Tonic GABA\textsubscript{A} receptor conductance in medial subnucleus of the tractus solitarius neurons is inhibited by activation of \( \mu \)-opioid receptors

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Herman MA, Gillis RA, Vicini S, Dretchen KL, Sahibzada N. Tonic GABA\textsubscript{A} receptor conductance in medial subnucleus of the tractus solitarius neurons is inhibited by activation of \( \mu \)-opioid receptors. J Neurophysiol 107: 1022–1031, 2012. First published November 23, 2011; doi:10.1152/jn.00853.2011.—Our laboratory previously reported that gastric activity is controlled by a robust GABA\textsubscript{A} receptor-mediated inhibition in the medial nucleus of the tractus solitarius (mNTS) (Herman et al. 2009), and that \( \mu \)-opioid receptor activation inhibits gastric tone by suppression of this GABA signaling (Herman et al. 2010). These data raised two questions: 1) whether any of this inhibition was due to tonic GABA\textsubscript{A} receptor-mediated conductance in the mNTS; and 2) whether \( \mu \)-opioid receptor activation suppressed both tonic and phasic GABA signaling. In whole cell recordings from rat mNTS neurons, application of three GABA\textsubscript{A} receptor antagonists (gabazine, bicuculline, and picrotoxin) produced a persistent reduction in holding current and decrease in population variance or root mean square (RMS) noise, suggesting a blockade of tonic GABA signaling. Application of gabazine at a lower concentration abolished phasic currents, but had no effect on tonic currents or RMS noise. Application of the \( \delta \)-subunit preferring agonist gadoxadol (THIP) produced a dose-dependent persistent increase in holding current and RMS noise. Pretreatment with tetrodotoxin prevented the action of gabazine, but had no effect on the THIP-induced current. Membrane excitability was unaffected by the selective blockade of phasic inhibition, but was increased by blockade of both phasic and tonic currents. In contrast, activation of tonic currents decreased membrane excitability. Application of the \( \mu \)-opioid receptor agonist DAMGO produced a persistent reduction in holding current that was not observed following pretreatment with a GABA\textsubscript{A} receptor antagonist and was not evident in mice lacking the \( \delta \)-subunit. These data suggest that mNTS neurons possess a robust tonic inhibition that is mediated by GABA\textsubscript{A} receptors containing the \( \delta \)-subunit, that determines membrane excitability, and that is partially regulated by \( \mu \)-opioid receptors.

Brain stem; tonic; \( \gamma \)-aminobutyric acid; opioid; vagus

Ours laboratory previously reported that GABA\textsubscript{A} receptor-mediated signaling in the medial subnucleus of the tractus solitarius (mNTS) is a major determinant of vago-vagal reflex activity in the basal state (Herman et al. 2009). These findings compelled us to investigate the types of GABA\textsubscript{A} receptor signaling in the mNTS present and their impact on neurons that comprise the circuitry controlling gastric activity. There are two types of GABA\textsubscript{A} receptor signaling that function in a cell- and region-specific manner. Phasic signaling involves the generation of inhibitory postsynaptic currents (IPSCs) that are the result of “point to point” transmission that occurs at synapses and results in 10–100 ms of inhibition. Tonic signaling is characterized by the presence of persistent inhibitory currents that are due to low levels of ambient GABA acting at highly sensitized extrasynaptic GABA\textsubscript{A} receptors (Glykys and Mody 2007a). GABA\textsubscript{A} receptors are heteromeric pentamers composed of specific subunit assemblies that confer the biophysical properties of each receptor class. Phasic GABA\textsubscript{A} receptors contain \( \alpha_{1-5}, \alpha_{5}, \) and/or \( \gamma \)-subunits. Tonic GABA\textsubscript{A} receptors are composed of the \( \alpha_{4}, \alpha_{5}, \alpha_{6}, \) and/or the \( \delta \)-subunits predominantly located at extrasynaptic sites (for review, see Belelli et al. 2009; Glykys and Mody 2007a). The charge transfer carried by tonic inhibition is over five times larger than that produced by synaptic inhibition (Semyanov et al. 2004), indicating that tonic GABA\textsubscript{A} receptor inhibition is a critical determinant of overall network tone and activity. Tonic inhibition is experimentally observed as a sustained reduction in holding current (\( I_{\text{hold}} \); or outward “shunt”) in response to pharmacological blockade by GABA\textsubscript{A} receptor antagonists such as bicuculline or gabazine (GBZ) (Bickley et al. 1996; Glykys and Mody 2007a). Another important pharmacological tool for studying tonic inhibition is the GABA\textsubscript{A} receptor agonist 4,5,6,7-tetrahydroisoxazolo(5,4-c)pyridin-3-ol (gadoxadol, THIP), which displays a dose-dependent preference for \( \alpha_{5} \), and/or \( \delta \)-subunit-containing GABA\textsubscript{A} receptors (Drasbek and Jensen 2006; Jensen and Lambert 1984; Stell and Mody 2002) and produces a sustained increase in \( I_{\text{hold}} \) (or inward “shunt”).

Although there is considerable evidence for direct synaptic (i.e., phasic) inhibition of mNTS neurons (Glatzer and Smith 2005; Travagli et al. 1991), the possibility exists that a significant proportion of the ongoing inhibition of mNTS neurons is mediated by ambient GABA in the mNTS acting on extrasynaptic receptors (i.e., tonic inhibition). Despite immunocytochemical evidence that the \( \delta \)-subunit of the GABA\textsubscript{A} receptor is expressed in the nucleus of the tractus solitarius (NTS) (Pirker et al. 2000), no studies have demonstrated the presence of tonic inhibition in NTS neurons in the basal state. One study that examined the contributions of different GABA\textsubscript{A} receptor subunits to currents in NTS neurons found no evidence for the \( \delta \)-subunit (Huang and Dillon 2001). However, this study was performed in neonatal rats (postnatal days 1–14). Since expression of the \( \delta \)-subunit is developmentally regulated, it is not likely to be fully expressed at this early age (Peden et al. 2008). Another study did report the presence of GBZ-insensitive tonic currents (\( I_{\text{tonic}} \)) in second-order NTS neurons. These currents were enhanced by the anesthetic agent propofol, but were not observed in the basal state (McDougall et al. 2008). Indirect evidence for \( I_{\text{tonic}} \) in NTS neurons comes from a recent report...
by Gao and Smith (2009), who characterized $J_{\text{ionic}}$ in the adjacent dorsal motor nucleus of the vagus (DMV). They found that the GABA$_A$ receptor agonist THIP decreased the frequency of spontaneous IPSCs (sIPSCs) in DMV neurons, an effect that was dependent on action potential generation. This finding led the authors to propose that the decrease in sIPSCs was most likely due to activation of extrasynaptic GABA$_A$ receptors on mNTS neurons that are synaptically coupled to DMV neurons (Gao and Smith 2009).

Electrophysiological studies demonstrate that $\mu$-opioid receptor agonists alter the synaptic activity of NTS neurons (Glatzer and Smith 2005; Rhim et al. 1993), and that activation of $\mu$-opioid receptors on the somatodendritic region of NTS GABA neurons suppresses their activity, including local inhibitory transmission within the NTS (Glatzer et al. 2007). In our laboratory’s recent in vivo microinjection study, $\mu$-opioid receptor stimulation decreased gastric motility by suppressing local GABAergic signaling on a synaptic level. As a result, it was not possible to determine the relative contributions of different types of GABA$_A$ receptor signaling on the peripheral endpoints measured or the specific effects of $\mu$-opioid receptors on the two types of GABA$_A$ receptor signaling.

Based on the importance of GABA$_A$ signaling demonstrated by our in vivo studies and the high levels of ambient GABA present in the NTS (Sved and Sved 1990), we hypothesized that 1) NTS neurons possess tonic GABA$_A$ receptor-mediated currents that dynamically alter neuronal excitability; and 2) $\mu$-opioid receptor activation would suppress tonic as well as phasic GABA$_A$ receptor signaling in the mNTS.

**MATERIALS AND METHODS**

All procedures were performed in accordance with National Institutes of Health guidelines and with the approval of the Animal Care and Utilization Committee of Georgetown University, Washington, DC.

**Identification of gastric-projecting NTS neurons.** In some experiments, a fluorescently labeled transneuronal virus, a recombinant adeno-associated virus type 2 lacking the packaging signal (NVB-2), was injected into the gastroesophageal region of Sprague-Dawley rats (postnatal days 12–20; n = 6) (provided by Dr. Gregg Homanics, Department of Anesthesiology, University of Pittsburgh, Pittsburgh, PA). These mice were generated on a C57/BL6 background strain and were genotyped with Southern blot, as previously described (Mihalek et al. 1999).

Brains were rapidly removed, subsequent to decapitation, and placed in an ice-cold high-sucrose solution (pH 7.3–4.4) that contained the following (in mM): sucrose 206.0; KCl 2.5; CaCl$_2$ 0.5; MgCl$_2$ 7.0; NaH$_2$PO$_4$ 1.2; NaHCO$_3$ 26; glucose 5.0; HEPES 5. The brain stem was then separated, blocked, and glued to the stage of a vibrating microtome (Vibratome Series 1000, Technical Products, St. Louis, MO). The brain stem was then cut into transverse sections (250–300 $\mu$m) and placed in an oxygenated (95% O$_2$/5%CO$_2$) artificial cerebrospinal fluid (aCSF) solution composed of the following (in mM): NaCl 120; KCl 2.5; EGTA 5; CaCl$_2$ 2.0 MgCl$_2$ 1.0; NaH$_2$PO$_4$ 1.2; NaHCO$_3$ 26; glucose 1.75; HEPES 5. Slices were incubated in this solution for 30 min at 35–37°C, followed by 30-min equilibration at room temperature (21–22°C). Following equilibration, a single slice was transferred to a recording chamber (volume 500 $\mu$l) mounted on the stage of an upright microscope (Nikon E600FN), where it was continually superfused with room-temperature oxygenated aCSF.

**Electrophysiological recording.** Neurons were visualized in brain slices using infrared differential interference contrast optics and a charge-coupled device camera (Nikon MTICCD725S). A ×60 magnification water immersion objective (Nikon) was used for identifying and recording individual neurons. NTS neurons, PRV-152 labeled neurons were visualized using fluorescence optics. To avoid photolytic damage, initial exposure to episcopic fluorescence illumination was brief (<2 s). Fluorescent images were captured using Scion Image software (Scion, Frederick, MD). Whole cell recordings (voltage-clamp and current clamp) and juxtacellular (cell-attached) recordings were made with patch pipettes (4–6 MΩ; Warner Instruments) coupled to an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), low-pass filtered at 2–5 kHz, digitized (Digidata 1200C; Axon Instruments), and stored on a computer using pClamp 9 software (Axon Instruments). Series resistance was typically <10 MΩ and was continuously monitored with a hyperpolarizing 5-mV pulse.

The intracellular solution used for voltage-clamp recording was composed of the following (in mM): potassium chloride (KCl) 145; EGTA 5; MgCl$_2$ 5; HEPES 10; Na-ATP 2; Na-GTP 0.2. The intracellular solution used for current clamp recording was composed of the following (in mM): potassium gluconate 145; EGTA 5; MgCl$_2$ 5; HEPES 10; Na-ATP 2; Na-GTP 0.2. The intra-cellular solution used for current clamp recording was composed of the following (in mM): potassium gluconate 145; EGTA 5; MgCl$_2$ 5; HEPES 10; Na-ATP 2; Na-GTP 0.2. The pipette solutions for juxtacellular recordings were as follows (in mM): potassium chloride (KCl) 145; EGTA 5; MgCl$_2$ 5; HEPES 10; Na-ATP 2; Na-GTP 0.2; or aCSF. Drugs were constituted in aCSF and applied by Y-tubing application for local perfusion primarily on the neuron of interest and surrounding area (Murase et al. 1989) via a series of solenoid valves (NRI electronics). As Y-tubing application involves focal application of a drug solution during regular bath superfusion, it is possible that differences in y-tube placement, in fluid exchange in the y tube, and/or in fluid exchange in the bath could alter actual concentration of drug delivered and subsequent response of the neuron. In all cases, every effort was made to standardize drug application as much as possible. We were particularly cognizant of placing the y-tube along the “z” axis in approximately the same focal plane as that of the cell from which we were recording. In the “x” and “y” axes, the tip of the y-tube was placed at a distance of ~30 $\mu$m from each recorded cell. Nevertheless, any recordings indicating errors in y-tube application (i.e., compromised fluid exchange, etc.) were excluded from the analysis.

To isolate only the inhibitory currents mediated by GABA$_A$ receptor recordings (holding potential = −60 mV) were performed in the presence of the ionotropic glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX; 10 $\mu$M) and 2-amino-5-phosphonovaleric acid (50 $\mu$M) and the glycine receptor antagonist strychnine (2 $\mu$M). A photocmicrograph of a gastric mNTS neuron (asterisk) visualized using infrared differential interference contrast optics appears as Fig. 1A. The same gastric mNTS neuron visualized using fluorescence optics to demonstrate PRV-152 green fluorescent protein label appears as Fig. 1B. A photocmicrograph of a transverse
microtome (Reichert-Jung) into sequential 30-

section through the medulla at the level of the mNTS showing the position of both the recording electrode (left) and the Y-tubing (right). Scale bar equals 200 μm.

RESULTS

GABA<sub>A</sub> receptor-mediated tonic inhibition in rat mNTS neurons. Phasic inhibition (I<sub>phasic</sub>) has been well documented in mNTS neurons, including neurons in the circuitry controlling gastric motor function (Glatzer et al. 2007; Glatzer and Smith 2005). To determine whether GABA<sub>A</sub> receptor-mediated tonic inhibition is present in mNTS neurons, GABA<sub>A</sub> receptor antagonists were focally applied to mNTS neurons in a whole cell voltage-clamp configuration. Focal application of bicuculline (100 μM) produced an outward shift in I<sub>hold</sub> and reduced the RMS noise (RMS). The GABA<sub>A</sub> receptor-mediated I<sub>tonic</sub> was defined as the difference in I<sub>hold</sub> before and after application of GABA<sub>A</sub> antagonist drugs. Focal application of bicuculline revealed an I<sub>tonic</sub> of 16.2 ± 2.7 pA and a reduced RMS noise of 6.2 ± 0.9 pA (Table 1), providing evidence of a I<sub>tonic</sub> in mNTS neurons. This was confirmed using the chloride channel blocker, PTX (100 μM), and a second specific GABA<sub>A</sub> receptor blocker, GBZ (100 μM) (Table 1). Representative traces depicting the effects of the two GABA<sub>A</sub> receptor blockers and the chloride channel blocker on I<sub>hold</sub> and RMS noise appear as Fig. 2.

As indicated in MATERIALS AND METHODS, both PRV labeled (i.e., gastric-related mNTS neurons; n = 4) and non-PRV-labeled mNTS neurons (n = 21) were studied. Effects of all three drugs on I<sub>hold</sub> and RMS noise level were similar, and, therefore, the data were grouped and analyzed (see Table 1). Positive data (i.e., the presence of I<sub>tonic</sub>) were obtained from 25 out of a total of 28 neurons examined, and I<sub>tonic</sub> was equally as
likely to be observed in gastric-related mNTS neurons as in unlabeled neurons.

To differentiate the $I_{\text{tonic}}$ from $I_{\text{phasic}}$, sIPSCs were also examined. sIPSCs were present in all mNTS neurons examined and occurred at a mean frequency of 1.9 ± 0.2 Hz, mean amplitude of 30.6 ± 4.6 pA, and mean decay time course of 12.9 ± 0.8 ms. All sIPSCs, in addition to $I_{\text{tonic}}$, were blocked by 100 μM of either bicuculline, PTX, or GBZ (Fig. 2). In five neurons, focal application of a lower concentration of GBZ (10 μM) completely blocked sIPSCs, but had no effect on $I_{\text{hold}}$ or RMS noise (Table 1, Fig. 2).

The source of GABA$_A$ receptor-mediated $I_{\text{tonic}}$ in mNTS neurons. The source of GABA that activates $I_{\text{tonic}}$ was investigated by examining the role of action potential-dependent synaptic activity on $I_{\text{tonic}}$. This was experimentally assessed by determining whether the Na$^+$ channel blocker TTX (10 μM) affected baseline and drug-induced changes in $I_{\text{hold}}$ and RMS noise. Data on the effect of TTX on baseline $I_{\text{hold}}$ and baseline RMS were inconsistent. In some cells, TTX alone produced a persistent reduction in $I_{\text{hold}}$ and decrease in RMS noise, but this effect was variable and, therefore, not significant. However, prior exposure to TTX prevented GBZ-induced changes in $I_{\text{hold}}$ and RMS (Table 2). The loss of ongoing $I_{\text{tonic}}$ with the blockade of action potential-dependent GABA release suggests that the source of the $I_{\text{tonic}}$ in the mNTS is spillover of synaptically released GABA into the extrasynaptic space.

Table 1. Effects of GABA$_A$ antagonists and THIP on $I_{\text{hold}}$ and RMS noise

<table>
<thead>
<tr>
<th>Drug Studied and Concentration Used</th>
<th>Change in $I_{\text{hold}}$</th>
<th>Change in RMS Noise</th>
</tr>
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<tbody>
<tr>
<td>Bicuculline (100 μM)</td>
<td>16.2 ± 2.7* (10)</td>
<td>−6.2 ± 0.9* (10)</td>
</tr>
<tr>
<td>Picrotoxin (100 μM)</td>
<td>19.1 ± 5.1* (5)</td>
<td>−7.5 ± 1.6* (5)</td>
</tr>
<tr>
<td>GABAzine (10 μM)</td>
<td>0.7 ± 0.5 (5)</td>
<td>−0.6 ± 0.3 (5)</td>
</tr>
<tr>
<td>GABAzine (100 μM)</td>
<td>16.5 ± 2.9* (10)</td>
<td>−6.0 ± 0.9* (10)</td>
</tr>
<tr>
<td>THIP (2 μM)</td>
<td>−8.3 ± 1.3* (13)</td>
<td>4.6 ± 0.2* (13)</td>
</tr>
<tr>
<td>THIP (5 μM)</td>
<td>−18.3 ± 2.1* (7)</td>
<td>9.6 ± 1.0* (7)</td>
</tr>
<tr>
<td>THIP (10 μM)</td>
<td>−42.1 ± 6.6* (11)</td>
<td>12.8 ± 1.6* (11)</td>
</tr>
</tbody>
</table>

Values are means ± SE in pA; nos. in parentheses, no. of neurons. $I_{\text{hold}}$ holding current; RMS, root mean square; THIP, 4,5,6,7-tetrahydroisoxazo-lo(5,4-c)pyridin-3-ol. *P < 0.05.

Fig. 2. Representative whole cell voltage clamp recording from mNTS neurons. A: focal application of bicuculline [bicuculline methiodide (BMI), 100 μM; upper trace], picrotoxin (PTX, 100 μM; middle trace), or gabazine (GBZ, 100 μM; lower trace) blocked phasic inhibitory postsynaptic currents (IPSCs) and inhibited tonic currents ($I_{\text{tonic}}$). B: application of 10 μM GBZ inhibited IPSCs, but had no effect on $I_{\text{tonic}}$.
current (Fig. 2, Table 1). However, the impact of the ionic on the overall activity of mNTS neurons (and by extension, the activity of the mNTS as a whole) cannot be determined in the voltage-clamp configuration. To determine the effect of the ionic on mNTS excitability, current-clamp recordings were performed to examine the impact of tonic and phasic GABA_ receptor signaling on the generation of action potentials.

We began by asking whether GBZ at a concentration selective for blocking only I^phasic (10 μM) and at a concentration that blocks both I^phasic and I^tonic (100 μM) alters the excitability of mNTS neurons. All cells were subjected to a step protocol consisting of a series of incremental current injections (25 pA, 600-ms duration), starting with a 100-pA hyperpolarizing current injection. Membrane resistance was measured by injecting a hyperpolarizing current (50 pA). At a membrane potential of ~60 mV, mNTS neurons were usually silent but exhibited action potential firing in response to the second depolarizing current step (i.e., a current injection of 50 pA).

In five mNTS neurons, application of 10 μM GBZ did not significantly change either the average resistance (0.65 ± 0.07 MΩ compared with a baseline value of 0.63 ± 0.07 MΩ) or the average number of action potentials in response to a 50-pA current injection (5.6 ± 1.3 compared with a baseline of value 5.6 ± 1.5). When the resistance was normalized to baseline values, application of 10 μM GBZ produced a change in normalized resistance of 1.03 ± 0.03 MΩ, which was not statistically significant (n = 5, P > 0.05; Fig. 4A).

In six mNTS neurons, application of 100 μM GBZ significantly increased the average resistance from the baseline value 0.65 ± 0.07 to 1.0 ± 0.2 MΩ (P < 0.05; Fig. 4B). Similarly, GBZ (100 μM) also significantly increased the average action potentials from a baseline value of 4.0 ± 0.9 to 5.2 ± 0.8 in response to a 50-pA current injection (P < 0.05; Fig. 4C).

When the resistance was normalized to baseline values, application of 100 μM GBZ increased the normalized resistance to 1.2 ± 0.07 MΩ (P < 0.05; Fig. 4, B and C).

We next examined the effect of stimulation of tonic GABA_A receptor currents on mNTS neuron excitability using the δ-subunit-prefering GABA_A receptor agonist THIP. The experimental protocol used was identical to that used in the GBZ studies (described above).

Recordings were made in seven mNTS neurons that displayed an average baseline resistance of 0.55 ± 0.07 MΩ and an average of 5.3 ± 0.7 action potentials in response to a 50-pA current injection. Application of 10 μM THIP significantly decreased the average resistance of the same neurons to 0.44 ± 0.04 MΩ and significantly decreased the average action potentials to 4.0 ± 0.7 in response to a 50-pA current injection (n = 7, P < 0.05; Fig. 4, D and E). When the resistance was normalized to baseline values, application of 10 μM THIP decreased the normalized resistance to 0.8 ± 0.04 MΩ (P < 0.05, n = 7; Fig. 4E).

Tonic GABA_A receptor currents alter the firing discharge of mNTS neurons. To examine the effect of blocking tonic GABA_A receptor currents on firing discharge, mNTS neurons were locally perfused with 100 μM GBZ using an extracellular cell-attached configuration. Recordings were made only in neurons that displayed spontaneous firing, but had a relatively low firing discharge and a high interevent interval so that excitatory effects could be reliably detected. In the 10 mNTS neurons recorded, application of 100 μM GBZ significantly decreased the interevent interval from a baseline value of 3.7 ± 1.4 s to 0.85 ± 0.26 s (P < 0.05; Fig. 5A). In addition, it
motility by suppressing local GABA activity in the mNTS (Herman et al. 2010). This observation led us to hypothesize that μ-opioid receptor activation would suppress tonic as well as phasic GABA_A receptor signaling in the mNTS. To determine the effect of μ-opioid receptor activation on tonic as well as phasic GABA_A receptor signaling, the μ-opioid receptor agonist DAMGO was locally perfused onto mNTS neurons during whole cell voltage clamp recordings. Local perfusion of DAMGO (100 nM) significantly decreased the frequency of sIPSCs by 1.6 ± 0.5 Hz (P < 0.05; n = 8), but did not produce a significant change in amplitude or decay of sIPSCs. Application of DAMGO also produced a persistent reduction in I_\text{\text{tonic}} (13.7 ± 2.5 pA, P < 0.05; n = 8) with a decrease in RMS noise of 4.9 ± 0.7 Hz (P < 0.05; n = 8) in 8/10 mNTS neurons (Fig. 6A). DAMGO was tested in both PRV-labeled (i.e., gastric-related mNTS neurons; n = 3) and non-PRV-labeled mNTS neurons (n = 8). The effects of DAMGO on I_\text{\text{hold}} and RMS noise level were similar, and, therefore the data were grouped and analyzed. The persistent reduction in I_\text{\text{hold}} produced by DAMGO was not statistically different from the reduction in I_\text{\text{hold}} produced by focal application of 100 μM GBZ (Table 1).

To determine whether the effects of DAMGO occurred across species and to further examine its effect on I_\text{\text{hold}} produced by DAMGO, experiments were performed in C57Bl6 mice to determine whether blockade of I_\text{\text{tonic}} would interfere with the reduction in I_\text{\text{hold}} produced by DAMGO. In these experiments, mNTS neurons were first perfused with GBZ or bicuculline (100 μM), followed by DAMGO (100 nM). While the initial exposure to GBZ or bicuculline produced a significant reduction in I_\text{\text{hold}} (8.8 ± 2.0 pA, P < 0.05; n = 11), subsequent perfusion of DAMGO (100 nM) in the presence of GBZ or bicuculline did not have a significant effect on I_\text{\text{hold}} (0.7 ± 0.9 pA, n = 11; Fig. 6B, lower panel). To then ascertain if the reduction in I_\text{\text{tonic}} produced by DAMGO would interfere with the reduction in I_\text{\text{hold}} produced by blockade of I_\text{\text{tonic}}, mNTS neurons were first perfused with DAMGO (100 nM) and then exposed to GBZ or bicuculline (100 μM). DAMGO significantly reduced sIPSCs by 2.7 ± 0.7 Hz (P < 0.05; n = 16) and produced a persistent reduction in I_\text{\text{hold}} (13.0 ± 1.7 pA, P < 0.05; n = 16). However, subsequent perfusion with bicuculline or GBZ (100 μM) produced a significantly attenuated reduction in I_\text{\text{hold}} (5.9 ± 2.5, n = 8 and 5.1 ± 2.4 pA, n = 8, respectively). See Fig. 6B, lower panel, right histogram.

μ-Opioid receptor suppression of tonic but not phasic GABA_A receptor currents is absent in mice lacking the δ-subunit. To further examine the specificity of DAMGO effects on tonic GABA_A receptors in mNTS neurons, we investigated the changes in I_\text{\text{hold}} and RMS noise produced by DAMGO in mice lacking the δ-subunit and wild-type controls. To confirm the absence of δ-subunits, the δ-subunit-prefering agonist THIP was focally applied to neurons from mice lacking the δ-subunit, and responses were compared with those in neurons from wild-type controls. THIP (10 μM) produced an average stimulated I_\text{\text{tonic}} of 17.1 ± 4.1 pA (P < 0.05; n = 7) in wild-type mice that was absent in mice lacking the δ-subunit (n = 5). In addition, perfusion of bicuculline or GBZ to mNTS neurons from mice lacking the δ-subunit did not produce a significant change in I_\text{\text{hold}} (2.8 ± 2.3 pA, n = 7).

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Focal application of DAMGO in wild-type mice produced an average reduction in $I_{\text{hold}}$ of 17.3 ± 3.2 pA ($P < 0.05; n = 11$) and decrease in RMS noise of 1.8 ± 0.8 Hz ($P < 0.05; n = 11$) that was absent in mice lacking the δ-subunit (Fig. 7A). In wild-type controls, DAMGO reduced sIPSC frequency by 1.6 ± 0.6 Hz ($P < 0.05; n = 10$) that was still present in mice lacking the δ-subunit with a reduction of 0.7 ± 0.2 Hz ($P < 0.05; n = 7$; Fig. 7B).

**DISCUSSION**

Data from these studies demonstrate that mNTS neurons possess a significant $I_{\text{tonic}}$ that affects both excitability and synaptic activity. The source of the tonic conductance is most likely ambient GABA that results from action potential-mediated presynaptic spillover, as the $I_{\text{tonic}}$ was significantly blunted by prior application of TTX (Table 2). In addition, the GABA$_\text{A}$ receptor responsible for the tonic conductance is likely extrasynaptic and contains an α$_3$- or α$_6$- and δ-subunit, as evidenced by the dose-dependent $I_{\text{tonic}}$ generated by the δ-selective agonist THIP (Belelli et al. 2009) and the absence of $I_{\text{tonic}}$ produced by THIP in mice lacking the δ-subunit. The $I_{\text{tonic}}$ was differentiated from phasic currents using dose-dependent effects of the GABA$_\text{A}$ receptor antagonist GBZ. Application of GBZ in a concentration of 10 μM abolished all phasic currents but did not significantly affect the $I_{\text{hold}}$. Application of GBZ in a concentration of 100 μM abolished all phasic currents and significantly reduced the $I_{\text{hold}}$. These results suggest that tonic and phasic inhibitory currents in mNTS neurons are mediated by specific GABA$_\text{A}$ receptors, which is consistent with the recent work of Bright et al. (2011), who report that inhibitory tonic conductance in thalamic relay neurons of the lateral geniculate nucleus and in cerebellar granule cells is reflective of the presence of δ-subunit-containing GABA$_\text{A}$ receptors.

**Differentiation of tonic and phasic currents in mNTS neurons.** The ability to differentiate phasic and tonic inhibition using concentration-specific effects of GABA$_\text{A}$ receptor antagonists is consistent with studies performed in the nucleus ambiguus and hippocampus (Bouairi et al. 2006; Stell and Mody 2002), where a higher concentration of antagonist was required to reveal the $I_{\text{tonic}}$. Conversely, one study performed in thalamic neurons found that a lower concentration of GBZ selectively abolished the $I_{\text{tonic}}$, leaving the phasic currents intact (Cope et al. 2005). The authors proposed that the inverse concentration-dependent relationship that they observed with regard to the $I_{\text{tonic}}$ was due to lower concentrations of ambient GABA in their preparation. This proposal was supported by the observation that the low concentration of GBZ was unable to selectively block the $I_{\text{tonic}}$ when they increased ambient GABA in their preparation (Cope et al. 2005). Microdialysis studies have shown that the ambient GABA concentration in the mNTS is relatively high (∼2.5 nmol/mg tissue) (Sved and Sved 1990), so the necessity of a high concentration of GBZ to block the $I_{\text{tonic}}$ in mNTS neurons is consistent with this proposal.

**Tonic GABA$_\text{A}$ receptor currents determine the excitability and firing discharge rate of mNTS neurons.** After demonstrating the presence of $I_{\text{tonic}}$ in mNTS neurons, the next question was what impact the $I_{\text{tonic}}$ had on the excitability of these neurons (and, by extension, activity of the mNTS network as a whole). To address this issue, we performed current-clamp recordings and examined the impact of blockade and stimulation of the $I_{\text{tonic}}$ on input resistance and firing frequency of mNTS neurons. Blockade of the phasic currents using 10 μM GBZ produced no significant change in either resistance or firing frequency. However, blockade of both the tonic and phasic currents using 100 μM GBZ significantly increased the resistance and firing frequency of mNTS neurons (Fig. 4B). Conversely, stimulation of the $I_{\text{tonic}}$ using 10 μM THIP significantly decreased the input resistance and firing frequency of mNTS neurons (Fig. 4E). The results of these studies indicate that tonic conductance is a powerful regulator of individual excitability of mNTS neurons. These results are consistent with findings in the thalamus where taurine, an agonist of extrasynaptic GABA$_\text{A}$ receptors, has been shown to reduce the excitability of thalamocortical relay neurons (Jia et al. 2008).

In addition to the effects on individual neuron excitability, data from these studies indicate that the $I_{\text{tonic}}$ alters overall synaptic activity at mNTS neurons. Similar to the findings on individual neuronal excitability, blockade of the $I_{\text{tonic}}$ with 100 μM GBZ resulted in an increase in spike frequency observed in cell-attached recordings. Conversely, stimulation of the $I_{\text{tonic}}$ with 10 μM THIP produced a decrease in spike frequency.

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**Fig. 5.** A: representative cell-attached recording from a mNTS neuron demonstrating changes in spontaneous action potentials in response to application of 100 μM GBZ. B: representative cell-attached recording from a mNTS neuron demonstrating changes in spike frequency with application of 10 μM THIP. C: graphical representations of changes in interevent interval (IEI) in response to application of 100 μM GBZ ($* P < 0.05; n = 10$). D: graphical representations of changes in IEI with application of 10 μM THIP ($* P < 0.05; n = 10$).
A representative whole cell voltage clamp recording from a mNTS neuron. Focal application of [d-Ala(2),MePhe(4),Gly(ol)(5)]enkephalin (DAMGO; 100 nM) suppressed the \( I_{\text{tonic}} \) and spontaneous IPSC (sIPSC) frequency, but produced no change in sIPSC amplitude or decay (upper panel). Graphical representation of the \( I_{\text{tonic}} \) and root mean square (RMS) produced by DAMGO and changes in frequency, amplitude, and decay following DAMGO administration (lower panel). *\( P < 0.05; n = 8 \). B: representative whole cell voltage clamp recording from a mNTS neuron. Focal application of GBZ (100 \( \mu \)M) significantly reduced the \( I_{\text{tonic}} \) and subsequent perfusion of DAMGO (100 nM) produced only a small further suppression of \( I_{\text{tonic}} \) (upper panel). Graphical representation is shown of the \( I_{\text{tonic}} \) produced by GBZ alone compared with GBZ following DAMGO pretreatment (lower left panel; *\( P < 0.05; n = 10 \) and 8, respectively) and of the \( I_{\text{tonic}} \) produced by GBZ alone compared with DAMGO following GBZ pretreatment (lower right panel; *\( P < 0.05; n = 8 \) and 11, respectively).

However, the ability to detect these changes in action potential firing was dependent on the baseline activity. GBZ was only able to produce an increase in firing rate in recordings with low baseline activity. Application of GBZ in recordings with high baseline activity did not appear to result in an increase in firing rate, but it was not possible to determine whether this was due to a lack of effect per se or because the baseline activity was already in a maximal state. The same was true for application of THIP to recordings with low baseline activity. The different levels of baseline activity in individual recordings may reflect differences in the basal level of GABA\(_A\) receptor activity and/or intracellular chloride concentration at different mNTS neurons, suggesting potential cell-type-specific effects of \( I_{\text{tonic}}\). However, more studies are required to fully examine cell-type-specific effects.

\( \mu \)-Opioid receptor-mediated suppression of tonic and phasic currents in mNTS neurons. Numerous studies have shown that activation of \( \mu \)-opioid receptors in the NTS decreases glutamatergic transmission, most likely through receptors located on the presynaptic terminals of afferent neurons (Appleyard et al. 2005; Glatzer and Smith 2005; Rhim et al. 1993). However, few studies have examined the impact of \( \mu \)-opioid receptor activation on local GABAergic transmission, and none has examined the impact of \( \mu \)-opioid receptor activation on tonic GABA signaling in the mNTS. Following our laboratory’s previous studies demonstrating the importance of GABA signaling in the mNTS on resting gastric tone (Herman et al. 2009), and the suppression of GABAergic signaling by microinjection of \( \mu \)-opioid receptor agonists into the mNTS (Herman et al. 2010), we examined the effect of \( \mu \)-opioid receptor activation on local inhibitory transmission in the mNTS, specifically on tonic as well as phasic GABA\(_A\) receptor signaling.

Smith and colleagues previously demonstrated that activation of \( \mu \)-opioid receptors in the mNTS decreased phasic GABA transmission. In two important studies on the impact of \( \mu \)-opioid receptor activation on both excitatory and inhibitory transmission, Smith and colleagues showed that superfusion of the \( \mu \)-opioid receptor agonist endomorphin-1 (EM-1) decreased the frequency of sIPSCs as well as IPSCs evoked from activation of local inhibitory mNTS neurons (Glatzer and Smith 2005). These effects on inhibitory transmission (as well as separate effects on excitatory transmission) were observed in GABAergic mNTS neurons (Glatzer et al. 2007). These observations led the authors to conclude that EM-1 reduces inhibitory transmission in the mNTS by acting on \( \mu \)-opioid receptors located on the cell bodies of local mNTS GABA neurons. Data obtained in the present study are consistent with the findings and conclusions of Smith and colleagues. However, in the present study, suppression of tonic as well as phasic GABA signaling was observed. Smith and colleagues did report an outward current with superfusion of EM-1 that they attributed to direct hyperpolarization of GABA neurons (Glatzer et al. 2007), which was consistent with an earlier study (Poole et al. 2007). Both studies attribute this direct hyperpolarization to the activation of postsynaptic K\(^+\) channels, but neither study performed additional experiments to characterize this current. Activation of K\(^+\) channels is one possible source of the reduction in \( I_{\text{hold}} \) observed with DAMGO; however, if this were the case, then DAMGO would still produce a reduction in \( I_{\text{hold}} \) in the presence of a GABA\(_A\) receptor antagonist, which was not observed in our study.

To examine whether the reduction in \( I_{\text{hold}} \) that we observed with application of DAMGO was due to action on extrasynaptic receptors, we applied it after blockade of the \( I_{\text{tonic}} \) with perfusion of either GBZ or bicuculline. Prior application of GBZ or bicuculline significantly attenuated the outward current observed with DAMGO, indicating a common pathway by which both GABA\(_A\) receptor antagonists and DAMGO produce their effects on \( I_{\text{hold}} \). This proposal was further substantiated by a separate set of experiments in which prior application of DAMGO attenuated the reduction in \( I_{\text{hold}} \) observed with bicuculline or GBZ. Altogether, the data from these experi-
ments strongly suggest that extrasynaptic GABA<sub>δ</sub> receptors are potentially involved in the effects of DAMGO on I<sub>hold</sub>. We also performed studies in mice with a genetic deletion of the δ-GABA<sub>δ</sub> receptor subunit. Activation of µ-opioid receptors induced a sustained reduction in I<sub>hold</sub> that was not observed in mice lacking the δ-subunit of the GABA<sub>δ</sub> receptor. However, a suppression of phasic GABA<sub>δ</sub> receptor signaling (as measured by a decrease in IPSC frequency) was still present in mice lacking the δ-subunit (Fig. 7B). These data suggest that postsynaptic δ-subunit-containing GABA<sub>δ</sub> receptors are involved in the suppression of tonic signaling by DAMGO, while the suppression of phasic signaling (presumably through a presynaptic mechanism) does not involve δ-subunit-containing GABA<sub>δ</sub> receptors, as it is unaltered in mice lacking the δ-subunit. These results suggest that µ-opioid receptors may represent a potential endogenous mechanism for suppression of tonic as well as phasic GABA<sub>δ</sub> receptor signaling in the mNTS.

Taken together, the results of these studies indicate that GABA<sub>δ</sub> receptor-mediated inhibition in the mNTS is composed of both tonic and phasic forms, and that both types of inhibition are suppressed by activation of µ-opioid receptors. These results are particularly relevant to understanding the role of GABA activity in the mNTS when the impact of both types of inhibition is considered. Phasic signaling provides a rapid means of inhibiting the mNTS on a cellular level, whereas the tonic inhibition likely influences the overall “gain” of the network, as is the case for other brain regions, such as the hippocampus and cerebellum (for review, see Semyanov et al. 2004). Thus changes in synaptic inhibition may be important for communicating point-to-point signals (such as the vago-vagal reflexes), and changes in tonic inhibition may be important for maintaining the overall activity of the vago-vagal reflex circuitry within a homeostatic range. By extension, modulation of tonic GABA<sub>δ</sub> receptor signaling by µ-opioid receptor stimulation may manifest as a contributing factor to the negative gastric side-effects of pharmacological agents, such as morphine, that include abdominal pain, constipation, nausea and vomiting, and decreased appetite (Becker and Blum 2009).

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

Author contributions: M.A.H., R.A.G., S.V., K.L.D., and N.S. conception and design of research; M.A.H. and N.S. performed experiments; M.A.H., S.V., and N.S. analyzed data; M.A.H., R.A.G., S.V., and N.S. interpreted results of experiments; M.A.H. prepared figures; M.A.H. drafted manuscript; M.A.H., R.A.G., S.V., and N.S. edited and revised manuscript; M.A.H., R.A.G., S.V., K.L.D., and N.S. approved final version of manuscript.

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