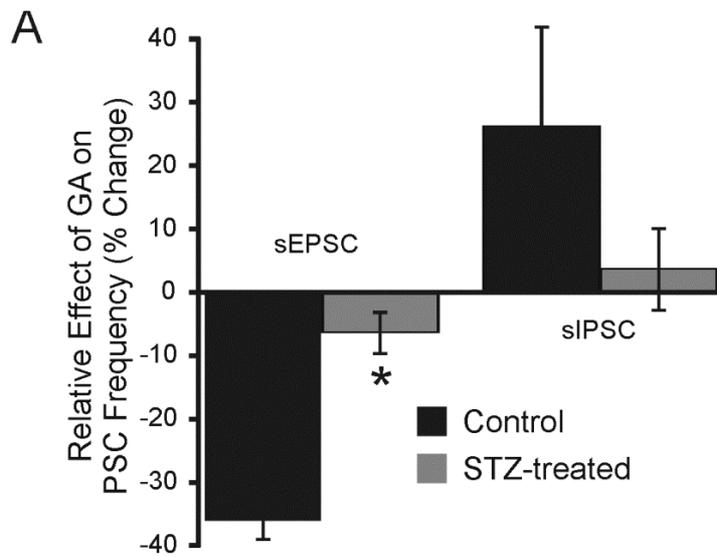


Figure 2

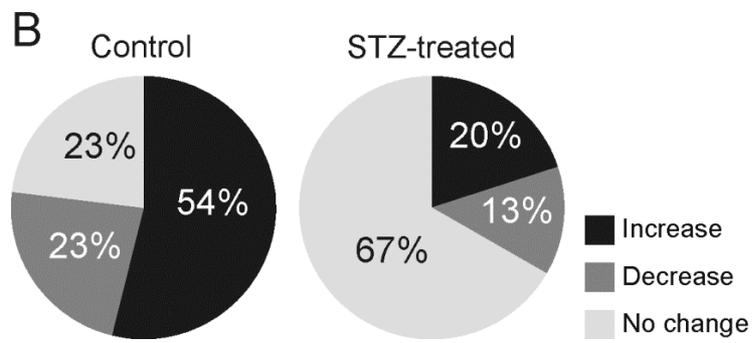
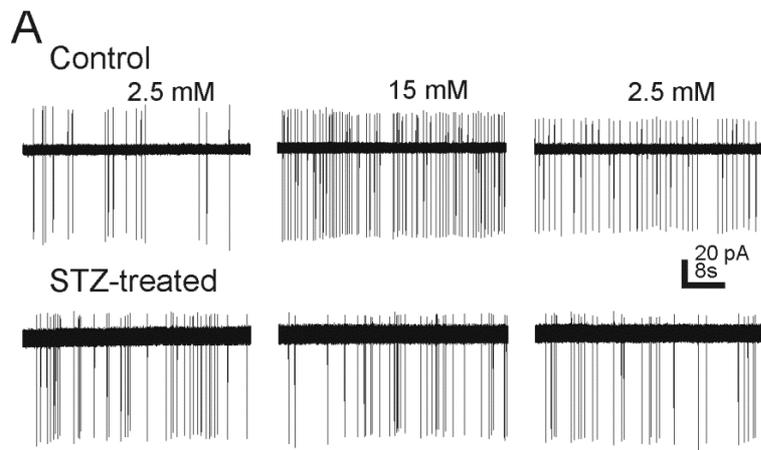


Figure 3

**II.**

**III.**