Diabetes induces GABA receptor plasticity in murine vagal motor neurons

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Diabetes induces GABA receptor plasticity in murine vagal motor neurons. J Neurophysiol 114: 698–706, 2015. First published May 20, 2015; doi:10.1152/jn.00209.2015.—Autonomic dysregulation accompanies type-1 diabetes, and synaptic regulation of parasympathetic preganglionic motor neurons in the dorsal motor nucleus of the vagus (DMV) is altered after chronic hyperglycemia/hypoinsulinemia. Tonic gamma-aminobutyric acid A (GABA_A) inhibition prominently regulates DMV neuron activity, which contributes to autonomic control of energy homeostasis. This study investigated persistent effects of chronic hyperglycemia/hypoinsulinemia on GABA_A receptor-mediated inhibition in the DMV after streptozotocin-induced type-1 diabetes using electrophysiological recordings in vitro, quantitative (q)RT-PCR, and immunohistochemistry. Application of the nonspecific GABA_A receptor agonist muscimol evoked an outward current of significantly larger amplitude in DMV neurons from diabetic mice than controls. Results from application of 4,5,6,7-tetrahydroisoaxazole[5,4-c]pyridin-3-ol hydrochloride (THIP), a δ-subunit agonist, suggested that GABA_A receptors containing δ-subunits contributed to the enhanced inducible tonic GABA current in diabetic mice. Sensitivity to THIP of inhibitory postsynaptic currents in DMV neurons from diabetic mice was also increased. Results from qRT-PCR and immunohistochemical analyses indicated that the altered GABAergic inhibition may be related to increased trafficking of GABA_A receptors that contain the δ-subunit, rather than an expression change. Overall these findings suggest increased sensitivity of δ-subunit containing GABA_A receptors after several days of hyperglycemia/hypoinsulinemia, which dramatically alters GABAergic inhibition of DMV neurons and could contribute to diabetic autonomic dysregulation.

GABA; tonic current; diabetes

Regulation of central autonomic function has thus been suggested as a novel therapeutic option to control diabetic hyperglycemia (Breen et al. 2013).

The preganglionic parasympathetic motorneurons innervating most of the subdiaphragmatic visera are located in the brainstem dorsal motor nucleus of the vagus (DMV). Together with sensory relay neurons in the nucleus tractus solitarius (NTS), the DMV regulates visceral function via the vagus nerve. Inhibitory, GABAergic neurotransmission is arguably the most prominent regulator of ongoing vagal motorneuron activity (Travaglione et al. 2006). GABA_A receptor antagonist application into the vagal complex significantly alters gastrointestinal and pancreatic function (Feng et al. 1990; Mussa and Verberne 2008; Washabau et al. 1995). Vagally mediated visceral functions related to energy homeostasis are thus tightly regulated by GABAergic signaling in the vagal complex.

The most typical GABA_A receptor isoforms in the brain contain a heteropentameric arrangement of two α-, two β-, and a γ-subunit(s) (Fritschy and Brunig 2003) and are considered to mediate the spatially and temporally distinct GABA signaling at the synaptic cleft, known as phasic inhibition. GABA has also been implicated in sustained neuronal modulation, specifically excitability and gain setting, through GABA_A receptors likely located peri- and extrasynaptically (Nusser et al. 1998; Semyanyov et al. 2003). Unlike phasic inhibition, tonic GABA inhibition is largely mediated by GABA_A receptors containing a δ-subunit in place of the γ-subunit (Brickley et al. 2001). Receptors containing δ-subunits confer a relatively high affinity for GABA and slow rates of desensitization, allowing for tonic activation at ambient GABA concentrations (Bai et al. 2001; Haas and Macdonald 1999; Yeung et al. 2003). In the DMV, tonic GABA currents are mainly mediated by δ-subunit containing receptors, but receptors containing γ-subunits also contribute in a subset of neurons when GABA concentration is increased (Gao and Smith 2010a,b). Both phasic and tonic forms of GABAergic inhibition contribute significantly to vagal motorneuron membrane potential and cellular activity (Bouairi et al. 2006; Gao and Smith 2010a,b).

Acutely elevated glucose concentration hyperpolarizes the membrane of gastric-related DMV neurons in a manner consistent with activation of tonic GABA currents, possibly due to elevated GABA release in the DMV (Ferreira et al. 2001). Diabetes-associated plasticity of GABA signaling in the DMV, however, has not been investigated adequately. We tested the hypothesis that GABA_A receptor-mediated inhibition is persistently altered in DMV neurons after chronic hyperglycemia/hypoinsulinemia in a mouse model of type-1 diabetes. Sustained changes in GABAergic inhibition of DMV neurons could contribute to diabetic vagal hypotonias.

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There are over 23 million people living with diabetes in the United States (Scully 2012), and the incidence is increasing (Bytzer et al. 2001). Recent studies have begun to focus on the importance of regulating blood glucose levels, independent of insulin, for treating diabetes (Breen et al. 2013; Ryan et al. 2014). Consistent with disrupted autonomic function, diabetes compromises several indexes of parasympathetic dysregulation, including altered gastric function (Rayner et al. 2001; Saltzman and McCallum 1983), reduced insulin secretion (Ahren 2000; Mussa and Verberne 2008; Yamatani et al. 1998), and dysregulated hepatic glucoseogenesis (Pociari et al. 2005), all of which are linked to increased mortality (Thayer and Lane 2007). Previously, these complications were attributed to neuropa thy (Horowitz et al. 2002), but diminished parasympathetic drive precedes degeneration of the vagus nerve (Mabe and Hoover 2011), implicating centrally mediated dysregulation of visceral motor control before neuronal degeneration.
METHODS

All experiments were performed on juvenile male (29.19 ± 1.23 g; 40 ± 1 days old) CD-1 mice (Harlan, Indianapolis, IN) housed in the University of Kentucky Division of Laboratory Animal Resources facilities under normal 14:10-h light-dark conditions, with food and water available ad libitum. The University of Kentucky Animal Care and Use Committee approved all animal procedures.

To induce type-1 diabetes with fructose hyperglycemia, mice were fasted for 5–6 h before receiving an intraperitoneal injection of either streptozotocin (STZ; 200 mg/kg), to eliminate insulin-secreting pancreatic β-cells, or the saline vehicle (0.1 ml). After injection, mice were returned to their home cages for 5–10 days. Blood glucose levels were monitored daily by tail snip and animals were considered diabetic after ≥3 consecutive days of sustained blood glucose levels ≥300 mg/dl.

Electrophysiology. On the day of experimentation, mice were anesthetized with isoflurane to effect (i.e., lack of tail-pinch response) and decapitated while anesthetized. The brainstem was rapidly removed and submerged in ice-cold (0–4 °C), oxygenated artificial cerebrospinal fluid (aCSF) with the following composition (in mM): 124 NaCl, 3 KCl, 26 NaHCO₃, 1.4 NaH₂PO₄, 11 glucose, 1.3 CaCl₂, 1.3 MgCl₂, and 1 kynurenic acid (KYN). The osmolarity of all solutions was 290–305 mosM, pH 7.3–7.4. The brainstem was mounted and slices (300 μm) were cut in the coronal plane using a vibratome. The slices were transferred to a holding chamber and incubated in warmed (30–33°C), oxygenated aCSF for 1 h before being transferred to a recording chamber mounted on the fixed stage of an upright microscope (BX51WI; Olympus, Melville, NY) where they were continually superfused with warmed (30–33°C), oxygenated aCSF of identical composition to that used for slice preparation. KYN was included in all recordings to block non-NMDA ionotropic glutamate receptors.

Whole cell patch-clamp recordings were performed under visual control on an upright, fixed-stage microscope equipped with infrared illumination and differential interference contrast (IR-DIC). Glass recording pipettes (2–5 MΩ; King Precision Glass, Claremont, CA) were filled with a solution containing the following (in mM): 130 Cs⁺-glucuronate, 1 NaCl, 5 EGTA, 10 HEPES, 1 MgCl₂, 1 CaCl₂, and 2–3 Mg₂-ATP; pH 7.3–7.4, adjusted with 5 M CsOH. Cs⁺ was used as the primary cation carrier to block K⁺ currents, which allowed consistent voltage clamp at depolarized membrane potentials and also diminished any influence of postsynaptic GABA_A receptors during recordings. Inhibitory GABA_A receptor-mediated currents (both tonic and phasic) were examined at a holding potential of 0 mV to isolate chloride currents. Recordings were discarded if series resistance was >25 MΩ or changed by >20% throughout the course of the experiment; mean series resistance was 13.5 ± 0.6 MΩ. Electrophysiological signals were recorded using an Axoclamp 700B amplifier (Molecular Devices, Union City, CA), acquired at 20 kHz, low-pass filtered at 3 kHz, and stored to a computer using a Digidata 1440A A-D converter and pClamp 10.2 software (Molecular Devices).

All drugs were bath applied until a steady state was reached, at which time analyses were performed. Drugs used included the GABA_A receptor antagonist bicuculline methiodide (BIC; 30 μM), which blocks both tonic and phasic GABA currents (Gao and Smith 2010a), the GABA_A receptor agonist muscimol (MUS; 75 nM), and 4,5,6,7-tetrahydrodioxoxazol[5,4-c]pyridin-3-ol hydrochloride (THIP; 1–10 μM), an agonist with preference for δ-subunit-containing GABA_A receptors. MUS and THIP were received from Sigma-Aldrich (St. Louis, MO). BIC was received from R&D Systems (Minneapolis, MN).

Quantitative real-time PCR. In a separate group of animals, three to four slices were generated in a manner similar to the procedure used for electrophysiological recordings to determine mRNA expression of GABA_A receptor subunits α4 and δ. From each slice, a 1-mm diameter punch (Miltex, York, PA) was taken containing most of the dorsal vaginal complex, with minimal tissue sampled from other surrounding structures. Punches from one animal were pooled into one sample. Each sample was immediately homogenized, placed in 0.5 ml of Trizol (Sigma, St. Louis, MO), and centrifuged according to manufacturer’s instructions. Spectrophotometry (NanoDrop, Wilmington, DE) was used to determine mRNA concentration and purity. RNA samples (2 μg) were then used to create cDNA. All quantitative RT-PCR reactions were run in triplicate in 96-well optical grade plates using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The abundance of all cDNA was determined using RT-PCR master mix (Applied Biosystems). Total volume for each run was 20 μl containing 80 ng of cDNA. The reaction times and temperature were 50°C for 2 min, 95°C for 10 min, followed by 50 cycles of 95°C for 15 s, and 60°C for 1 min. Primer and Taqman probe sets were purchased from Applied Biosystems. The sequences for each were generated from the listed references within GenBank (α4: Mm00802631; δ: Mm01266203; and β-actin: Mm00607939). All reactions used forward and reverse primer concentrations of 100 nM. Probe concentrations for all reactions were 50 nM. No-template and no-RT controls were run for each plate. Fold change in receptor subunit expression was calculated by the formula 2^ΔΔCt using β-actin as an internal reference (Livak and Schmittgen 2001). Relative mRNA abundance was presented as fold change; the ΔCt was used to determine statistical significance with an unpaired, two-tailed t-test (Wood and Giroux 2003).

Immunohistochemistry. Mice were anesthetized with isoflurane and perfused transcardially with phosphate-buffered saline (pH 7.4) followed by 4% paraformaldehyde (Sigma) and 0.1% picric acid (Sigma). Serial coronal sections (20 μm) were cut using a cryostat and processed for choline acetyltransferase (ChAT) and GABA_A receptor β-subunit immunoreactivity. Sections were rinsed with 0.05 M Tris-buffered saline (TBS; pH 7.4) and nonspecific immunoreactivity blocked with 10% normal donkey serum (Jackson Immunoresearch, West Grove, DE) was used to determine mRNA concentration and purity. RNA samples (2 μg) were then used to create cDNA. All quantitative RT-PCR reactions were run in triplicate in 96-well optical grade plates using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The abundance of all cDNA was determined using RT-PCR master mix (Applied Biosystems). Total volume for each run was 20 μl containing 80 ng of cDNA. The reaction times and temperature were 50°C for 2 min, 95°C for 10 min, followed by 50 cycles of 95°C for 15 s, and 60°C for 1 min. Primer and Taqman probe sets were purchased from Applied Biosystems. The sequences for each were generated from the listed references within GenBank (α4: Mm00802631; δ: Mm01266203; and β-actin: Mm00607939). All reactions used forward and reverse primer concentrations of 100 nM. Probe concentrations for all reactions were 50 nM. No-template and no-RT controls were run for each plate. Fold change in receptor subunit expression was calculated by the formula 2^ΔΔCt using β-actin as an internal reference (Livak and Schmittgen 2001). Relative mRNA abundance was presented as fold change; the ΔCt was used to determine statistical significance with an unpaired, two-tailed t-test (Wood and Giroux 2003).

Data analysis. To determine drug effects on phasic [i.e., inhibitory postsynaptic current (IPSCs)] and tonic inhibitory currents, 2 min of continuous steady-state activity (5–10 min after drug equilibration within the chamber) were analyzed offline using Clampex 10.2 (Molecular Devices) and MiniAnalysis 6.0.3. (Synaptosoft, Decatur, CA). Tonic GABA currents are significantly dependent on ambient GABA concentration resulting from action potential-dependent GABA release, so tetrodotoxin was not used to block action potentials in this analysis (Gao and Smith 2010a). All synaptic events were used to assess spontaneous (sIPSCs) frequency, but only unitary events (i.e., single peak) were used for sIPSC amplitude and decay time constant measurements. Charge transfer for the averaged sIPSCs (Qpc) was calculated with the equation Qpc = f × QsIPSCs × t, where f is the sIPSC frequency (Hz), QsIPSCs is the mean charge transfer/sIPSC during the 2 min, and t is the duration (120 s) (Ataka and Gu 2006). The effects of drug application on IPSC characteristics are expressed as the percent change from baseline.
Tonic currents were measured either as agonist-induced (the difference in holding current between control aCSF and steady state in the presence of agonist) or total current (the holding current difference between agonist application and steady state in the presence of BIC). Changes in tonic current amplitude were considered significant if they were greater than twice the average root mean square value; the minimum tonic current amplitude measurement to be considered significant was therefore ≥5.8 pA. Tonic currents were corrected for neuronal size by normalizing to whole cell capacitance to account for any slight variability in space-clamp (Glykys and Mody 2007) and are therefore presented as current density (pA/pF). There was no difference in whole cell capacitance between groups measured by the algorithm present within the pClamp protocol (46.7 ± 2.3 pF in saline treated vs. 45.2 ± 2.8 pF in STZ treated; \( P = 0.68 \)). For all electrophysiological experiments, only one cell was used per slice. Data are presented as means ± SE. Statistical measurements were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Two-tailed student t-tests were used to determine statistical differences between groups (i.e., saline- and STZ-treated animals) for tonic and phasic current and RT-PCR analyses. The nonparametric Mann-Whitney U-test was used to determine whether the number of neurons exhibiting inducible currents changed after STZ-treatment. A one-tailed Student’s t-test was used when analysis was restricted to only the neurons with significant agonist-induced tonic currents (i.e., unidirectional change). \( P < 0.05 \) was considered significant.

**RESULTS**

**Characteristics of STZ-induced diabetes.** There was no difference in the age of animals used (39 ± 2 days for saline-treated vs 41 ± 4 days for diabetic animals; \( P > 0.05 \)). The average blood glucose concentration of saline-treated CD-1 mice was 164 ± 7 mg/dl (\( n = 16 \)). The average blood glucose of STZ-treated mice was 507 ± 15 mg/dl (\( n = 17 \)). Animals were maintained in a diabetic state (blood glucose >300 mg/dl) for 3–7 days, with an average diabetic duration of 5.7 ± 0.4 days. By the day of experimentation, STZ-treated mice weighed less (27.6 ± 1.3 g) than saline-treated mice (32.4 ± 0.8 g; \( P = 0.004 \)), which is similar to previous reports (Rerup and Tarding 1969). However, weight did not correlate with any of the tonic GABA current density measures, including baseline current (\( R^2 = 0.12 \)), inducible MUS current (\( R^2 = 0.01 \)), or inducible THIP current (\( R^2 = 0.34 \)).

**Phasic currents.** sIPSC parameters were measured to determine phasic current parameters in DMV neurons from saline- and STZ-treated mice. Mean sIPSC frequency in DMV neurons was 4.5 ± 1.2 and 5.2 ± 0.9 Hz in saline- and STZ-treated animals, respectively (\( P = 0.62 \)). Mean amplitude tended to be elevated in STZ animals but was not significantly different (35.0 ± 2.7 pA in saline vs. 42.3 ± 2.6 pA in STZ; \( P = 0.06 \)). Mean decay time constant (11.4 ± 0.6 ms, saline treated vs. 12.7 ± 0.7 ms, STZ treated; \( P = 0.21 \)) and total phasic current charge transfer (514.1 ± 151.2 pA/s, saline-treated vs. 758.3 ± 252.2 pA/s, STZ-treated animals) were also not different between groups. There was no statistical difference in phasic current parameters between DMV neurons from saline- and STZ-treated mice under baseline conditions.

**Inducible tonic currents after MUS application.** Previous investigations of tonic currents in the DMV identified a receptor pool that is not saturated under normal baseline conditions and could be evoked by applying GABA\(_A\) receptor agonists (Gao and Smith 2010a,b). Therefore, the GABA\(_A\) receptor agonist MUS (75 nM) was applied to DMV neurons from control (\( n = 9 \)) and diabetic (\( n = 7 \)) mice to characterize the contribution of the global GABA\(_A\) receptor pool mediating the tonic current (Fig. 1A). An increase in the holding current after MUS application compared with baseline was defined as the inducible tonic current that likely results from activation of a receptor pool that is available but not bound by GABA under baseline conditions. Neurons from diabetic mice had a larger mean MUS-induced tonic current amplitude (19.3 ± 4.8 pA; \( n = 7 \); Fig. 1C) and density (0.43 ± 0.08 pA/pF; Fig. 1D) compared with those from saline-treated mice (6.3 ± 2.2 pA; \( P = 0.04 \); 0.12 ± 0.04 pA/pF; \( P = 0.007 \); \( n = 9 \)). The proportion of neurons expressing the MUS-inducible current (\( n = 6 \) of 9 neurons in control and 5 of 7 neurons in STZ-treated mice) was not significantly different between groups (Fig. 1B; \( P = 0.45 \)). Nevertheless, to ensure that the increased MUS-induced response in cells from STZ-treated mice was not related to differences in numbers of responsive neurons between groups, neurons demonstrating a MUS-induced tonic current (\( n = 6 \) saline, \( n = 5 \) STZ treated) were examined independently (Fig. 1E). An increased MUS-induced current density was also detected when only the responding subset of cells was analyzed (0.54 ± 0.10 pA/pF in STZ treated; \( n = 5 \); vs. 0.20 ± 0.01 pA/pF in saline-treated; \( n = 6 \); \( P = 0.02 \); Fig. 1E). For all neurons measured, there was no difference in frequency (4.0 ± 1.6 in saline vs. 5.5 ± 1.2 Hz in STZ; \( P = 0.5 \)), amplitude (36.1 ± 4.5 vs. 38.5 ± 3.6 pA; \( P = 0.7 \)), decay time constant (11.7 ± 0.8 vs. 13.9 ± 1.0 ms; \( P = 0.1 \)), or charge transfer (429.8 ± 195.0 vs. 771.7 ± 284.0 pA/s) of phasic currents after MUS application. No differences existed for IPSC frequency (\( P = 0.4 \)), amplitude (\( P = 0.8 \)), decay time (\( P = 0.3 \)), or charge transfer (\( P = 0.3 \)) when only those neurons demonstrating a MUS-induced tonic current were examined.

To assess whether resting tonic current (i.e., the current activated by ambient GABA in the slice) was different between saline- and STZ-treated mice, the baseline tonic current was compared with the current induced by the addition of BIC. The BIC-induced tonic current amplitude (32.9 ± 3.9 pA, saline treated; \( n = 18 \); 34.8 ± 4.1 pA, STZ treated; \( n = 16 \); \( P = 0.7 \)) and density (0.70 ± 0.07 pA/pF, saline treated; 0.83 ± 0.12 pA/pF, STZ treated; \( P = 0.4 \)) were not different between the groups. Total tonic current was assessed by comparing the MUS-induced current to the current measured after blocking GABA\(_A\) receptors with BIC (i.e., \( I_{MUS} - I_{BIC} = I_{tonic(G)} \)). Although the total tonic current amplitude was not significantly different between groups (45.5 ± 5.6 pA, saline treated; \( n = 9 \); 59.3 ± 7.2 pA, STZ treated; \( n = 7 \); \( P = 0.2 \)), the total tonic current density was significantly larger in neurons from STZ treated (1.42 ± 0.23 pA/pF; Fig. 1D) compared with saline-treated mice (0.90 ± 0.08 pA/pF; \( P = 0.04 \)). Taken together, these data suggest that DMV neurons from diabetic mice have a larger pool of unoccupied, agonist-inducible receptors that may potentially contribute to tonic GABA\(_A\) receptor-mediated current than controls.

**THIP-induced tonic GABA current.** The GABA\(_A\) receptor agonist THIP (1–10 \( \mu \)M), which has preference for receptors containing the \( \delta \)-subunit (Drasbek and Jensen 2006; Ebert et al. 1994; Meera et al. 2011; Ranna et al. 2006), was applied to assess sensitivity of the tonic current to THIP in DMV neurons from saline- and STZ-treated mice (Fig. 2). At concentrations <10 \( \mu \)M, THIP is relatively selective for GABA\(_A\) receptors
containing δ-subunits (Meera et al. 2011; Mtchedlishvili et al. 2010) and the THIP-induced tonic current in rat DMV neurons was previously shown to be maximal at 10 μM (Gao and Smith 2010a), so responses to 1, 3, and 10 μM were determined. At 1 μM THIP, 20% (n = 5) of neurons from saline-treated mice and 33% (n = 3) of neurons from STZ-treated mice displayed a THIP-inducible tonic current density of 0.02 ± 0.08 and 0.3 ± 0.2 pA/pF, respectively. At 3 μM, 33% (n = 9) of neurons from saline-treated mice and 78% (n = 9) of neurons from STZ-treated mice responded with inducible tonic current density of 0.11 ± 0.05 and 0.54 ± 0.12 pA/pF, respectively (P = 0.0008). At 10 μM, 75% of neurons from saline-treated controls (n = 4) and all (n = 7) neurons from the STZ-treated mice displayed inducible currents. The average inducible current in these neurons was 0.28 ± 0.08 in DMV neurons from saline-treated controls and 0.54 ± 0.12 pA/pF (P = 0.01) from neurons in STZ-treated mice. Sensitivity of the tonic current to THIP was increased in neurons from STZ-treated mice, determined by both the magnitude and prevalence of the response.

For the remaining analyses, a concentration of 3 μM was used. This concentration can be used to determine responses of putative δ-containing receptors (Drasbek and Jensen 2006; Ebert et al. 1994; Meera et al. 2011; Mortensen et al. 2010; Mtchedlishvili et al. 2010) and is less than that necessary to induce activity attributable to receptors containing γ2-subunits (Ebert et al. 1994; Meera et al. 2011; Mortensen et al. 2010). Similar to MUS, THIP induced an outward current in most DMV neurons (Fig. 3A). Overall, the THIP-induced current was significantly larger in amplitude and density in DMV neurons from STZ-treated mice compared with saline-treated controls (6.5 ± 6.7 pA; P = 0.008; and 0.54 ± 0.12 pA/pF; P = 0.01; n = 9; Fig. 3C) compared with saline-treated mice (5.4 ± 2.5 pA; 0.11 ± 0.05 pA/pF; n = 9). Total tonic current induced after THIP application was assessed by measuring the maximum THIP-induced current before and after addition of BIC (i.e., I_{THIP} - I_{BIC}). The total tonic current amplitude (57.2 ± 5.9; P = 0.008) and density (1.25 ± 0.10 pA/pF; Fig. 3D) were significantly elevated in
neurons from diabetic vs. saline-treated animals (31.9 ± 6.4 and 0.73 ± 0.13 pA/pF, respectively). The STZ-treated group also demonstrated significantly more neurons (7/9) with inducible currents than saline-treated mice (3/9; P = 0.04; Fig. 3B). THIP-responsive neurons were examined independently to determine whether the greater current magnitude in cells from STZ-treated mice was related to the relatively higher proportion of responding cells after STZ treatment or was due to an increase in inducible current amplitude in the subset of cells expressing the current. Although relatively few cells from controls responded (n = 3), the increase in THIP-induced current density in neurons from diabetic animals (n = 7) was maintained for these subgroups of responding neurons (0.67 ± 0.11 pA/pF in STZ treated; 0.30 ± 0.04 pA/pF in saline treated; Fig. 3E; P = 0.02). Thus the proportion of DMV cells expressing a THIP-induced current was higher in the STZ-treated group, and the current amplitude and density were greater in cells from STZ-treated mice.

In all neurons examined, phasic sIPSC frequency (2.9 ± 1.0, saline treated; 3.6 ± 1.1 Hz, STZ treated; P = 0.7), amplitude (32.1 ± 4.0 vs. 39.7 ± 3.2 pA; P = 0.1), decay time constant (12.9 ± 1.2 vs. 13.8 ± 0.8 ms; P = 0.6), and total phasic current charge transfer (275.4 ± 97.0 vs. 550.3 ± 293.7 pA/s; P = 0.4) were not significantly different between treatment groups during THIP application. However, DMV neurons from STZ-treated animals demonstrated a significantly larger percent increase in sIPSC decay time constant after THIP application compared with baseline conditions (2.7 ± 3.3% saline treated; 13.9 ± 3.3% STZ treated; Fig. 4; P = 0.02). STZ treatment did not significantly alter the percent change in THIP-sensitivity of sIPSC frequency (P = 0.2), amplitude (P = 0.8), or phasic current charge transfer (P = 0.5) in DMV neurons. Therefore, the effect of THIP on tonic current was accompanied by an increased sIPSC decay time constant, suggesting that functional THIP-sensitive GABA receptors also increased at the synapse in DMV neurons from STZ-treated mice. No differences existed when only those neurons demonstrating a THIP-induced tonic current were examined for frequency (P = 1.0), amplitude (P = 0.1), decay time (P = 0.6), or charge transfer (P = 0.6). Percent change was also not different in frequency (P = 0.2), amplitude (P = 1.0), decay time (0.6), or charge transfer (P = 0.9).

Real-time quantitative PCR. Quantitative RT-PCR was employed to determine whether the increase in THIP-induced tonic inhibition reflected an increase in the transcription of the δ-subunit of the GABAA receptor. The δ-subunit’s traditional, predominant partner, α4, was also examined because this subunit can influence the membrane expression of the δ-subunit (Abramian et al. 2010). Fold change in mRNA expression is illustrated in Fig. 5 for the α4- and δ-GABAA receptor.
subunits. The α4 (P = 0.09) - and δ-subunit (P = 0.65) mRNA expression levels were not different between the groups.

**Immunohistochemistry.** Immunohistochemically labeled δ-subunits associated with the membrane and increased functional expression appear as clustered or enlarged puncta on or close to the surface of the neuron (Abramian et al. 2014; Mangan et al. 2005). Regardless of treatment, δ-containing receptor labeling in the DMV was less robust than in the cerebellum, which has high δ-subunit expression. In general, somatic staining for the δ-subunit was diffuse in DMV neurons from saline-treated control mice (n = 6; Fig. 6), being distributed lightly throughout the soma and proximal dendrites. Labeling in the DMV from STZ-treated diabetic mice (n = 6) was characterized by bright immunoreactive puncta in DMV neurons from all mice (Fig. 6), which was qualitatively different than what was observed in controls. These findings imply that receptor trafficking and/or clustering may be associated with the increased functional expression demonstrated in electrophysiological recordings from DMV neurons in diabetic mice.

**DISCUSSION**

The present study investigated effects of STZ-induced type 1 diabetes on tonic GABA currents in the DMV and identified significant modification of the GABAergic inhibitory regulation of DMV neuronal function in diabetic mice relative to controls, even after normalizing glucose concentrations in vitro. An agonist-induced, GABA<sub>Α</sub> receptor-mediated tonic current was observed in DMV neurons, which was significantly increased in both magnitude and proportion of neurons expressing it after several days of sustained hyperglycemia. This increase is likely mediated by functional changes in δ-subunit containing receptors, since THIP sensitivity was increased. Molecular expression of δ-subunits was not increased, but changes in immunohistochemical labeling patterns inferred a redistribution of the receptors. Taken together, these results indicate a functional upregulation of GABA<sub>Α</sub> receptors due to an increased contribution of δ-subunit containing GABA<sub>Α</sub> receptors in DMV neurons from mice with type 1 diabetes.

Acutely elevated glucose concentration in the dorsal brainstem induces an increase in GABAergic inhibition of DMV neurons, resulting in significant changes in gastrointestinal function (Ferreira et al. 2001). With an increased agonist-inducible component of tonic inhibition, glucose-stimulated release of GABA onto DMV neurons would be expected to result in a significant membrane hyperpolarization and decreased action potential firing in diabetic mice, effects that exceed those in neurons from normoglycemic animals. The sustained plasticity of receptors mediating tonic inhibition in the DMV suggests that the enhanced response to GABA could outlast the glucose-stimulated effect on GABA release and influence cellular functions persistently, independent of responses to acute changes in glucose concentration. The relatively larger effect of GABA in the DMV of mice with diabetes...
may provide a mechanism for underlying disturbances in parasympathetic signaling that precede overt neuropathy. The persistence of this effect in the presence of the standardized glucose concentration used in our recordings implies that DMV neuron, and thus vagus nerve, responsiveness may remain altered long after recovery from hyperglycemic events.

Under standard recording conditions, parameters of phasic GABA currents were similar between neurons from saline- and STZ-treated mice, suggesting that synaptic GABA release is approximately equivalent in the two groups. Alterations in THIP sensitivity of IPSC decay time constant are subtle in neurons from mice with diabetes, but even subtle changes in synaptic GABA receptor responses can significantly affect overall rodent behavior (Crestani et al. 1999; Shen et al. 2010). Since THIP sensitivity of sIPSC frequency was unchanged, neither a sustained increase in activity of intact, upstream GABAergic neurons projecting to the DMV nor an increase in total number of GABAergic synapses seems likely to underlie the increase in decay time constant. The THIP effects on sIPSC amplitude were also not different between groups, suggesting that the total number of GABA receptors at the synapse is not altered. Therefore, it seems likely that the increase in THIP-sensitive sIPSC decay time constant is due to plasticity in the postsynaptic receptor, possibly involving insertion of additional THIP-sensitive GABA<sub>A</sub> receptors in the synaptic membrane. The THIP-sensitive, total phasic current charge transfer in DMV neurons, however, was not different between saline- and STZ-treated animals. Therefore, they may not contribute as much to membrane potential control as those that occur in the tonic current receptor pool.

Consistent with previous reports (Gao and Smith 2010a), the present study uncovered a significant tonic current that is likely mediated by δ-containing receptors in DMV neurons, based on THIP sensitivity. This effect may not be related to a specific organ projection since retrograde labeling from periphery does not predict tonic current expression in the DMV (Gao and Smith 2010a). Most DMV neurons from saline-treated animals had a significant MUS-inducible current (67%). Neurons from STZ-treated animals, however, demonstrated significantly larger MUS-inducible currents with no change in resting tonic current (i.e., receptors active at ambient GABA concentration) or number of neurons with a MUS-inducible current. Although DMV neurons from diabetic animals may be normally inhibited under most conditions, they are capable of responding robustly when ambient GABA concentration increases (Ferreira et al. 2001).

Unlike MUS, a general GABA<sub>A</sub> receptor agonist that activates synaptic and extra-synaptic receptors, THIP had minimal effects on IPSCs and was probably mainly active at extrasynaptic receptors contributing to tonic currents, as has been described in this and other systems (Meera et al. 2011; Mortensen et al. 2010; Nusser et al. 1998). The lack of THIP-sensitive inducible current in most DMV neurons from saline-treated animals suggests that any δ-subunit containing receptors mediating tonic currents are likely saturated at normal GABA concentrations in most neurons from control mice, where tonic current activation contributes significantly to resting membrane potential in DMV neurons (Gao and Smith 2010a). Conversely, most neurons (78%) from STZ-treated mice demonstrated large-amplitude, THIP-inducible currents, although the resting tonic current (i.e., receptors active at ambient GABA concentration) was not significantly different. It is possible that the enhanced inducible component of tonic current allows DMV neurons from diabetic mice to discriminate between wider ranges of inhibitory input, especially when ambient GABA concentration increases, (e.g., during hyperglycemic events). The larger THIP-induced currents suggest increased function of THIP-sensitive, putatively δ-subunit-containing, receptors in neurons from diabetic mice. Studies in heterogeneous expression systems suggest that THIP can activate receptors containing subunits in addition to δ, including γ- or ρ- or θ-containing receptors at high concentrations (≥10 μM) (Ebert et al. 1994; Meera et al. 2011; Ranna et al. 2006), whereas 1–3 μM used here is relatively selective for GABA<sub>A</sub> receptors containing δ-subunits (Drasbek and Jensen 2006; Ebert et al. 1994; Meera et al. 2011). Tonic currents in the DMV can also be mediated in part by other receptor subunits (Gao and Smith 2010a,b), so receptor subunits other than the δ-subunit might also contribute to elevated tonic currents. Although the potential contribution of other subunits remains to be elucidated, the current data suggest elevations in the functional expression of the δ-subunit.

The precise mechanism for changes in putative δ-subunit containing receptor-mediated responses (i.e., increased effects of THIP) remains to be elucidated. It is possible that GABA release or reuptake are altered, but no change was observed in phasic current frequency or resting tonic current, suggesting that ambient GABA concentrations are not different. Rather, functional regulation of δ-subunits often results from posttranslational modification (Abramian et al. 2014; Goodkin et al. 2008). A serine residue in the α<sub>1</sub>-subunit serves as a phosphorylation site on the δ-containing receptor (Abramian et al. 2010; Joshi and Kapur 2009), which mediates trafficking of the receptor in the membrane. In particular, PKC activity can stabilize δ-containing receptors in the membrane (Joshi and Kapur 2009). Since transcription was not significantly altered, the diabetes-associated increase in functional responsiveness to THIP in DMV neurons may be associated with increased phosphorylation and/or receptor redistribution. Consistent with this hypothesis, clustering of immunolabeling was observed in DMV neurons from STZ-treated mice, which was similar to labeling patterns associated previously with increased receptor function in other brain regions (Abramian et al. 2010, 2014; Joshi and Kapur 2009).

Enhanced GABA currents in neurons from diabetic mice persisted after standardization of glucose levels, implying plasticity of the receptors that outlasts the hyperglycemic period. It is likely that the enhanced tonic GABA current in DMV neurons from diabetic mice actively inhibits DMV motor neuron activity, and this may be particularly relevant when GABA release is increased (e.g., by elevated glucose concentration). Modulation of GABA release has indeed been proposed as a state-setting mechanism in the DMV, modifying responses to other physiological stimuli (Browning et al. 2004). Speculatively, the agonist-inducible tonic GABA current may also counter the increased excitatory synaptic drive reported in this model (Bach et al. 2015; Zsombok et al. 2011). Since chronic hyperglycemia is associated with increased agonist-inducible currents (i.e., MUS and THIP), several days of diabetes in this model shifts the balance of inhibitory control of vagal neuron function by a significant increase in tonic inhibition. This elevated inhibitory capacity of DMV neurons may

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underlie the reduced parasympathetic drive seen in diabetes before overt vagal neuropathy and is a potential mechanism underlying diminished gastric motility, vagally mediated insulin release, and postprandial suppression of hepatic glucoseogenesis observed in diabetic patients.

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

AUTHOR CONTRIBUTIONS

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