Characterization of noradrenergic transmission at the Dorsal Motor Nucleus of the Vagus involved in reflex control of fundus tone

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Short Title: Alpha 2-adrenoreceptors at the DMV inhibit fundus-projecting neurons

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Abstract

Quantitative analysis of innervation to Dorsal Motor Nucleus of the Vagus (DMV) fundus-projecting neurons indicates that approximately 17% of input neurons are noradrenergic. To determine whether this small percentage of neurons innervating DMV output to the stomach is physiologically relevant, we evaluated the role of norepinephrine at the DMV in mediating a vago-vagal reflex controlling the fundus. A strain gauge was sutured onto the fundus of isoflurane-anesthetized rats to monitor changes in tone evoked by esophageal distension (ED). ED produced a decrease in fundus tone of $0.31 \pm 0.02$ g ($p<0.05$) which could be reproduced after a 30 minute interval between distensions. Bilateral cervical vagotomy and/or pretreatment with intravenous atropine methylbromide prevented the reflex-induced fundus relaxation. In contrast, intravenous L-NAME had no effect. Bilateral microinjection of alpha 2-adrenoreceptor antagonists (yohimbine and RS-79948) into the DMV also prevented the response. Prior to microinjection of alpha 2-adrenoreceptor antagonists, ED decreased fundus tone by $0.33 \pm 0.05$ g ($p<0.05$). After antagonist microinjection, ED decreased fundus tone by only $0.05 \pm 0.06$ g ($p>0.05$). Bilateral microinjection of prazosin into the DMV had no effect on the response. Microinjection of norepinephrine into the DMV mimicked the effect of ED, and was also prevented by prior microinjection of an alpha 2-adrenoreceptor antagonist. Our results indicate that noradrenergic innervation of DMV fundus-projecting neurons is physiologically important, and suggest that norepinephrine released at the DMV acts on alpha 2-adrenoreceptors to inhibit activity in a cholinergic-cholinergic excitatory pathway to the fundus.
Introduction:

We recently reported ultrastructural evidence for selective noradrenergic innervation of fundus-projecting neurons in the dorsal motor nucleus of the vagus (DMV) of the rat (22). Examination of synaptic contacts onto DMV fundus-projecting neurons that were identified by injection of CTB-HRP, a retrograde neuronal tracer into the fundus, revealed that 17.4±2.7% of the terminals showed dopamine-beta hydroxylase immunoreactivity (DBH-ir). In contrast, synaptic contacts onto DMV antrum-projecting neurons were devoid of DBH-ir terminals.

These findings raised the question of whether noradrenergic afferent synaptic input to DMV fundus-projecting neurons that comprise only 17.4±2.7% of the total synapses is physiologically important? To determine this, we employed a vago-vagal reflex that controls fundus tone in the rat. This gastric relaxation reflex has been recently described by Rogers and colleagues (13, 24). An important aspect of this reflex is that it allows a change in fundus tone, as measured by a miniature strain gauge transducer, to be the experimental endpoint. The reflex is activated by distension of the thoracic esophagus with a fluid-filled balloon for 1 min, which produces consistent relaxation of the fundus.

The first purpose of our study was to utilize this “esophageal-gastric reflex” (EGR) (13) and determine whether blockade of norepinephrine receptors in the DMV via microinjection of adrenoreceptor antagonists would prevent EGR-induced fundic relaxation. In performing these studies, we also sought to determine the identity of the adrenoreceptor that mediates the reflex-induced synaptic transmission at the DMV. In an earlier study, Fukuda et al. (10) demonstrated that excitation of the NTS
noradrenergic pathway to the DMV in a rat brain slice preparation affected alpha 2- but not alpha 1-adrenoreceptors on DMV neurons. However, both alpha 2- and alpha 1-adrenoreceptors have been shown to be present on the same DMV neuron (10, 18). Hence, it is plausible that the alpha 2-adrenoreceptor is in the synapse whereas the alpha 1-adrenoreceptor is extrasynaptic (data of Fukuda et al, (10)).

The second purpose of our study was to assess whether microinjection of norepinephrine into the DMV would mimic the effects of the EGR on fundus tone by activating the same adrenoreceptor at the DMV. According to Rogers and colleagues (13, 24), the EGR that is evoked by esophageal distension with a 0.16 mL fluid-filled balloon is mediated by dual vagal innervation of the fundus consisting of DMV pathways containing inhibitory nitrergic- and excitatory cholinergic enteric neurons. Norepinephrine released at the DMV has been proposed by them to activate the inhibitory nitrergic pathway by excitation of alpha 1-adrenoreceptors and to inhibit the excitatory cholinergic pathway by activation of alpha 2-adrenoreceptors on DMV neurons (13, 24). The evidence for this proposal is indirect. It is based, in part, on data obtained with antagonists applied to the floor of the fourth ventricle (24) and, in part, on data obtained with tests of IV administered atropine methylnitrate and L-NAME on the EGR (13). To date, no data have been reported on the effect of microinjection of drugs that block the alpha 1-adrenoreceptors at the DMV on the EGR.

A third purpose of our study was to repeat tests of IV quaternary atropine and L-NAME administration and observe their effect on the EGR. By pursuing this third purpose, our aim was to obtain data that would define the role of the dual inhibitory
nitrergic and excitatory cholinergic pathways in noradrenergic-induced effects on DMV neurons.
Methods:

Animals and Surgical Preparation: All experiments were performed on adult male Sprague Dawley rats (250-350g) (Taconic, MD) that were fasted overnight (16-24h) with water available *ad libitum*. Animals were anesthetized with isoflurane administered via a nose cone (5% induction; 2.5% maintenance) vaporized with 95% oxygen and 5% CO₂. Adequate depth of anesthesia was monitored via toe pinch and corneal reflex for the duration of the experiment. Body temperature was maintained at 37 ± 1° C with an infrared heating lamp. After induction of anesthesia, the nose cone was switched to an intubation tube subsequent to tracheotomy. We chose isoflurane for our studies because of our recent success in using this anesthetic to study hindbrain control of upper GI function in the ferret (20).

The carotid artery was cannulated with polyethylene tubing (PE-50) connected to a pressure transducer (sensitivity: 5 µV/V/mmHg) for continuous monitoring of arterial blood pressure. Blood pressure was measured for the purpose of monitoring the physiological state of the animal during the course of the experiment. The pressure transducer was connected to a bridge amplifier and data acquisition system (Powerlab; AD Instruments, Colorado Springs, CO). The external jugular vein was cannulated with polyethylene tubing (PE-50) for intravenous administration of drugs. Both cervical vagus nerves were carefully isolated and individually looped with a silk suture for selective sectioning during the course of the experiment.

The stomach and duodenum were then exposed via a midline abdominal incision. To measure changes in fundus tone, a miniature strain gauge transducer (2x4 mm; RBI Products, Inc.), calibrated prior to each experiment, was then sutured to the
fundus oriented along the circular smooth muscle fibers. The strain gauge was coupled to a bridge amplifier and data acquisition system (Powerlab; AD Instruments, Colorado Springs, CO). To provide uniform preload conditions, a low-compliance balloon constructed from a latex condom was inserted through the pylorus via a small incision in the distal duodenum. The balloon was placed in the fundus and secured with ligatures around the duodenum. At the start of each experiment the balloon was inflated with 2 ml of warm water to produce a mild and consistent preload strain (24) against which the fundus could relax. This preload strain was maintained for the duration of the experiment.

**Esophageal Distension Technique:** The esophageal distension balloon was constructed similar to that described by Hermann et al. (13). It was fabricated from a silicone tubing (Length 15mm x I.D. 0.5mm x O.D. 1mm) that was attached to a noncompliant 5F Fogarty occlusion catheter. The catheter was in turn connected to a 1 ml syringe. The balloon was orally inserted into the thoracic esophagus and positioned approximately 1 cm above the esophageal hiatus for the duration of the experiment. At the end of each experiment, the location of this esophageal distension balloon was reconfirmed visually. During distension the balloon was inflated with 0.1 ml of water, increasing the outer diameter of the balloon to 4 mm. According to Hermann, et al. (9), a similar degree of distension (i.e., 0.16 ml) increases the outer diameter of the esophagus from ~2 to 4 mm. In all experiments, the balloon was distended for a period of 1 min.

All experiments were conducted 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.

**Microinjection Procedure:** All animals were placed in a small animal stereotaxic frame (Kopf Instruments, Tujunga, CA) in a prone position. Prior to stereotaxic surgery all animals were administered dexamethasone (0.8 mg sc) to minimize swelling of the brain. A partial craniotomy was performed, the dura was reflected and the cerebellum was partially retracted to expose the dorsal medulla. In all studies, the calamus scriptorius (CS) (i.e. the caudal tip of the area postrema) was used as a reference point for determining the coordinates for micropipette placement. The stereotaxic coordinates for the DMV were as follows: AP = 0.5mm, ML = 0.5mm, and DV = 0.6mm. The precise location of the DMV in our microinjection studies was functionally assessed by the L-glutamate microinjection method, which is described in detail in our earlier work (4, 8).

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

**Histological Verification of Microinjection Sites:** Upon completion of each experiment, animals were euthanized with a lethal dose of pentobarbital. The brain of each animal was rapidly removed and placed in a 4% buffered paraformaldehyde/20% sucrose solution for at least 24 hrs. Following fixation/cryoprotection, brains were then cut on a cryostat into serial 50 μm coronal sections and mounted on slides.
Subsequently, tissue was stained with neutral red, dehydrated, cleared and coverslipped. The location of each microinjection site was identified and a camera lucida drawing of it was made in relation to the nuclear groups as defined by the atlas of Paxinos and Watson (21).

**Experimental Design and Protocols:** Fundus tone and blood pressure were monitored and recorded in all the experiments performed. In all studies, there was a minimum interval of 10 minutes between application of a gastric preload and the first esophageal distension. At least 2 reproducible esophageal-gastric reflex (EGR) responses were obtained prior to assessing the effects of experimental manipulations (e.g. vagotomy, agonist and antagonist pretreatments) on EGR responses. A 30-minute interval between distensions was found to be sufficient to provide a consistent reproducible EGR response. At the end of each experiment, IV sodium nitroprusside (50 µg/kg) was routinely administered to confirm both the direction of the strain gauge transducer signal recorded and to determine that the stomach was capable of further relaxation following an experimental intervention (Fig 2B, inset).

**Bilateral cervical vagotomy:** After two reproducible EGR responses were obtained, the cervical vagus nerves were bilaterally severed. To obtain a stable baseline before repeating esophageal distension, an interval of at least 10 minutes post-vagotomy was allowed.

**Intravenous N\(^{\text{G}}\)-nitro-L-arginine methyl ester (L-NAME) and atropine methylbromide:** L-NAME was administered as an intravenous bolus injection of 10 mg/kg. This dose was used in previous studies (13, 16, 27), including those conducted by Hermann et al. Focusing on the details of one of these studies, [Takahashi and Owyang (27)], vagal
pathways mediating the gastric accommodation reflex were characterized in rats (*in vivo*). Gastric distension using 6 ml volume produced an increase of $9.0 \pm 1.0$ cmH₂O of intragastric pressure. After vagotomy gastric distension produced an increase of $16.8 \pm 1.9$ cmH₂O. These results were interpreted as indicating that the loss of vagus nerves interfered with the ability of the stomach to relax and to accommodate a volume of 6 ml. Pretreatment with a bolus IV injection of L-NAME (10mg/kg) had a similar effect (intragastric pressure rose to $13.5 \pm 1.6$ cmH₂O), which was nearly identical to that seen with hexamethonium pretreatment ($13.9 \pm 1.2$ cmH₂O). The effect of L-NAME on the pressure increase evoked by gastric distension was antagonized by pre-administration of L-arginine.

To further justify the dose of L-NAME (10 mg/kg, IV) in the present study, this dose of L-NAME was shown by us to be effective in blocking inhibitory nitric transmission at the lower esophageal sphincter (LES) as this dose altered the LES response to DMV stimulation (20).

In the present study, after two reproducible EGR responses were obtained, antagonists to nitric oxide synthase or muscarinic cholinergic receptors were administered. Esophageal distension was then repeated 5-7 minutes after L-NAME administration. The choice of a 5-7 minute interval between L-NAME administration and esophageal distension was dictated by the need to obtain a stable baseline and to decrease the probability of significant CNS effects of L-NAME (17).

Atropine methylbromide was administered as an intravenous bolus injection of 0.1 mg/kg. This dose was chosen based on its effective block of muscarinic receptors
in our earlier study in the rat (4). Esophageal distension was then repeated 10 minutes after atropine methylbromide administration.

**Bilateral microinjection of alpha-adrenoreceptor antagonists into the DMV:** After two reproducible EGR responses were obtained, the micropipette was inserted into the DMV. L-glutamate evoked responses were then evaluated to confirm the placement of the micropipette tip in the DMV (4). For the alpha 2-adrenoreceptor studies on the EGR, yohimbine hydrochloride (500 pmol/30nl) or RS-79948 (100 pmol/30 nl) was bilaterally microinjected into the DMV. The dose of yohimbine was selected based on our previous work (8). The dose of RS-79948 was initially determined based on its relative potency as compared to yohimbine (29), and then by further testing against two doses of norepinephrine microinjected into the DMV. Specifically, a dose of 100 pmol RS-79948 completely prevented 10 pmol of norepinephrine from decreasing fundus tone. One hundred pmol of RS-79948 only partially counteracted a large dose of 100 pmol of norepinephrine microinjected into the DMV from decreasing the fundus tone. Since 100 pmol of RS-79948 was effective against 10 pmol of norepinephrine, we used this dose in the studies of EGR.

For the alpha 1-adrenoreceptor studies, prazosin hydrochloride (100 pmol/30 nl) was bilaterally microinjected into the DMV. This dose of prazosin was determined based on functional antagonism of the alpha 1-adrenoreceptor selective agonist phenylephrine. Phenylephrine (100 pmol/30 nl) was microinjected into the medial subnucleus of the tractus solitarius (mNTS) where it has been reported to exert inhibitory effects on gastric motility (12). Two reproducible responses to phenylephrine were obtained with a 1 hr interval between microinjections. In 4 rats phenylephrine
decreased fundus tone by 0.34 ± 0.03 g (p<0.05). In an additional two rats, prazosin (100pmol/30nl) was microinjected following two phenylephrine microinjections. In these two rats, phenylephrine initially decreased fundus tone by 0.44 ± 0.3 g. Five minutes following prazosin microinjection, a repeat phenylephrine microinjection resulted in an + 0.01 ± 0.00 g EGR response. This was the basis for using a dose of 100pmol/30nl of prazosin in our study. After microinjection of alpha-adrenoreceptor antagonists into the DMV, an interval of 5-10 minutes was used to establish a stable baseline prior to testing the EGR.

**Unilateral microinjection of norepinephrine into the DMV:** Procedures for microinjection into the DMV were the same as described for alpha-adrenoreceptor antagonists. Norepinephrine was unilaterally microinjected and the effect on fundus tone was observed. Two doses of norepinephrine were studied. In studies characterizing the response to norepinephrine microinjection, a dose of 100 pmol/30 nl was used. After two reproducible responses to norepinephrine were obtained, both cervical vagus nerves were sectioned and norepinephrine was again microinjected after an interval of 10 min. Studies characterizing the antagonism of norepinephrine microinjection by the alpha 2-adrenoreceptor antagonist RS-79948 were carried out using a dose of 10 pmol/30 nl norepinephrine. Both doses of NE produced similar decreases in fundus tone. In these studies, L-glutamate was not used because our microinjection techniques utilized double-barreled pipettes. As such, studies of the interaction of two drugs, namely norepinephrine and antagonists of norepinephrine, left no room for identifying the DMV with L-glutamate. Instead, we identified the DMV with norepinephrine, which we had learned would produce a characteristic decrease in
fundus tone. Two reproducible responses to unilateral norepinephrine microinjection were obtained with an hour interval between microinjections. RS-79948 (100pmol/30 nl) was then microinjected into the same site. Unilateral microinjection of norepinephrine (10 pmol/30nl) was then repeated after an interval of 10 minutes.

**Drugs and Chemicals:** All drugs were purchased from Sigma (St Louis, MO), with the exceptions of RS-79948 hydrochloride (Tocris, Ellisville, MO); sodium nitroprusside (Abbott Labs, Chicago, IL); isoflurane (Baxter, Deerfield, IL); and dexamethasone (Elkins-Sinn, Cherry Hill, NJ). All drugs were dissolved in 0.9% saline and the solution was brought to a pH of 7.0-7.4 except yohimbine and prazosin, which were dissolved in double-distilled water and the solution was brought to a pH of 6.0-6.5.

**Data Analysis:** The end point used to evaluate the EGR was the maximum change in fundus tone. Maximum change was determined by subtracting the gram-tension at the point of maximum decrease in fundus tone from the average fundus tone over the 3-minute interval prior to distension. In experiments where a phasic motility pattern was present, baseline values for tone were derived from averaged maximum decreases over 3 min prior to experimental manipulation. Values for maximum change during experimental manipulations were derived in a similar way. A graphical representation of this technique is shown in Figure 1. The statistical significance of this response was determined by a one-sample t-test with a criterion for statistical significance of p<0.05. In all cases the EGR was performed twice prior to any experimental manipulation. The average value of both pre-experimental manipulation responses was used to determine the control EGR value. Statistical significance between pre- and post-treatment EGR responses was determined by a paired t-test and the criterion for statistical significance
was set at p<0.05. Comparison of norepinephrine microinjection responses before and after each experimental intervention was determined in the same way.
Results:

Effect of bilateral cervical vagotomy, intravenous L-NAME, and intravenous atropine methylbromide on the esophageal-gastric reflex (EGR)

In the first 4 animals studied, balloon inflation with 0.1 ml of water produced a relaxation of the fundus. The average relaxation response measured as a decrease in gram-tension was $0.31 \pm 0.02$ g ($p<0.05$). A repeat esophageal distension performed 30 minutes after the initial distension produced a near identical response ($0.32 \pm 0.05$ g, $p<0.05$; see Figure 2B). This degree of fundus relaxation is slightly larger than that reported by Hermann et al. (13) using 0.16 ml to distend the esophageal balloon (see control responses in Figure 2 of their paper).

Our first goal before testing alpha-adrenoreceptor antagonists in the DMV on the EGR was to determine if this response was mediated by the vagus nerves. Thus, after obtaining two repeatable fundus relaxation responses, bilateral cervical vagotomy was performed and the data are summarized in Figure 2A. As can be noted, bilateral cervical vagotomy completely prevented the EGR. A representative experiment appears as Figure 2B. Bilateral cervical vagotomy per se significantly increased baseline fundus tone by $0.28 \pm 0.06$ g ($p<0.05$).

Our second goal was to obtain information that would help us decide which alpha-adrenoreceptor antagonist (alpha 1 or alpha 2) to test at the DMV to determine the role of noradrenergic synapses in this nucleus in mediating the EGR. Rogers and colleagues (13, 24) have concluded from their studies that DMV neurons containing alpha 1-adrenoreceptors synapse in the stomach with nitric oxide-releasing enteric...
neurons. They have also concluded that DMV neurons containing alpha 2-adrenoreceptors synapse in the stomach with acetylcholine-releasing enteric neurons. We reasoned that if intravenous L-NAME blocked the major part of the EGR (as described by Hermann et al.,(13)), we would first test the alpha 1-adrenoreceptor antagonist, prazosin, at the DMV. Alternatively, if atropine methylbromide blocked the major part of the EGR, we would first test alpha 2-adrenoreceptor antagonists (e.g. yohimbine, RS-79948) at the DMV. To determine this, we first investigated the effect of intravenous L-NAME on the EGR.

Data from 5 rats are summarized in Figure 2C and indicate that intravenously administered L-NAME in a dose of 10mg/kg had no effect on the EGR. A representative experiment appears as the upper traces of Figure 2D. L-NAME per se significantly increased baseline fundus tone by 0.11 ± 0.04 g (p<0.05). Finally, the effect of intravenous atropine methylbromide was tested in a total of 5 rats (2 of which were also administered L-NAME with no effect). The data are also summarized in Figure 2C and indicate that atropine methylbromide in a dose of 0.1 mg/kg completely blocks the EGR. A representative experiment appears as the lower traces in Figure 2D. Atropine methylbromide per se significantly decreased fundus tone by 0.12 ± 0.03 g (p<0.05). To determine whether some of the antagonistic effects of atropine methylbromide might be due to the decrease in baseline fundus tone by atropine per se, we administered sodium nitroprusside (50 ug/kg, IV) at the end of each experiment. Sodium nitroprusside always produced a robust decrease in fundus tone (see Fig 2B, inset) indicating that the fundus still had the capacity to relax after atropine methylbromide administration.
**Effect of bilateral microinjection of alpha 2-adrenoreceptor antagonists into the DMV on the EGR**

Data obtained with IV L-NAME and IV atropine methylbromide suggested to us that relaxation of the fundus is due to activation of inhibitory postsynaptic alpha 2-adrenoreceptors on DMV fundus-projecting neurons and not the result of activation of excitatory postsynaptic alpha 1-adrenoreceptors on DMV fundus-projecting neurons. To test this, we employed two antagonists of alpha 2-adrenoreceptors, namely yohimbine and RS-79948, to see whether they could counteract the decrease in fundus tone produced by the EGR. As shown in Figure 3A, bilateral microinjection of either yohimbine (500 pmol, n = 4) or RS-79948 (100 pmol, n = 2) into the DMV counteracted the EGR. Also shown in Figure 3C is the degree of antagonism of the reflex when data from the studies of both alpha 2-adrenoreceptor blockers are combined (n = 6). Using these combined data, control relaxation of the fundus with esophageal distension was 0.33 ± 0.05 g; p<0.05. After bilateral microinjection of the alpha 2-adrenoreceptor antagonists into the DMV, relaxation of the fundus during the EGR was only 0.05 ± 0.06 g (p>0.05). Hence, the magnitude of blockade with alpha 2-adrenoreceptor antagonists was similar to the magnitude of blockade observed with either bilateral cervical vagotomy (Figure 2A) or with IV atropine methylbromide (Figure 2C). Representative experiments performed with yohimbine and RS-79948 are shown in Figure 3B. The microinjection sites for the yohimbine experiment shown in Figure 3B appear in Figure 3D. The microinjection sites for the six experiments summarized in Figure 3C are shown in Figure 4. Alpha 2-adrenoreceptor blockade per se decreased baseline fundus tone by 0.15 ± 0.03 g (p<0.05). Control experiments were performed investigating the
effects of bilateral microinjection of vehicle for yohimbine into the DMV (double-distilled water brought to a pH of 6.0; n=3). In these control studies, no effect was observed on the EGR (data not shown).

**Effect of unilateral microinjection of norepinephrine into the DMV on fundus tone**

If norepinephrine is the neurotransmitter at the DMV mediating esophageal distension-induced fundus relaxation, then norepinephrine microinjected into the DMV should mimic the alpha 2-adrenoreceptor mediated relaxation of the fundus evoked by esophageal distension. To test this, 2 doses of norepinephrine were unilaterally microinjected into the DMV and changes in fundus tone were noted. The first dose tested was 100 pmol and the data are summarized in Figure 5A. As can be noted, norepinephrine unilaterally microinjected into the DMV of 4 rats produced a decrease in fundus tone. The magnitude of the decrease was similar to that observed with esophageal distension. In each of the 4 animals, ipsilateral vagotomy was performed after obtaining two reproducible responses with 100 pmol norepinephrine. Ipsilateral vagotomy completely prevented norepinephrine-induced fundus relaxation (Figure 5A). Figure 5B shows a representative tracing of this effect. The microinjection site for the experiment shown in Figure 5B appears in Figure 5C. Unilateral microinjection sites were confirmed for each experiment in this series (data not shown-- available on request).

In another 4 animals we tested a 10 pmol dose of norepinephrine at the DMV. This lower dose produced a similar decrease in fundus tone (Figure 6A), and a representative experiment illustrating the time action curve of its effect is shown in Figure 6B. Ipsilateral vagotomy was not tested on the fundus relaxation produced by
the lower dose of norepinephrine because it was assumed that since ipsilateral vagotomy blocked the fundus relaxation produced by 100 pmol of norepinephrine, it would also block the fundus relaxation produced by 10 pmol of norepinephrine.

We did test the ability of RS-79948 (100 pmol) to block the fundus relaxation produced by 10 pmol norepinephrine in 4 rats. After two reproducible responses were obtained with unilateral microinjection of norepinephrine, RS-79948 was microinjected into the same site. Repeat microinjection of 10 pmol norepinephrine was performed and had no significant effect on fundus tone (Figure 6A and 6B). The microinjection site for the experiment shown in Figure 6B appears as Figure 6C. RS-79948 unilaterally microinjected into the DMV per se produced a significant reduction in fundus tone (0.15 ± 0.02 g; p<0.05). All microinjection sites were confirmed for each experiment in this series (data not shown-- available on request).

Effect of bilateral microinjection of an alpha 1-adrenoreceptor antagonist into the DMV on the EGR

We tested the alpha 1-adrenoreceptor antagonist prazosin (100 pmol) bilaterally microinjected into the DMV to determine whether this agent would influence the EGR. Four rats were tested and the results are summarized in Figure 7A. As can be noted, prazosin pretreatment did not significantly alter the reflex-induced fundus relaxation. A representative experiment is presented as Figure 7B. The microinjection sites for the experiment shown in Figure 7B appear as Figure 7C. Prazosin per se did produce a significant decrease in fundus tone (0.28 ± 0.03 g; p<0.05). All microinjection sites were confirmed for each experiment (data not shown-- available on request). Control experiments were performed investigating the effects of bilateral microinjection of
vehicle for prazosin into the DMV (double-distilled water brought to a pH of 6.0; n=3). In these control studies, no effect was observed on the EGR (data not shown).
Discussion:

The purposes of our study were to: (1) utilize the EGR described by Rogers and colleagues (13, 24) and determine whether blockade of approximately 17% of the synaptic contacts with DMV fundus-projecting neurons exerts a physiologically relevant effect; (2) assess whether microinjection of norepinephrine into the DMV would mimic the effects of the EGR; and (3) assess the role of dual inhibitory nitrergic- and excitatory cholinergic transmission in noradrenergic reflex-induced effects on DMV fundus-projecting neurons.

We accomplished the first purpose by determining the effect of bilateral microinjection of alpha 2-adrenoreceptor antagonists on the EGR. Two chemically different alpha 2-adrenoreceptor antagonists were tested, namely, yohimbine and RS-79948, and both were found to prevent approximately 85% of the fundus relaxation evoked by esophageal distension. Hence, we conclude that blockade of approximately 17% of the synaptic contacts with DMV fundus-projecting neurons is physiologically relevant. Indeed, our data indicate that the approximately 17% of synaptic contacts which use norepinephrine as the chemical messenger carry almost all of the information necessary to carry out the EGR.

In performing these studies we were able to obtain definitive information as to the type of adrenoreceptor that is important for the activation of the EGR. Data derived from microinjecting yohimbine, RS-79948 and prazosin bilaterally into the DMV demonstrates the prominence of the alpha 2-adrenoreceptor in synaptic transmission associated with EGR. No significant role of alpha 1-adrenoreceptors was observed in
this vago-vagal response. In addition, since alpha 2-adrenoreceptor blockade inhibited the EGR, there was no reason to assess the role of beta 1-adrenoreceptors at the DMV.

It is our opinion that norepinephrine is a neurotransmitter, not a neuromodulator, at the fundus-projecting DMV neurons that take part in the receptive relaxation reflex. This is based on our observations that norepinephrine is contained in nerve terminals synapsing with DMV fundus-projecting neurons (22) and that esophageal distension-induced decrease in fundus tone is virtually blocked by microinjection of alpha 2-adrenoreceptor antagonists in the DMV. Moreover, microinjection of norepinephrine into the DMV mimics the effect of esophageal distension and this effect is similarly blocked by alpha 2-adrenoreceptor antagonists.

Rogers et al. (24) evoked the EGR with a physiological stimulus similar to ours, but chose to deliver their alpha-adrenoreceptor antagonists by way of the floor of the fourth ventricle. In doing this, yohimbine reduced EGR-induced fundic relaxation to 56% of control, and prazosin reduced EGR-induced fundic relaxation to 55% of control. However, a combination of the two blockers reduced the distension-induced response to 28% of control. This is in contrast with our present study in which blockade of alpha 2-adrenoreceptors at the DMV abolished approximately 85% of the EGR response. In addition, blockade of alpha 1-adrenoreceptors in this nucleus was without any significant effect on this response. These contrasting results are most likely reflective of the differences in the method of delivery of the adrenoreceptor antagonists. In choosing to deliver the antagonists via the floor of the fourth ventricle, Rogers et al. (24) surmised that it would "maximize the exposure of the DMV to the antagonist...." However, as they report later (13), this method of delivery also has a direct impact on the area
postrema and the solitary nucleus. Both these nuclei have alpha 2-adrenoreceptors (1, 6), and, in the case of the solitary nucleus, alpha 1-adrenoreceptors as well (12). Therefore, it is conceivable that blocking these adrenoreceptors via the fourth ventricle produced the confounding effects on the EGR. Furthermore, the inability of yohimbine to produce a more complete block of the EGR may have been due to insufficient diffusion of the drug to the DMV in a high enough concentration.

It should be noted that alpha 2-adrenoreceptors exist not only on postsynaptic membranes but also on axon terminals of the neuron that releases norepinephrine (i.e. autoreceptors) (3). Therefore, it is conceivable that norepinephrine synaptically released at the DMV could interact with presynaptic alpha 2-adrenoreceptors to inhibit neural release of norepinephrine. Hence administering alpha 2-adrenoreceptor antagonists in our study would increase the amount of norepinephrine released during the EGR thereby augmenting fundus relaxation. However, this was not the case as the EGR response was blocked. Thus, we conclude that blockade of presynaptic alpha 2-autoreceptors did not contribute to the results of our study.

Another point for consideration is that not all of the afferent input to DMV gastric-projecting neurons synapse within the DMV. Several investigators have reported the presence of DMV dendrites that extend into the NTS, primarily into the subnucleus gelatinosus; just rostral to the obex (see Rinamen et al. (23)). Ultrastructural analysis of these dendrites show that they form synaptic contacts with vagal afferents in the NTS (23). It is therefore possible that alpha 1-adrenoreceptors are present on these NTS projecting DMV dendrites. Hence, our lack of effect with prazosin might be explained by its microinjection into the DMV and not into the NTS.
Focusing on the second purpose of our study, namely, whether microinjection of norepinephrine into the DMV mimics the effects of the EGR, our data indicate that it does in all respects. Norepinephrine microinjected unilaterally into the DMV reduced fundus tone that was prevented by ipsilateral vagotomy. This indicates that the drug, prior to ipsilateral vagotomy, was acting on DMV and not on NTS neurons (4). Similarly, blockade of the alpha 2-adrenoreceptor significantly attenuated the effect of norepinephrine microinjection into the DMV on fundus tone. Thus, fundus relaxation evoked by either EGR or norepinephrine is largely prevented by blockade of alpha 2-adrenoreceptors. It should be noted here that the lack of a significant degree of fundus relaxation produced by norepinephrine after alpha 2-adrenoreceptor blockade in the DMV precluded testing of an alpha 1-adrenoreceptor antagonist at this site.

Our third purpose was to assess the contribution of dual inhibitory nitrergic- and excitatory cholinergic transmission in the EGR response. Our data demonstrate that it is the noradrenergic-induced inhibition of excitatory cholinergic transmission that is responsible for the EGR. This is based on our observations that: (1) bilateral microinjection of alpha 2-adrenoreceptor antagonists but not an alpha 1-adrenoreceptor antagonist largely prevents the EGR; (2) norepinephrine microinjected into the DMV mimics the effect of the EGR, and does so by activating an alpha 2-adrenoreceptor, and (3) IV atropine methylbromide but not IV L-NAME blocks the EGR.

We find ourselves in a similar situation to Hermann et al. (13) trying to reconcile differing results from two independent laboratories. Hermann and colleagues attempted to resolve their findings for evidence that two pathways are involved in this reflex (EGR) with our earlier finding (7) that the EGR is determined exclusively by the withdrawal of
cholinergic tone induced by alpha 2-adrenoreceptor mediated inhibition of DMV neurons. In attempting to resolve these disparate findings, they raised the following criticisms vis-à-vis our studies: (1) we did not employ the same reflex-stimulating techniques used by them and were probably studying a different vago-vagal reflex, namely, a gastro-gastric reflex (which we refuted — see Ferreira et al., (9)); (2) we used an esophageal balloon distension measuring 10 mm in diameter whereas their esophageal balloon distension measured approximately 4 mm in diameter; (3) we used an esophageal stimulation volume of 0.7 ml whereas theirs was 0.16 ml; (4) we used an intragastric balloon for measuring the endpoint of gastric motility whereas they used a gastric wall strain gauge (applied to the fundus); and (5) we used alpha-chloralose as an adjunct to urethane anesthesia, which they report as inducing “adynamic ileus.” They used Inactin (thiobutabarbital) for anesthesia in their two studies (13, 24).

The question is whether these same criticisms can be leveled against the disparate findings of the present study. The answer is no, as the design of the present study addresses all 5 criticisms. We used a very similar reflex-stimulating technique as used by Rogers and colleagues (13, 24). Indeed, our distension volume was less than that used by this research group (0.10 ml vs 0.16 ml), although our end point of fundus relaxation was larger (0.34 g) than that reported by them (0.22 g — see their Figure 2 (13)). The diameter of our esophageal balloon (4mm) when distended was in the range of that described by Hermann, et al. (9, 13). Similarly, to record fundus tone, we used a miniature strain gauge applied to the fundus. Finally, we used isoflurane anesthesia in place of the alpha-chloralose-urethane cocktail that was employed by us in our earlier work (7).
Why are our results different than those of Rogers and colleagues? There are several possible explanations. One is the issue of anesthetics used. We employed isoflurane in our current study whereas they employed a relatively high dose of Inactin (150-200 mg/kg IP). The usual dose of this anesthetic is 80-125 mg/kg IP (2, 5, 14, 15, 25, 26, 28, 30).

A further possibility involves the use of L-NAME. By administering this inhibitor of nitric oxide synthase (10 mg/kg, IV), Hermann et al. (13) reported that it reduced the amplitude of fundus relaxation produced by esophageal distension to 26.3±7.2% of the original EGR response. The interval between L-NAME administration and a test of the EGR was 15 minutes (IV drug treatment preceded the gastric preload by 10 min; and gastric preload required 5 min to establish for a total of 15 minutes). Hermann et al. (13) raise the possibility that the L-NAME could be acting centrally but discount this by stating that “previous work has shown that the central neuropharmacological components of the EGR circuit (does) not utilize nitrergic or cholinergic transmission” citing four references. They fail to cite two references showing that nitrergic transmission at the NTS is involved in mediating a vago–vagal reflex that produces inhibition of gastric motility (7, 8). Another point to consider here is the earlier findings of Ma et al. (17) on the effect of IV administration of L-NAME (10 mg/kg) on medial NTS neurons in the rat. They showed that in 12 of the 14 neurons recorded, L-NAME decreased their activity, an effect that was maximal 12-15 minutes after its administration. Hence, L-NAME does affect hindbrain neural activity in one of the two major hindbrain nuclei comprising the vago-vagal reflex. In our study, the interval between L-NAME administration and the EGR test was 5-7 mins. At this time interval,
L-NAME appeared to have exerted a maximal effect as confirmed by the consistent rise in blood pressure. However, it was ineffective in blocking the EGR response.

The differences in findings between our study and the findings of Hermann et al. (13) may also be due to their dose of atropine methylnitrate. The dose used by them (50 µg/kg, IV) reduced the amplitude of gastric relaxation to 52.0±4.4% of control. The rationale given for using the atropine methylnitrate dose is based on two cited references in their paper. One of these, Takahashi and Owyang (27) used a similar, but not identical dosing regimen (50 µg/kg bolus and continuous infusion of 20 µg/kg/hr), and found that atropine had no effect on the gastric accommodation reflex in their study. Hence, the data of Takahashi and Owyang cannot be used as a source for indicating that 50 µg/kg atropine is a full muscarinic receptor blocking dose because these receptors are not involved in mediating the accommodation reflex. The other citation is to P. Millard's chapter in a textbook on clinical veterinary nursing (19). Millard lists the dose range of atropine (0.02-0.05 mg/kg) that is found in the contents of an anesthetic emergency kit. However, no reference is given for how the atropine dose was determined and/or whether the dose range applied to treating an anesthetic emergency in rats. We used a dose of atropine methylbromide (i.e., 0.1 mg/kg, IV) that was twice as high as that used by Hermann et al. (13). In a previous study, this dose was effective in preventing L-glutamate microinjection at the DMV from increasing intragastric pressure (4). In the present study, we found that administration of atropine methylbromide (0.1 mg/kg, IV) prevented approximately 85% of the EGR response.

Reflecting on these latter two differences, namely, the time to peak effect of L-NAME on the EGR response and the atropine dose, we suggest that the different
findings of Hermann et al (13) may be due to a combination of an inadequate atropine dose and an NTS effect of L-NAME.
Perspectives and Significance:

We employed the "esophageal-gastric reflex" described by Rogers and colleagues (24) as our test system for examining the role of noradrenergic transmission at the DMV in controlling fundus tone. We presume that the components of this reflex consist of afferent vagal neurons with sensory elements in the thoracic esophagus, second order noradrenergic neurons originating in the NTS (22) and efferent vagal neurons projecting to the fundus. This reflex, commonly known as the receptive relaxation reflex (11), is partly responsible for the maintenance of an unchanging intragastric pressure after eating. Swallowing of food stretches the esophagus and reflexively (by receptive relaxation) reduces fundus tone, thus keeping intragastric pressure stable. Data from the present study suggest that this reflex operates by utilizing less than 20% of synaptic input to the fundus-projecting DMV neurons. Norepinephrine, acting at the NTS-DMV synaptic interface inhibits ongoing activity of DMV motor neurons leading to the EGR reflex. This effect is mediated entirely by alpha 2-adrenoreceptors; alpha 1-adrenoreceptors do not play a role. Our current data also show that the efferent vagal pathway involved in the receptive relaxation reflex is comprised solely of cholinergic-cholinergic neurons. Enteric nitrergic neurons responding to preganglionic release of ACh do not contribute to this reflex.

In perspective, the view that central noradrenergic neurons "determine the brain's global orientation concerning events in the external world and within the viscera" (3) is applicable to the vago-vagal pathway that we studied. In this pathway, brainstem noradrenergic neurons respond to mechanical stimuli within the esophagus that in turn
causes the DMV fundus-projecting neurons to inhibit the fundus, thus making room for ingested food without a rise in intragastric pressure.
References:


Figure Legends

Figure 1: A. Graphical representation of the technique used in analyzing fundus tone. To compare pre- and post-EGR fundus tone, average minimum values of the points (y) during the EGR (bar) were subtracted from those points over a 3 minute interval prior to distension (x). B. Similarly, per se effects of experimental interventions (e.g., drug administration or bilateral vagotomy) were calculated from the average minimum points over a 3 minute interval following baseline stabilization. Note: In the figure, experimental intervention (arrow) causes an increase in baseline. Both this and interventions that produced a decrease in baseline were similarly calculated.

Figure 2: A. Histogram representation of averaged control responses of the EGR as compared to averaged EGR responses following bilateral cervical vagotomy, *p<0.05, n = 4. B. Representative experimental tracing depicting fundus tone changes during the two EGRs (first and second panels) and after bilateral cervical vagotomy (third panel). A typical response to IV administration of sodium nitroprusside (50 µg/kg; SNP) is shown in B, lower right panel. The 3 horizontal dark lines above each tracing indicate the time period of esophageal distension. C. Histogram representation of averaged control responses of the EGR as compared to averaged EGR responses following either IV L-NAME or IV atropine methylbromide, *p<0.05, n = 5. D. Representative experimental tracings depicting fundus tone changes during the EGR before (top left panel) and after (top right panel) IV L-NAME; and before (lower left panel) and after (lower right panel) IV atropine methylbromide. Horizontal dark lines above each tracing indicate the time period of esophageal distension.
**Figure 3:** A. Histogram representation of averaged control responses of the EGR as compared to averaged EGR responses following either yohimbine or RS-79948 microinjection [*p<0.05, n=4 (yohimbine)]; [n=2 (RS-79948)]. B. Representative experimental tracings depicting fundus tone changes during EGR before (top left panel) and after (top right panel) bilateral yohimbine microinjection into the DMV; and before (lower left panel) and after (lower right panel) bilateral RS-79948 microinjection into the DMV. Horizontal dark lines above each tracing indicate the time period of esophageal distension. C. Histogram representation of averaged responses of the EGR as compared to averaged EGR responses following microinjection of either alpha 2-adrenoreceptor antagonist [*p<0.05, n=6 (averaged values for all the data obtained with yohimbine and RS-79948)]. D. Photomicrograph of bilateral DMV microinjection sites for the yohimbine experiment shown in Figure 3B. The stippled boxes depict the pipette tracks, and the dashed ‘ovals’ show the outline of both DMVs. Abbrev: DMV: Dorsal Motor Nucleus of the Vagus; cc: central canal; NTS: Nucleus of the Tractus Solitarius; XII: Hypoglossal nucleus.

**Figure 4:** Camera lucida drawings depicting bilateral microinjection sites for all the alpha 2-adrenoreceptor antagonist studies summarized in Figure 3C. Paired symbols represent each experiment performed. Filled symbols and x's represent studies performed with yohimbine. Open symbols represent studies performed with RS-79948. Abbrev: DMV: Dorsal Motor Nucleus of the Vagus; cc: central canal; NTS: Nucleus of the Tractus Solitarius; XII: Hypoglossal nucleus; AP: area postrema. Numbers on the
left side of each coronal section indicate the distance of each section from calamus scriptorius.

**Figure 5:**  
**A.** Histogram representation of averaged responses to norepinephrine microinjection before and after ipsilateral vagotomy, \( *p<0.05, n = 4 \).  
**B.** Representative experimental tracing depicting fundus tone during the two control unilateral norepinephrine microinjections into the DMV (first and second panel) and after (third panel) ipsilateral cervical vagotomy. Arrows above each trace indicate the times when norepinephrine microinjections occurred.  
**C.** Photomicrograph of unilateral DMV microinjection site. The stippled box depicts the pipette track, and the dashed ‘ovals’ show the outline of both DMVs.  
Abbrev: DMV: Dorsal Motor Nucleus of the Vagus; cc: central canal; NTS: Nucleus of the Tractus Solitarius; XII: Hypoglossal nucleus.

**Figure 6:**  
**A.** Histogram representation of averaged responses to norepinephrine microinjection before and after microinjection of RS-79948, \( *p<0.05, n = 4 \).  
**B.** Representative experimental tracing depicting fundus tone changes during unilateral norepinephrine microinjection into the DMV (first panel) and after (second panel) unilateral microinjection of RS-79948. Arrows above each trace indicate the times when norepinephrine microinjections occurred. As can be seen in the figure, bilateral microinjection of RS-79948 prevented norepinephrine from decreasing fundus tone. It can also be noted that RS-79948, *per se*, decreased baseline fundus tone. The antagonism observed with RS-79948 was not due to the decrease in fundus tone as subsequent administration of sodium nitroprusside retained its robust effect (data not
shown). **C.** Photomicrograph of unilateral DMV microinjection site. The stippled box depicts the pipette track, and the dashed ‘ovals’ show the outline of both DMVs. Abbrev: DMV: Dorsal Motor Nucleus of the Vagus; cc: central canal; NTS: Nucleus of the Tractus Solitarius; XII: Hypoglossal nucleus.

**Figure 7:** **A.** Histogram representation of averaged control responses of the EGR as compared to averaged EGR responses following bilateral prazosin microinjection, n = 4  
**B.** Representative experimental tracing depicting fundus tone changes during EGR before (left panel) and after (right panel) bilateral prazosin microinjection into the DMV. Horizontal dark lines above each tracing indicate the time period of esophageal distension. **C.** Photomicrograph of bilateral DMV microinjection sites. The stippled boxes depict the pipette tracks, and the dashed ‘ovals’ show the outline of both DMVs. Abbrev: DMV: Dorsal Motor Nucleus of the Vagus; cc: central canal; NTS: Nucleus of the Tractus Solitarius; XII: Hypoglossal nucleus.