GABA signaling in the nucleus tractus solitarius sets the level of activity in dorsal motor nucleus of the vagus cholinergic neurons in the vagovagal circuit

Melissa A. Herman,1 Maureen T. Cruz,1 Niaz Sahibzada,2 Joseph Verbalis,3 and Richard A. Gillis2

1Interdisciplinary Program in Neuroscience, 2Department of Pharmacology, and 3Department of Medicine, Georgetown University, Washington, DC

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Herman MA, Cruz MT, Sahibzada N, Verbalis J, Gillis RA. GABA signaling in the nucleus tractus solitarius sets the level of activity in dorsal motor nucleus of the vagus cholinergic neurons in the vagovagal circuit. Am J Physiol Gastrointest Liver Physiol 296: G101–G111, 2009. First published November 13, 2008; doi:10.1152/ajpgi.90504.2008.—It has been proposed that there is an “apparent monosynaptic” connection between gastric vagal afferent nerve terminals and inhibitory projection neurons in the nucleus tractus solitarius (NTS) and that two efferent parallel pathways from the dorsal motor nucleus of the vagus (DMV) influence peripheral organs associated with these reflexes (6). The purpose of our study was to verify the validity of these views as they relate to basal control of gastric motility. To test the validity of a direct connection of vagal afferent terminals (known to release L-glutamate) directly impacting second-order projection neurons, we evaluated the effect of GABA receptors on gastric motility. Microinjection of bicuculline methiodide into the mNTS produced robust decreases in gastric motility (–1.6 ± 2 mmHg, P < 0.05, n = 23), which were prevented by cervical vagotomy and by pretreatment with kynurenic acid microinjected into the mNTS. Kynurenic acid per se had no effect on gastric motility. However, after GABA receptors were blocked in the mNTS, kynurenic acid produced a robust increase in gastric motility. To test for the contribution of two parallel efferent DMV pathways, we assessed the effect of either intravagal atropine methylbromide or Nω-nitro-L-arginine methyl ester on baseline motility and on decreases in gastric motility induced by GABA receptor blockade in the mNTS. Only atropine methylbromide altered baseline motility and prevented the effects of GABA receptor blockade on gastric motility. Our data demonstrate the presence of intra-NTS GABAergic signaling between the vagal afferent nerve terminals and inhibitory projection neurons in the NTS and that the cholinergic-cholinergic excitatory pathway comprises the functionally relevant efferent arm of the vagovagal circuit.

gastric; vagus; afferent; inhibition; rat

THE IMPORTANCE OF THE VAGOVAGAL reflexes in the regulation of gastrointestinal (GI) function has been highlighted in a recent series of articles published under the theme “Musings on the Wanderer: What’s New in Our Understanding of Vagovagal Reflexes?” (6, 37). The central nervous system component of this reflex circuit is located in the hindbrain and consists of a sensory nucleus, the nucleus tractus solitarius (NTS), and a motor nucleus, the dorsal motor nucleus of the vagus (DMV). Currently it is thought that signals from sensory receptors in the GI tract are received in the NTS and conveyed to DMV preganglionic vagal nerves (28, 36). These impulses produce functional changes in thoracic and abdominal viscera (28, 36). Critical to conveying sensory signals to vagal efferent impulses are synaptic connections between NTS and DMV neurons. There is considerable evidence that GABAergic neurons are responsible for a significant part of this communication (9, 35). There is also evidence that noradrenergic neurons take part in this communication process (10, 11, 18, 26, 29). Both the GABAergic and noradrenergic neurons are considered to be NTS projection neurons that synapse onto DMV gastric-projecting neurons (9, 10, 29). Glutamatergic neurons have also been proposed to be involved (9, 35, 39), but to our knowledge there is no functional evidence of this involvement in an in vivo experimental preparation.

Two views expressed in the series of “themed” articles are that 1) there is an “apparent monosynaptic” connection between gastric vagal afferent fibers and inhibitory NTS neurons projecting to the DMV (Travagli et al., Figs. 1, 2, and 5 (37); Chang et al., Fig. 7 (6)); and 2) dual parallel pathways from the DMV participate in the vagovagal reflexes, a cholinergic-cholinergic excitatory pathway and a nonadrenergic, noncholinergic (NANC) inhibitory pathway (Travagli et al., Fig. 5; Chang et al., Fig. 3). The first view persists despite both anatomical and functional in vitro evidence of local inhibitory processing within the NTS (13, 15, 17, 21). The second view lacks functional evidence for the NANC pathway as it relates to gastric motility (8, 18). Nevertheless, these two views continue to dominate this field of research (5, 19, 28, 36, 41, 42).

Data from the studies presented here suggest that both of these views may be incorrect with respect to vagovagal control of gastric motility. In regards to the view that there is an apparent monosynaptic connection between gastric vagal afferent nerve fibers and inhibitory NTS neurons that project to the DMV, our data indicate that local inhibitory signaling by NTS interneurons is interposed between vagal afferent nerve fibers and NTS projection neurons that in turn regulate the activity of DMV neurons. With regard to the view that two parallel pathways from the DMV participate in resting gastric motility, our data are not supportive of this position. Consistent with our previous reports (8, 10, 11, 18, 32), the present studies provide further evidence that in two additional conditions, namely baseline conditions and GABA receptor blockade in the medial subnucleus of the tractus solitarius (mNTS), inhibition of gastric motility produced under these conditions is not due to activation of NANC postganglionic neurons.

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Address for reprint requests and other correspondence: r. A. Gillis, Dept. of Pharmacology, Georgetown Univ. Medical Center, 3900 Reservoir Rd., NW, Washington, DC 20057 (e-mail: gillisr@georgetown.edu).

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MATERIALS AND METHODS

Animals and Surgical Preparation

All experiments were performed on adult male Sprague-Dawley rats (250–350 g; Taconic, MD) in accordance with the National Institutes of Health guidelines for the care and use of animals in research and with the approval of the Animal Care and Use Committee of Georgetown University, Washington, DC. Animals were fasted overnight (18–24 h) with water available ad libitum. Anesthesia was induced by an intraperitoneal injection of urethane (1.5 g/kg). Adequate depth of anesthesia was monitored via toe pinch and corneal reflex for the duration of the experiment. Supplemental anesthesia (0.3 g/kg) was administered as needed. Body temperature was maintained at 37 ± 1°C with an infrared heating lamp. All animals were intubated to maintain a patent airway and to administer artificial respiration, if necessary. The carotid artery was cannulated with polyethylene tubing (PE-50) connected to a pressure transducer (sensitivity 5 μV·V⁻¹·mmHg⁻¹) for continuous monitoring of arterial blood pressure as an indicator of the physiological state of the animal. The pressure transducer was connected to a bridge amplifier and a data-acquisition system (PowerLab; ADInstruments, Colorado Springs, CO). The external jugular vein was cannulated with polyethylene tubing (PE-50) for intravenous administration of drugs. Both cervical vagus nerves were isolated and individually looped with silk suture for selective sectioning during the course of the experiment. To monitor tone and gastric motility, a low-compliance balloon constructed from a latex condom (connected to PE-60 tubing) was inserted into the stomach by way of the fundus and positioned toward the corpus/antrum area. The balloon was inflated with 3–5 ml of warm distilled water [see Cruz et al. (8) for the rationale for the volume of water used] to produce global distension of the stomach. The tubing attached to the balloon was connected to a pressure transducer (sensitivity 5 μV·V⁻¹·mmHg⁻¹) that in turn was connected to a bridge amplifier and data-acquisition system (PowerLab; ADInstruments).

Microinjection Procedure

All animals were placed in a small animal stereotaxic frame (Kopf Instruments, Tujunga, CA) in a prone position. Following a partial craniotomy, the dura was reflected, and the underlying cerebellum was retracted to expose the dorsal medulla. Our goal was to place the micropipette tip in the area of the mNTS where the majority of gastric vagal afferents terminate (2, 31). The densest area of innervation is the caudal tip of the area postrema. The stereotaxic rationale for our use of the term “the area of the mNTS”). In all studies, the stereotaxic coordinates used are in reference to calamus scriptorius (i.e., the caudal tip of the area postrema). The stereotaxic coordinates were as follows: anteroposterior = 0.5 mm, mediolateral = 0.4–0.6 mm, and dorsoventral = 0.3–0.6 mm. The location of the mNTS was functionally confirmed by microinjection of L-glutamate, as described in detail in our earlier studies (8, 12), and histologically confirmed in all studies (see Histological Verification of Microinjection Sites).

All microinjections into the mNTS were performed with a double-barreled glass micropipette (inner diameter 0.3 mm, outer tip diameter 30–60 μm; Frederick Haer, Bowdoinham, ME) positioned at a 30° angle from the perpendicular. At one end, the micropipette was connected to polyethylene tubing (PE-50) that was attached to a syringe for loading and unloading of drugs via negative or positive pressure, respectively. Drugs were microinjected within 5–10 s in 30-nl volumes determined by calibration tape (Formaline 9006B, Wheeling, IL) that was affixed to the micropipette.

Histological Verification of Microinjection Sites

Upon completion of each experiment, animals were euthanized with a lethal dose of pentobarbital sodium. The brain of each animal was rapidly removed and placed in a 4% buffered paraformaldehyde-20% sucrose solution for at least 24 h. After fixation and cryoprotection, brains were cut on a cryostat into serial 50–μm coronal sections and mounted on slides. Subsequently, the tissue was stained with neutral red, dehydrated, cleared, and coverslipped. The location of each microinjection site was identified, and a camera lucida drawing was made in relation to nuclear groups as defined by the atlas of Paxinos and Watson (25). In addition, microinjection sites were more specifically identified by comparison with the established locations of various subnuclei of the NTS as defined by Kalia and Sullivan (20).

Experimental Protocols

General protocol. In all experiments, a stable baseline intragastric pressure (IGP) (reflecting the level of gastric tone and phasic motility) and a stable arterial blood pressure recording were obtained for at least 10 min before any experimental manipulations were initiated. The micropipette was inserted unilaterally, and each animal was allowed to stabilize for a period of at least 2 min. Thereafter, in most animals (exceptions are indicated below in the description of protocols), L-glutamate (500 pmol/30 nl) was used as a pharmacological tool to identify pipette location. When a decrease in IGP (i.e., reduction in gastric tone) was observed with L-glutamate microinjection, we assumed that the micropipette tip was placed in the area of the mNTS. This observation allowed us to proceed with our experiment (i.e., test other experimental substances at this l-glutamate-responsive site). Prior to the microinjection of other drugs into the mNTS, at least two reproducible l-glutamate responses were elicited. To allow full recovery from the reproducible l-glutamate responses, a minimum interval of 10 min was used between each l-glutamate injection. When drugs other than L-glutamate were studied, the time interval between injections was determined for each agent. All microinjected drugs were compared with vehicle (0.9% saline) microinjection performed under identical conditions as the experimental treatments.

Experimental model. An experimental model that would provide interpretable physiological data was required to relate drug-induced perturbations in synaptic events in the area of the mNTS to downstream changes in peripheral organ function (i.e., changes in IGP). To create this model we needed to avoid the confounding effect of microinjected drugs from affecting the nearby DMV, located ~100 μm away from the mNTS (23). To avoid any possible DMV effects of microinjected drugs, we performed unilateral microinjection into the area of the mNTS of animals with the ipsilateral cervical vagus nerve sectioned but with the contralateral vagus nerve still intact. This was physiologically possible because l-glutamate unilaterally microinjected into the area of the mNTS can evoke vagally mediated decreases in IGP via activation of an inhibitory pathway originating in the microinjected NTS that projects to the contralateral DMV (Fig. 1). This connection has been functionally demonstrated (Ref. 8 and the present study; see RESULTS).

In sectioning the ipsilateral cervical vagus nerve, we lose significant sensory signaling into the mNTS that could potentially confound the extrapolation of drug-induced effects evoked from unilateral microinjection to a fully intact preparation. However, we do not feel that this is the case because contralateral vagal afferents also terminate in the area of the mNTS (14, 20).

A diagram of our experimental model depicting the neurocircuitry of the vagovagal pathways based on the above discussion and based on current thinking expressed in the themed articles is shown in Fig. 1.

Protocol for unilateral microinjection of GABA<sub>A</sub> receptor antagonists. Subsequent to ipsilateral vagotomy and two reproducible responses of l-glutamate microinjection into the unilateral mNTS, bicuculline methiodide (BMI; 20 pmol/30 nl) was microinjected at the same site into the mNTS. This dose of BMI was chosen on the basis
of our previous studies of GABA<sub>A</sub> receptor blockade in the rat NTS (38). Unilateral microinjection of BMI could be repeated following a 30-min period. This interval allowed for a complete recovery to baseline activity.

There is evidence that quaternary salts of bicuculline can block calcium-gated potassium channels in addition to blocking GABA<sub>A</sub> receptors (30). To verify that the BMI effects seen in these experiments were due to an action on the GABA<sub>A</sub> receptor, the GABA<sub>A</sub> receptor selective antagonist gabazine (GBZ; 20 pmol/30 nl) was unilaterally microinjected into the mNTS.

Protocol for unilateral microinjection of a GABA<sub>A</sub> receptor agonist. The protocol for microinjecting the GABA<sub>A</sub> receptor agonist muscimol into the mNTS was similar to that of the antagonists described above. Muscimol (100 pmol/30 nl) was unilaterally microinjected into the mNTS and could be repeated following a 30-min interval to allow complete recovery to baseline activity. This dose was chosen on the basis of our previous work (38).

Protocol for unilateral microinjection of an ionotropic glutamate receptor antagonist. After ipsilateral vagotomy, two reproducible responses to unilateral microinjection of l-glutamate were obtained from the area of the mNTS. Kynurenic acid (Kyn; 1 nmol/30 nl) was then microinjected into the mNTS at the same site. This dose was chosen on the basis of our previous studies (38). Microinjection of Kyn was not repeated.

Protocols for studying the interaction between GABA<sub>A</sub> receptor blockade and ionotropic glutamate receptor blockade using unilateral microinjection. In these studies, l-glutamate was not microinjected because of the constraints of employing double-barrel micropipettes. Two types of studies were performed in ipsilaterally vagotomized rats. One type was to determine whether Kyn microinjected into the area of the mNTS would alter the effect of BMI microinjection in the same site. The other type was to determine whether blockade of GABA<sub>A</sub> receptors in the area of the mNTS with GBZ would alter the effect of Kyn microinjection at the same site.

In the first type of study, at least two reproducible responses to BMI were obtained with a recovery period of no less than 30 min between microinjections. The occurrence of a characteristic decrease in IGP with BMI microinjection suggested that the micropipette tip was in the area of the mNTS. Once the response to GBZ had stabilized (10–15 min after microinjection), Kyn was microinjected into the same site.

Protocols for intravenous administration of atropine methylbromide and L-NAME. These intravenously administered drugs were tested under two experimental conditions in ipsilaterally vagotomized rats. One was under basal or resting conditions, and the other was as a pretreatment to determine the DMV vagal pathway mediating the robust decrease in IGP produced by BMI microinjection into the area of the mNTS. For the study conducted under basal conditions, either atropine methylbromide (0.1 mg/kg) or N<sup>ω</sup>-nitro-l-arginine methyl ester (l-NAME; 10 mg/kg) was administered, and their effect on IGP was noted. For the study on BMI-induced decreases in IGP elicited from the mNTS, at least two reproducible responses to BMI microinjection were obtained. After a recovery interval of at least 30 min between microinjections, either atropine methylbromide (0.1 mg/kg) or l-NAME (10 mg/kg) was intravenously administered. Microinjection of BMI was then repeated after IGP and arterial blood pressure had stabilized from the intravenously administered drug (~7–10 min). The doses of atropine methylbromide and l-NAME were based on our previous studies (8, 18).

Data Analysis

Data were analyzed by use of Chart software (AD Instruments, Colorado Springs, CO). The end point used for gastric motility was IGP, which reflects the degree of tonic contraction of gastric smooth muscle and will be referred to in the RESULTS section as either IGP or gastric tone. Values for IGP were always taken as the lowest value of the experimental tracing, even when phasic contractions were present.

Changes in gastric tone in response to drug administration (microinjected or intravenous) were compared with a 3-min baseline recording just prior to microinjection. The 3 min of baseline were divided into three 1-min samples, averaged, and compared with the effect of the drug. For experiments in which a drug was microinjected twice (under identical experimental conditions), the two responses were averaged.

Data are presented as means ± SE. A paired t-test was performed when animals served as their own controls. When animals represented independent samples, the data were analyzed by an unpaired t-test between separate control and experimental groups. A one-sample t-test was performed to determine whether a group mean was significant from zero. In addition, a one-way ANOVA was performed when more than one experimental intervention was used (i.e., l-glutamate or BMI microinjection before vagotomy, after ipsilateral vagotomy, and after bilateral vagotomy) followed by the Newman-Keuls post hoc analysis. In all cases, P < 0.05 was the criterion used to denote statistical significance.

RESULTS

Validation of the Experimental Model for Assessing Neural Activity in the Area of the mNTS and Characterization of DMV Output Pathways Providing Baseline Neural Activity to the Stomach

Our initial goals were to validate our experimental model and to characterize the output pathways from the DMV to the...
stomach. First, we microinjected L-glutamate (500 pmol) into the area of the mNTS of four rats with both cervical vagus nerves intact. L-glutamate produced a decrease in IGP (Fig. 2A). Next, following ipsilateral cervical vagotomy, L-glutamate was microinjected into the same site in these animals. L-glutamate evoked similar decreases in IGP as that observed prior to ipsilateral vagotomy (Fig. 2A). Finally, L-glutamate was microinjected into the same site of four rats subjected to sectioning of the remaining (contralateral) cervical vagus nerve. Bilateral cervical vagotomy completely prevented L-glutamate from decreasing IGP (Fig. 2A). A representative experiment appears as part of Fig. 2A. The micropipette tip in each case was located in the area of the mNTS (data not shown).

To characterize the DMV output pathways to the stomach that are active under basal conditions, either atropine methyl bromide or L-NAME were administered intravenously to rats with the ipsilateral cervical vagus nerve sectioned. Intravenous administration of L-NAME (10 mg/kg) to five rats had no significant effect on baseline IGP (Fig. 2B). In contrast, intravenous administration of atropine methyl bromide (0.1 mg/kg) to four rats produced a significant reduction in IGP (Fig. 2B). Representative experiments appear as part of Fig. 2B.

The above data indicate that in the ipsilateral vagotomized rat we have a viable model in which to assess 1) the nature of the synapse(s) where vagal afferent terminals engage NTS inhibitory neurons that project to the DMV and 2) the nature of output activity from the DMV to the stomach. This model was used for all the studies described below except the “control” animals shown in Fig. 2A.

Studies of the Effects of GABA<sub>A</sub> Receptor Blockade in the Area of the mNTS

Studies were performed using the GABA<sub>A</sub> receptor antagonist BMI microinjected into the area of the mNTS. Microinjection of BMI (20 pmol/30 nl) in 23 rats produced a robust decrease in IGP (−1.6 ± 0.2 mmHg, P < 0.05; Fig. 3A) that could be repeated after a 30-min recovery period. In addition, microinjection of BMI produced a small but significant decrease in mean arterial blood pressure (−6.4 ± 1.4 mmHg, P < 0.05). The decreases in IGP and mean arterial blood pressure occurred immediately after microinjection and reached a nadir in 2–5 min. The average duration of the decrease in IGP was 10.3 ± 0.9 min. In four rats, the vehicle for BMI microinjected into the area of the mNTS had no effect on IGP (Fig. 3A). A representative experiment performed with BMI is shown in Fig. 3B. The microinjection sites for the 23 experiments, and the four vehicle experiments are summarized in Fig. 4. As can be noted, the micropipette tip in each case was located in the area of the mNTS.

We also studied a second GABA<sub>A</sub> receptor antagonist, GBZ. Similar to BMI, unilateral microinjection of GBZ (20 pmol/30
nl) in eight rats also produced a robust decrease in IGP ($-1.5 \pm 0.2$ mmHg; $P < 0.05$; Fig. 3C). Microinjection of GBZ produced a small but significant decrease in mean arterial blood pressure ($-2.6 \pm 0.6$ mmHg; $P < 0.05$). The time to onset and time to peak effects for GBZ on IGP were similar to those observed with BMI, whereas the duration of the decrease in IGP was more prolonged. The duration was $16.6 \pm 2.5$ min in four of the five rats (one never recovered to baseline values). In four rats, microinjection of the vehicle for GBZ into the mNTS had no effect on IGP (Fig. 3C). A representative experiment performed with GBZ is shown in Fig. 3D. The microinjection sites for the five GBZ experiments are summarized in Fig. 4. As can be noted, the micropipette tip in each case was located in the area of the mNTS.

Next, we studied the effect of sectioning the remaining (contralateral) cervical vagus nerve. This was performed in 4 of the 23 rats whose data are summarized in Fig. 3A. In these experiments, two BMI-induced decreases in IGP responses were obtained with only the vagus nerve contralateral to the mNTS site of microinjection intact. An interval of 30 min was used between the two responses. Both responses were similar and therefore averaged (Fig. 5A). After 30 min of recovery following the second BMI-induced decrease in IGP, the remaining (contralateral) cervical vagus nerve was sectioned. Microinjection of BMI was then repeated and had no effect on IGP (Fig. 5A). A representative experiment is shown as Fig. 5B.

Studies of the Effects of GABA$_A$ Receptor Activation in the Area of the mNTS on Gastric Tone

Studies were performed using the GABA$_A$ receptor agonist muscimol that was microinjected into the mNTS. Muscimol (100 pmol/30 nl), unilaterally microinjected into the mNTS of four rats, produced an increase in IGP ($+0.6 \pm 0.2$ mmHg; $P < 0.05$) that was blocked by bilateral vagotomy (data not shown). The change in IGP observed was less than one-half the magnitude of the change in IGP observed with either BMI or GBZ. Microinjection of muscimol produced a significant increase in mean arterial blood pressure ($12.0 \pm 0.6$ mmHg; $P < 0.05$), an effect that was approximately twofold greater in magnitude than that seen with BMI. The micropipette tip in each experiment was located in the area of the mNTS (data not shown).

Studies of the Effects of Blocking Ionotropic Glutamate Receptors in the Area of the mNTS on the Decrease in Gastric Tone Produced by GABA$_A$ Receptor Blockade

These studies were performed to determine whether the decrease in gastric tone produced by GABA$_A$ receptor blockade in the area of the mNTS was due to the action(s) of unopposed glutamate in this area. To assess this, we unilaterally microinjected the ionotropic glutamate receptor antagonist Kyn into the area of the mNTS to see whether it would prevent the decrease in gastric tone produced by microinjection of BMI. After two reproducible BMI-induced inhibitory IGP responses were obtained from the mNTS, Kyn (1 nmol/30 nl) was microinjected into the same site. Ten minutes after Kyn was microinjected, BMI was reinjected. Prior to microinjecting Kyn, microinjection of BMI into the mNTS produced a significant decrease in IGP ($-1.5 \pm 0.4$ mmHg; $P < 0.05$) in the five animals studied (Fig. 6A). After Kyn treatment, BMI microinjection into the mNTS did not produce a significant decrease in IGP ($-0.4 \pm 0.4$ mmHg; $P > 0.05$; Fig. 6A). A representative tracing of the Kyn pretreatment experiment appears as Fig. 6B. In each experiment the micropipette tip was located in the area of the mNTS (data not shown).
Studies of the Effects of Blocking Ionotropic Glutamate Receptors in the Area of the mNTS on Gastric Tone After GABA_A Receptor Blockade in the Area of the mNTS

We performed additional studies where Kyn was microinjected into the area of the mNTS after first blocking GABA_A receptors at the same site with microinjection of GBZ. Our choice of GBZ was dictated by its more stable effect and longer duration of action compared with BMI. In four animals, GBZ (20 pmol/30 nl) was first unilaterally microinjected into the area of the mNTS and produced the expected decrease in IGP (−1.4 ± 0.2 mmHg; P < 0.05). Once the response to GBZ had stabilized, microinjection of Kyn into the same site evoked a robust increase in IGP (Fig. 7B). These data are summarized in Fig. 7A (third histogram). The micropipette tip in each experiment was located in the area of the mNTS (data not shown).

Studies to Determine the Vagal Efferent Pathway to the Stomach That Mediates the Gastric Motility Effects Produced by Blocking GABA_A Receptors in the Area of the mNTS

The efferent limb mediating the baseline activity in the circuitry outlined in Fig. 1 was shown to be mediated by a cholinergic-cholinergic pathway on the basis of positive data obtained with intravenous atropine methyl bromide and negative data obtained with intravenous l-NAME (Fig. 2B). The purpose of these studies was to assess whether the efferent limb mediating the changes in gastric tone produced by blocking GABA_A receptors in the area of the mNTS was the cholinergic-cholinergic pathway. For this purpose, two reproducible BMI-induced inhibitory IGP responses evoked from the area of the mNTS were obtained in four rats. Following a 30-min recovery period after the second BMI response, atropine methyl bromide (0.1 mg/kg iv) was administered. Ten minutes after atropine methyl bromide was given, BMI was retested. Prior to administering atropine methyl bromide, microinjection of BMI into the mNTS produced a decrease in IGP (−1.2 ± 0.2 mmHg; P < 0.05) (Fig. 8A). After atropine methyl bromide treatment, BMI microinjected into the area of the mNTS did not produce a significant decrease in IGP (−0.1 ± 0.06 mmHg; P > 0.05; Fig. 8A). A representative atropine experiment appears as Fig. 8B. In each case, the micropipette tip was located in the mNTS (data not shown).

In a series of experiments separate from those described for atropine methyl bromide, we tested whether a NANC pathway involving nitric oxide played a role in the IGP decreases noted with GABA_A receptor blockade in the area of the mNTS. To examine this, two reproducible BMI-induced inhibitory IGP responses were obtained in five rats. After a 30-min recovery period from the second BMI-induced IGP response, l-NAME (10 mg/kg iv) was administered. Ten minutes after l-NAME

Fig. 6. A: histograms of averaged IGP responses to unilateral microinjection of BMI (20 pmol/30 nl) into the area of the mNTS before microinjection of kynurenic acid (Kyn; 1 nmol/30 nl) compared with microinjection of BMI after microinjection of Kyn into the area of the mNTS. *P < 0.05 paired Student’s t-test; #P < 0.05 1-sample t-test; n = 5. B: representative experimental tracing depicting changes in IGP following unilateral microinjection of BMI (20 pmol/30 nl, repeated microinjections) into the area of the mNTS and microinjection of Kyn (1 nmol/30 nl).
was given, BMI was retested. Prior to administering L-NAME, BMI microinjected into the area of the mNTS produced a significant decrease in IGP (−1.1 ± 0.1 mmHg; P < 0.05) (Fig. 8C). After L-NAME treatment, BMI microinjected into the area of the mNTS produced a significant decrease in IGP (−1.3 ± 0.3 mmHg; P < 0.05; Fig. 8C). This response was not significantly different from that observed with unilateral microinjection of BMI prior to L-NAME treatment. A representative tracing of the L-NAME experiment appears as Fig. 8D. In each experiment, the micropipette tip was located in the mNTS (data not shown).

**DISCUSSION**

The major finding obtained in these studies is that intra-NTS GABAergic signaling in the area of the mNTS is an important factor in regulating functional activity within the vagovagal reflex circuitry. Our strongest evidence is the data obtained with microinjection of GABA_A receptor antagonists into the area of the mNTS. Both BMI and GBZ produced robust decreases in gastric motility. This response could not occur if the only signaling in the NTS was from vagal afferent terminals releasing L-glutamate at the site where vagal afferent nerves terminate results in inhibition of gastric motility that is blocked by bilateral microinjection of GBZ (20 pmol/30 nl) into the area of the mNTS after atropine methylbromide (0.1 mg/kg iv), *P < 0.05 1-sample t-test; n = 4. B: representative experimental tracing depicting changes in IGP following unilateral microinjection of GBZ (20 pmol/30 nl) into the area of the mNTS after unilateral microinjection of GBZ (20 pmol/30 nl). C: representative experimental tracing depicting IGP responses following unilateral microinjection of Kyn (1 nmol/30 nl).

In interpreting the results of our studies in relation to related experimental studies, several methodological issues need to be addressed. First, it should be noted that the experimental model used in our studies incorporates within it all the elements of the vagovagal reflex circuitry. The area of the mNTS targeted for drug microinjection receives sensory input from the upper GI tract by way of vagal afferents (2, 31). In turn, neurons in the NTS project to the DMV, and this latter nucleus contains the preganglionic parasympathetic neurons that innervate the stomach (20). Evidence that the above neurons and hindbrain nuclei in the rat represent the vagovagal reflex circuitry is as follows: 1) activation of the vagal sensory neurons by distending the upper GI tract excites NTS neurons and inhibits DMV neurons (23, 41); 2) activation of vagal sensory neurons by distending the upper GI tract results in an inhibition of gastric motility that is blocked by either bilateral microinjection of tetrodotoxin into the mNTS (10), bilateral cervical vagotomy (10, 18), or intravenous administration of a quaternary form of atropine (18); 3) activation of NTS neurons with microinjection of L-glutamate at the site where vagal afferent nerves terminate results in inhibition of gastric motility that is blocked by bilateral cervical vagotomy (10); and 4) activation of DMV...
neurons with microinjection of L-glutamate increases gastric motility that is blocked by either cervical vagotomy (8, 10) or intravenous administration of a quaternary form of atropine (8).

Second, an important consideration for the experimental model used in these studies is the type of anesthesia. Some anesthetics are known to impact GABAergic signaling, specifically, to augment it (22). This would be a significant confound in our experimental design since it could artificially magnify the impact of GABA_A receptor signaling in the area of the mNTS on gastric motility. Hence, in the present study, we used urethane as our anesthetic of choice because it has been reported not to augment GABA_A receptor signaling in the NTS (1). Urethane does augment other types of signaling, e.g., signaling mediated by somatostatin (40), but how this altered signaling would influence our results is difficult to interpret without knowing what role, if any, somatostatin plays in the vagovagal reflex circuitry controlling gastric motility.

Third, a key advantage to the model employed is the observation that vagovagal reflex circuitry remains functional after sectioning of one of the cervical vagus nerves. This has been demonstrated by noting the same magnitude of inhibition of gastric motility with microinjection of excitatory substances into the NTS before and after unilateral vagotomy (8, 12). Knowledge that vagovagal circuitry is fully functional after sectioning of one of the cervical vagus nerves (see Fig. 2) enabled us to perform studies assessing whether the first synapse in the NTS acts as a “relay station,” or whether local processing takes place. In addition, it allowed us to reassess the nature of the DMV output pathways that regulate gastric motility by making it possible to selectively manipulate NTS neurons on one side of the brain stem that engage DMV neurons on the contralateral side of the brain stem (Fig. 1). This assured us that the vagovagal reflex circuitry could be engaged from the mNTS without the confounding effects of drug diffusion into the ipsilateral DMV, which is located ~100 μm from the targeted NTS site. The disadvantage of this model is that the ipsilateral vagal afferent input into the targeted NTS is missing owing to sectioning of the cervical vagus nerve on that side. However, some vagal afferent input is present owing to crossover of vagal sensory fibers from the contralateral intact vagus nerve (Fig. 1) (4, 7, 14, 20). Moreover, this disadvantage is far outweighed by the advantage of having gastric motility changes produced by selective NTS manipulation and not by contaminating effects of drug diffusion to the adjacent DMV producing direct stimulation of preganglionic gastric-projecting fibers. It is conceivable that the contralateral DMV could be affected by drugs microinjected into the area of the mNTS on the side of the medulla where the cervical vagus nerve was sectioned. Rinaman and colleagues (27) have reported that DMV dendrites cross the midline and enter the contralateral DMV. This potential site of drug action could not be ruled out in our study. However, it is unlikely that this site of drug action significantly contributes to the changes in intragastric pressure that we observed because direct drug action on DMV neurons would produce effects opposite to those that we obtained.

Evidence of GABAergic signaling in the area of the mNTS was demonstrated earlier by Smith et al. (33). These investigators used an in vitro cranial nerve explant preparation where vagus nerve fibers were stimulated ~3 mm from the brain stem during whole-cell voltage-clamp recordings of neurons in the dorsomedial NTS. Upon stimulation of vagal afferent fibers, both excitatory and inhibitory responses could be elicited from neurons in this area. Excitatory postsynaptic currents (EPSCs) that were evoked had constant latency, indicative of a monosynaptic connection with primary vagal afferents. These EPSCs were mediated by non-NMDA ionotropic glutamate receptors. Most importantly, with regards to the inhibitory postsynaptic currents (IPSCs), Smith and colleagues observed variable-latency responses, suggestive of a polysynaptic connection with primary vagal afferents. This occurred in ~50% of the neurons that initially displayed constant-latency EPSCs in response to vagal afferent stimulation. All IPSCs were blocked by bicuculline, indicating that vagal nerve stimulation had activated a local NTS GABAergic circuit (33). The data of Bailey et al. (3) confirm these results with the finding that solitary tract stimulation activates GABAergic inputs to GABA neurons in the NTS. On the basis of these results, Smith and colleagues suggest that stimulation of vagal sensory nerves synapsing in the NTS activate inhibitory neurons that then suppress excitatory vagal drive to a high proportion of NTS neurons.

About a fourth of the neurons displaying the initial EPSC also displayed variable-latency EPSCs. Most neurons that exhibited polysynaptic EPSCs also displayed polysynaptic IPSCs. Spontaneous IPSCs occurred in all neurons in which vagal stimulation evoked multisynaptic EPSCs. After blockade of GABA_A receptors with bicuculline, both the frequency of spontaneous EPSCs and the duration of evoked multisynaptic EPSCs increased. These observations allowed Smith and colleagues to suggest that NTS excitatory circuits (presumably glutamatergic interneurons) provide ongoing synaptic activity in the NTS but are ultimately controlled by local inhibitory interneurons. They conclude that “the results further emphasize the importance of local GABAergic inhibition in controlling and conditioning vagal input in the NTS” (33).

Additional evidence of GABA signaling within the NTS was reported by Davis et al. (9). In this study, the investigators used photolysis to focally release glutamate and depolarize local NTS neurons. The diameter of the stimulation area with photolysis was ~50 μm. Photolysis resulted in increased frequency of IPSCs that presumably were evoked from local GABAergic neurons. Photolysis of caged glutamate in the presence of a GABA_A receptor antagonist (picrotoxin) produced evoked EPSCs that were blocked by CNQX. The EPSCs were attributed to the release of transmitter from local glutamatergic neurons. These studies using focal glutamate photolysis demonstrate the presence of local GABAergic and glutamatergic circuits in the NTS.

Further evidence that local inhibitory signaling, presumably from interneurons, influences information from vagal sensory terminals to NTS neurons that project to the DMV is data obtained from transgenic mice expressing enhanced green fluorescent protein (EGFP) under the control of the GAD67 promoter. These EGFP-GABA neurons are identified in the NTS and recorded in the whole-cell, voltage-clamp configuration. Whole cell recordings made during electrical stimulation of the solitary tract exhibited constant-latency responses in the majority of neurons (3, 15), indicative of a monosynaptic connection. However, when EGFP-GABA neurons in the NTS were retrogradely labeled by pseudorabies virus (PRV) injected in the corpus of the stomach, none of the double-labeled neurons exhibited a constant-latency response. Indeed, in three
different studies performed by Smith and colleagues (15–17), none of the PRV-labeled NTS neurons exhibited a response suggestive of a direct monosynaptic innervation by vagal sensory nerve terminals. Instead, these neurons displayed variable-latency responses to solitary tract stimulation, suggesting the presence of local inhibitory signaling between vagal afferents and premotor DMV neurons.

An important question raised by our findings is how blockade of GABA<sub>A</sub> receptors in the area of the mNTS results in such a robust decrease in gastric motility. Our data indicate that prior blockade of ionotropic glutamate receptors in the area of the mNTS prevents GABA<sub>A</sub> receptor blockade from having an effect on gastric tone. This information, combined with data from previous studies discussed above, led us to propose the scheme shown in Fig. 9. Focusing on the NTS GABA neuron projecting to the DMV, there is considerable evidence for its existence. First, Davis and colleagues (9) reported that glutamate photolysis in the NTS results in GABA<sub>A</sub> receptor-mediated IPSCs in DMV neurons. Second, transneuronal retrograde viral label from the stomach sequentially labels DMV motor neurons followed by NTS GABAergic neurons (15). Third, electrical stimulation of the solitary tract results in evoked IPSCs in the DMV (35).

Focusing on the neural input to NTS GABA neurons that project to the DMV, Glatzer and Smith (17) reported that both inhibitory and excitatory synaptic input is present to premotor neurons retrogradely labeled with PRV from the stomach. What determines the ongoing activity of DMV gastric-projecting neurons [which are known to exhibit pacemaking activity (35)] is the balance between inhibition and excitation that is present in NTS interneurons. In our experimental preparation, the balance is weighted toward inhibition due to high GABAergic activity. Indeed, blockade of ionotropic glutamate receptor signaling with microinjection of Kyn into the area of the mNTS had no effect on gastric tone (Fig. 10, A and B), presumably because of the overwhelming GABAergic inhibition of GABA premotor neurons. Conversely, with microinjection of BMI and GBZ into the area of the mNTS, the input to the GABA premotor neurons is only excitatory and gastric tone is decreased. Finally, microinjection of Kyn into the mNTS after prior microinjection of a GABA<sub>A</sub> receptor blocker produces an increase in gastric tone and motility (Fig. 9, A and B). This is presumably due to loss of excitatory input to GABA premotor neurons, enabling the intrinsic pacemaking activity of the DMV neurons to take over and excite gastric smooth muscle to produce an increase in gastric motility.

To our knowledge, our results are the first to show that blockade of GABA<sub>A</sub> receptors in the area of the mNTS produces robust changes in gastric function. The significance of GABA<sub>A</sub> receptor signaling in the NTS on gastric function has been described in previous studies, but only in an in vitro cranial nerve explant-brain slice preparation or in a brain slice preparation (9, 33). However, data obtained with an in vitro preparation demonstrate changes in synaptic events that cannot be directly related to physiological changes in gastric function (as in the present study). In addition, it is not possible to know the impact, if any, of synaptic changes recorded in a brain slice on physiological changes in gastric motility.

Our findings from an intact, in vivo rat preparation highlight the importance of intra-NTS GABAergic signaling in regulating functional activity within the vagovagal reflex circuitry. Prior to our study, we had the perspective that the major factor in regulating functional activity of the vagovagal reflex circuitry was sensory signaling from the GI tract. Based on the robust changes in gastric tone brought about by blocking GABA<sub>A</sub> receptors in the area of the mNTS (changes that were even greater than those observed with L-glutamate microinjection), our view is that GABA<sub>A</sub> receptor signaling in the area of the mNTS is the major determinant of vagovagal reflex activity in the basal state. On the basis of our finding that blockade of ionotropic glutamate receptors with Kyn microinjection has no per se effect on measured gastric function, we suggest that ongoing GABA<sub>A</sub> receptor signaling functionally overwhelms all sensory input from the GI tract. The prominence that we give to the importance of GABA<sub>A</sub> receptor signaling in the area of the mNTS has already been advocated by Mifflin for the regulation of sensory input from baroreceptors and arterial blood pressure [see review by Mifflin (24)].

We also sought evidence for the presence of parallel excitatory and inhibitory DMV vagal pathways to the stomach proposed by others (6, 28, 36, 37, 42). The evidence we sought was under basal conditions, and experimental conditions during the decrease in gastric motility produced by GABA<sub>A</sub> receptor blockade in the area of the mNTS. Using intravenous administration of L-NAME or atropine methylbromide, we only obtained evidence for the functional presence of the cholinergic-cholinergic excitatory pathway. This was demonstrated by noting the positive effects of intravenous atropine methylbromide and the lack of effect of intravenous L-NAME under basal and experimental conditions. Thus, under the experimental conditions of baseline activity and GABA<sub>A</sub> receptor blockade in the area of the mNTS, inhibition of gastric motility is due to withdrawal of the cholinergic-cholinergic excitatory pathway and is not due to activation of NANC...
postganglionic neurons. According to data of Takahashi and Owyang (34), activation of NANC postganglionic neurons may occur when the stomach is distended by larger fluid volumes than used in the present study. In summary, our data suggest that local inhibitory signaling in the mNTS determines the impact of incoming sensory information on NTS neurons projecting to the DMV. Furthermore, our data do not fit with the view that two parallel pathways from the DMV participate in the resting gastric motility. On the contrary, it demonstrates that only one DMV pathway, namely the cholinergic-cholinergic excitatory pathway, is functionally relevant in the vagovagal reflex circuitry examined in our study.

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