Noradrenergic neurons in the rat solitary nucleus participate in the esophageal-gastric relaxation reflex

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Summary

Activation of esophageal mechanosensors excites neurons in and near the central nucleus of the solitary tract [NSTc]. In turn, NSTc neurons coordinate the relaxation of the stomach [i.e., the receptive relaxation reflex; RRR] by modulating the output of vagal efferent neurons of the dorsal motor nucleus of the vagus [DMN]. The NSTc area contains neurons with diverse neurochemical phenotypes, including a large population of catecholaminergic and nitrergic neurons. The aim of the present study was to determine if either one of these prominent neuronal phenotypes was involved in the RRR.

Immunohistochemical techniques revealed that repetitive esophageal distension caused 53% of tyrosine hydroxylase immunoreactive [TH-ir] neurons to co-localize cFos in the NSTc. No nitric oxide synthase [NOS-ir] neurons in the NSTc co-localized cFos in either distension or control conditions. Local brainstem application [2ng] of α-adrenoreceptor antagonists [i.e., α−1 [prazosin] or α−2 [yohimbine]] significantly reduced the magnitude of the esophageal-distension induced gastric relaxation to approximately 55% of control conditions. The combination of yohimbine and prazosin reduced the magnitude of the reflex to approximately 27% of control. In contrast, pretreatment with either the NOS-inhibitor [L-NAME] or the beta-adrenoceptor antagonist [propranolol] did not interfere with esophageal-distension induced gastric relaxation. Unilateral microinjections of the agonist, norepinephrine [0.3ng], directed at the DMN were sufficient to mimic the transient esophageal-gastric reflex.

Our data suggest that noradrenergic, but not nitrergic, neurons of the NSTc play a prominent role in the modulation of the RRR through action on α-1 and α-2 adrenoreceptors. The finding that esophageal afferent stimulation alone is not sufficient to activate NOS positive neurons in the NSTc suggest that these neurons may be strongly gated by other CNS inputs, perhaps related to the coordination of swallowing or emesis with respiration.
Introduction

The receptive relaxation reflex [RRR] is an important mechanism that increases gastric volume and reduces intragastric pressure to ensure that swallowed food is efficiently transported from esophagus to stomach (9;25;30;33). A number of investigators have shown that this potent proximal gastric relaxation is triggered by the activation of low threshold vagal afferent mechanosensors in the esophagus (33). The reflex requires intact vago-vagal connections between the esophagus, brainstem and stomach (30). Several anatomical tracing studies have shown that vagal afferent projections from the esophagus terminate in and near the central division of the nucleus of the solitary tract [NSTc] (1;4;13). Previous investigations have shown that neurons in the NSTc are intensely activated by low-level esophageal distension (7;30;33).

Much of the neurophysiological and anatomical work done on esophageal afferents and the NSTc has focused on the role these entities play in the production of the swallowing motor program and in coordinating the act of swallowing with respiration and cardiovascular function (6;27) Other studies have demonstrated a dense projection from the NSTc area to the nucleus ambiguus [NA]; the source of vagal motor neurons that project to the esophagus, lower esophageal sphincter, and heart (10;16;20). The NSTc-NA pathway is clearly involved in the production of swallowing as well as the regulation of the glottis and heart rate during deglutition (3;6;22).

The relationship between the NSTc and the vagal motor neurons that control the stomach has only recently been addressed. Physiologically guided nano-injections of retrograde and anterograde tracer onto NSTc neurons that respond to esophageal distension demonstrate that these neurons project heavily throughout the full anterior-posterior extent of the dorsal motor nucleus of the vagus [DMN], the primary source of preganglionic autonomic control over the stomach (30).

Vagal reflex control over gastric tone and motility is affected by modulating the activity of two antagonistic vagal efferent projections. Vagal efferent -mediated increases in gastric tone and motility occur following activation of cholinergic neurons in the gastric enteric plexus by loosely aggregated preganglionic neurons in the DMN (30;32). Conversely, rapid gastroinhibition can result from the inhibition of these DMN neurons. Indeed, it is well known that intestinal, gastric, and esophageal distension causes an abrupt cessation in the tonic firing of DMN neurons which coincides with the rapid onset of a reduction in gastric tone and motility (30;31). Activation of gastric and esophageal distension sensitive afferent fibers can also produce a potent gastroinhibition through the activation of a vagal non-adrenergic, non-
cholinergic [NANC] pathway to the fundus (30;34;35). Our previous neurophysiological studies suggest that putative NANC-path inhibitory neurons in the DMN are excited by esophageal distension (30).

Little else is known about the mechanisms by which NSTc neurons produce changes in DMN neurons that result in the RRR. The NSTc area contains a number of different neuronal phenotypes. Two neurochemical phenotypes that are especially prominent are noradrenergic and nitrergic (20;21). The core of the NSTc contains an especially dense concentration of nitric oxide synthase [NOS] neurons while TH-ir neurons are found throughout the NST. Of particular interest is the subset of TH-ir neurons found encircling the NSTc-NOS neurons. Previous immunohistochemical studies have shown that virtually all of these TH-containing neurons near the NSTc also express dopamine beta-hydroxylase and are, therefore, norepinephrine-producing neurons (37).

Both of these NST cell types [nitrergic and noradrenergic] have been implicated in the control of variety of autonomic functions (5), though not the RRR. Indeed, these TH-ir and NOS-ir neurons could receive a large amount of input from esophageal vagal afferents (1). With the combined use of immunohistochemical and in vivo physiological methods, we aimed to determine: 1] the distribution and neurochemical identification of NST neurons activated by esophageal distension; and 2] the relative influence of these pathways in the modulation of the RRR.

[Preliminary accounts of this work have been previously presented: Travagli RA, Rogers RC. A proposed brainstem circuitry for the receptive relaxation reflex. Digestive Disease week. May 16-22, 1998. New Orleans, LA; Rogers RC, Bantikyan A, Travagli RA. Involvement of catecholamines in the esophageal-gastric relaxation pathway. 30th Neuroscience Meeting. New Orleans, LA, 2000]
Methods

Drugs and chemicals

Animals were anesthetized with thiobutabarbital [Inactin®, Sigma; 150-200mg/kg, ip]. This long term anesthetic has been shown not to interfere with brainstem autonomic reflexes (8) or the activation of DVC neurons following exposure to cytokines (11;12;17).


The antagonists for α-1 adrenergic receptors [prazosin], α-2 adrenergic receptors [yohimbine], or beta-adrenergic receptors [propranolol] as well as the NOS synthesis inhibitor [N⁶-Nitro-L-arginine methyl ester hydrochloride; L-NAME] were purchased from Sigma [St Louis, MO]. These drugs were made fresh daily immediately prior to use at a concentration of 1mg/ml in sterile phosphate buffered saline [PBS]. The adrenergic agonist [norepinephrine, NE; Receptor Research Chemicals, Baltimore, MD] was made fresh daily at a concentration of 40mM in sterile PBS.

Experiment 1: Immunohistochemical procedures

All experimental protocols were performed according to the guidelines set forth by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committees at the Pennington Biomedical Research Center and the University of Michigan Health Sciences Center.

Male Long –Evans rats [Charles River Laboratories, Wilmington, MA; N = 37] weighing between 250 and 500g were anesthetized with thiobutabarbital [150-200 mg/kg, ip]. A tracheal catheter was placed to maintain a clear airway. Animals were mounted in a stereotaxic frame. An esophageal distension balloon was constructed from a 1.5cm length of 1mm OD, 0.5 mm ID silicone tubing [AM Systems, Seattle, WA]. The balloon tubing was connected via polyethylene
tubing [PE 50] to a modified small animal respirator [Harvard Instruments, Cambridge, MA]. The tubing and 1cc respirator cylinder were filled with water. The respirator was programmed to cyclically inflate the balloon to a final volume of 160µL [i.e., 2.5mm distended diameter]. Our previous study (30) showed that this distension produced a transmural pressure increase of less than 14mm Hg. This degree of distension activates vagal mechanosensors, but not spinal nociceptors (33). Surgically prepared, anesthetized rats were mounted in the stereotaxic frame.

A distal esophageal distension group [N=16] had the balloon placed orally in the esophagus such that the tip was located 1cm above the esophageal hiatus in the thoracic esophagus. The balloon was then distended and relaxed every 10 seconds [50% duty cycle] for 90 minutes. A control group of rats [N=13] did not receive the esophageal balloon but the oral cavity was manipulated as if the balloon/catheter were to be placed in the esophagus.

At the end of the 90min stimulation period, the animals rested for an additional 30min to allow for maximal Fos-activation of NST neurons. At that time, rats were transcardially perfused with PBS, followed by 4% paraformaldehyde in PBS. Brainstems were then removed to a solution of 4% paraformaldehyde plus 20% sucrose for overnight post fixation. Brainstems were cut on a freezing microtome into 40µm sections.

All sections through the medullary brainstem were saved and processed for the demonstration of nuclear cFos protein, a marker for prolonged and significant neuronal excitation (28). This protocol is available in detail elsewhere (18;28). Briefly, all tissue sections were rinsed in PBS and incubated in sodium borohydride and hydrogen peroxide to eliminate remaining fixative and to block endogenous peroxidase. Sections were then rinsed and blocked in 5% normal goat serum, re-rinsed in PBS and then incubated in rabbit, anti-rat cFos primary antibody overnight at room temperature on a shaker table. Sections were rinsed in PBS and incubated with biotinylated goat, anti-rabbit secondary antibody. Sections were rinsed in PBS and incubated in Vector ABC peroxidase reagent, followed by the Vector peroxidase chromogen Nova Red. cFos staining was revealed in this protocol as brick-red nuclei [Figures 1, 3]. Omission of primary antibody or incubation with inappropriate secondary antibody produced no cFos label.

Sections were then divided into three lots with each lot containing a full series of sections through the brainstem. One of the three lots that were processed for cFos was mounted on subbed slides, dried and coverslipped [see Fig. 1]. In addition to immunostaining for cFos activation, the two other lots were also processed for either tyrosine hydroxylase [TH] or neuronal nitric oxide synthase [NOS]. Sections were rinsed and blocked with 5% horse serum, re-rinsed and followed with either mouse, anti-rat TH primary antibody or mouse, anti-rat
NOS primary antibody. Sections were incubated on a shaker table overnight at 8 °C, then rinsed and incubated in Vector ABC reagent, re-rinsed and reacted with the Vector peroxidase chromogen SG. Cytoplasmic staining for TH or NOS is blue-black using this method. This cytoplasmic chromogen contrasts well with the nuclear Nova Red stain that identifies cFos activated neurons [Figure 3]. Occasionally, TH or NOS-ir material was processed with Nova Red and counterstained with Methyl Green [Vector Laboratories]. Sections were viewed, analyzed, and photographed with a Nikon E800 microscope coupled with a Zeiss Axiocam CCD camera.

Analysis of the location of cFos–positive neurons: For quantitative purposes, the subregions of the solitary nucleus were divided as shown in Figure 2 and subsequently schematized into row and column regions of interest. Anterior-posterior “rows” were sampled/binned at three levels: calamus [“0.0”], 0.5mm rostral to calamus at the level of the anterior portion of the area postrema [“+0.5”], and 1.0mm rostral to calamus [“+1.0”]. This rostralmost section, just anterior to the rostral edge of the area postrema, is a principal region of termination of esophageal vagal afferents, according to several earlier papers (1;30). The medial-lateral [“columns”] extent of the NST was divided into a “lateral” portion [i.e., all of the NST lateral to the mid-point of the solitary tract], a “central” portion [i.e., the NST between the mid-point of the solitary tract and the mid-point of the DMN] and a “medial” portion [i.e., between the mid-point of the DMN and the medial-most extreme of the NST; see scheme in Figs. 1 & 2].

In the cFos-only processed sets, all representative sections [i.e., those containing the NST] from anterior-posterior levels [rows] were evaluated for the presence of cFos labeled cells across the three subregions [as defined above]. Inclusion of cFos-labeled neurons required that the nuclei be a minimum of 6µm in diameter and had to exhibit a nucleolus. These criteria guaranteed that staining artifacts and nuclear fragments would not be included in the count. An investigator unaware of the experimental condition being analyzed counted cFos stained nuclei; a second observer verified counts. The agreement between counts of the two observers was within 10%. Data in Figure 1 represents the averaged total number [+ SEM] of cFos-activated neurons throughout the NST.

Given that the total number of cFos-activated cells in the NST in response to distal esophageal distension was markedly increased compared to the control condition, a plot of the distribution of cFos-positive neurons across and through the NST was constructed. A one-way analysis of variance was performed on the numbers of cFos-positive cells per row and column segment. Bonferroni post-tests on selected, appropriate, pairs of segments followed. Statistical
significance was defined at $P < 0.05$. [Fig. 2; Data represent the average number of cFos-labeled neurons within any given subregion per histological section per animal.]

Analysis and quantification of the cFos-double immunostained neurons [i.e., cFos+TH positive and cFos+NOS positive] was restricted to the central portion of the NST because: a) this region has been demonstrated to be the primary relay area for esophageal afferent information (1;30); and b) our analysis showed that the anterior, central divisions of the NST were significantly more activated by esophageal distension than the other divisions [Fig. 2]. Data from this analysis are reported as the average number [per histological section] of either TH- or NOS-identified neurons in the central NST and the average number of identified [TH or NOS] neurons that were also cFos-positive [Fig. 4].

The above experiments yielded unexpected results regarding the distribution of cFos-NOS-ir in the NSTc after esophageal distension. Thus, these results prompted us to add two additional experimental groups. A proximal esophageal distension group [N = 4], where the esophageal stimulation balloon was placed orally just below the larynx and was cyclically distended as described above. Another group of rats [N = 4] received acid stimulation of the distal esophagus. A length of PE 50 tubing was orally placed in the esophagus to end at the level of the hiatus (26); 10$\mu$L of a pH 1 solution of HCl was infused through this cannula into the esophagus every 10min for 90minutes.

All tissue processing and analyses of these groups were identical to those described above. Data in Figure 1 represents the averaged total number [± SEM] of cFos-activated neurons throughout the NST across all four groups. These data were subjected to a one-way analysis of variance; statistical significance defined at $P<0.05$. Dunnett’s post-test for comparisons against control followed. The distributions of cFos-activated neurons within [i.e., across and through the subregions of] the NST for these additional two groups were plotted and analyzed as described above for the control and distal esophageal stimulation groups.

Experiment 2: motility measurement methods [in vivo]

The following set of experiments was designed to either interrupt the integrity of the esophageal-gastric receptive relaxation reflex [via the use of presumptive antagonists] or fictively mimic the reflex [via the microinjection of presumptive agonists directly into the DVC]. The initial surgical and experimental preparations were identical for both groups with the exception of the delivery of the drugs in question.

All rats used in gastric motility measurement experiments were food deprived for 16hrs prior to instrumentation. Rats were anesthetized with thiobutabarbital and fitted with a tracheal
cannula, as described above. Uniformity in gastric pre-load conditions was accomplished by a latex balloon made from the small finger of a size 6 surgeon’s glove and attached to a 10 cm length of 2mm OD silicone tubing. The balloon was installed into the empty fundus through a purse-string suture-fistula in the greater curvature of the antrum. A miniature strain gauge [RB Company, Madison, WI] was sutured to the exterior of the fundus so as to align the strain measuring elements with circular smooth muscle fibers. Care was taken to assure that the strain gauges were attached to the fundus without stretching the stomach. Application of 2ml of water to the gastric balloon produced a mild, but consistent 1g distension pre-load strain on the fundus (34). This arrangement produced a consistent distension against which the fundus, equipped with a strain gauge, could relax. Strain gauge signals and balloon pressure signals were routed through a PC-based signal analysis system that digitized and stored the records.

The rat was positioned in a stereotaxic frame; removing the occipital skull plate exposed the dorsal brainstem. The dura and arachnoid meninges were carefully removed to facilitate delivery of antagonists or agonists.

Functional verification of the results of Experiment 1 required that we antagonize the potential effects of either NO or NE in the DVC. Previous work from this laboratory has shown that neurons in the NSTc area responsive to esophageal distension project throughout the entire DMN. The DMN is a relatively large, flattened tubular structure approximately 2 mm long [anterior-posterior; AP], 0.5 mm wide [mediolateral; ML] and 0.1 mm deep [dorso-ventral; DV]. Of course, the RRR vago-vagal reflex circuits are duplicated bilaterally in the medulla. A single pair of even relatively large [40nL] injections of antagonist would cover only a small fraction of the total volume of the DMN. This factor makes a “negative result” [i.e., no change in the RRR amplitude] uninterpretable. Further, antagonizing putative NSTc reflex inputs to the DMN with multiple microinjections is not realistic. To do so would require the nearly simultaneous application of four 40nL volumes of antagonist to either side. Therefore, we chose instead to deliver the antagonists [which did not have basal effects on gastric motility] via the floor of the 4th ventricle to maximize the exposure of the DMN to the antagonist and block [i.e., override] the endogenous pharmacological basis of a stimulated reflex.

Noradrenergic or nitrenergic antagonists:

Based on the results of Experiment 1, subpopulations of immunologically identified [i.e., TH or NOS] NST neurons are activated by esophageal distension and may play a role in the receptive relaxation reflex [RRR]. Therefore, these experiments were designed to interrupt the normal esophageal distension-induced gastric reflex by applying the presumptive antagonists
onto the floor of the fourth ventricle, thereby maximizing the exposure of the antagonist[s] to the full extent of the NST-DMN interface.

All stereotaxic and surgical preparations are as described above [N=25]. As described in Experiment 1, above, an esophageal balloon cannula was placed in the distal esophagus and connected to the dome of a pressure transducer. The balloon, tubing, and transducer dome were filled with water. A one ml syringe was connected with the transducer dome to distend the esophageal balloon [160µL final volume] during the esophageal stimulation.

Once all surgical preparations were completed and the esophageal cannula was in place, basal fundic activity was monitored for one hour before starting the stimulation protocol. The fundic balloon was then inflated to 2ml, producing a modest distension of the fundus [i.e., consistent 1g distension pre-load strain]. Simultaneous to the gastric pre-load, 2µl of PBS were microdropped onto the floor of the fourth ventricle directly above the dorsal vagal complex [DVC]. Ten minutes later, the esophageal balloon was distended [i.e., onset of esophageal stimulation] for one minute, and then released. The gastric balloon pre-load was relieved five minutes after the stimulation was delivered. This session constituted the control or basal RRR; thus, each animal served as its own control for comparison purposes. One hour after this control RRR was elicited, the protocol was repeated with the exception that one of the following antagonists or inhibitors were microdropped [2ng in a 2µL volume] onto the dorsal surface of the DVC:

- prazosin [α-1 adrenoceptor antagonist]
- yohimbine [α-2 adrenoceptor antagonist]
- propranolol [non-selective beta-adrenoceptor antagonist]
- L-NAME [NO synthesis inhibitor]
- prazosin + yohimbine [2ng each; total volume 2µL]

**Antagonists: in vivo data analysis**

The change in fundic tone elicited by the esophageal distension was evaluated by averaging the strain gauge output for the minute before and during the esophageal distension. The difference in fundic tone, before and during distension, represents the magnitude of the RRR.

Calculating a difference score between control and drug treatment conditions and multiplying this score by 100 determined the effects of antagonist application to the DVC on the elicited RRR. That is, a fundic relaxation index of “100%” is indicative of no change to the control reflex due to the drug, while an index of “50%” indicates a loss in the relaxation reflex by
50% [Fig. 5]. The raw data were subjected to a one-way analysis of variance; post-hoc comparisons were made against the set control value of “100” [i.e., “no change” in reflex magnitude] using the Dunnett’s test for comparisons to control. Statistical significance was defined at P < 0.05.

Noradrenergic agonists:

Our observations from the preceding set of experiments revealed that only noradrenergic antagonists interrupted the normal RRR. To test the hypothesis that NE action in the DMN can elicit the same drop in gastric tone as seen with esophageal distension, relatively small [40nL volume], unilateral injections of NE [50mM; total dose = 0.3ng = 2pmoles] into the DMN were made via micropipette. The logic of agonist application [via point microinjection] parallels that for antagonist application [via broad coverage]. Specifically, point microinjection of a presumptive agonist into small areas of the DMN that are affected by NST input should be expected to elicit the full reflex. This is due to the extraordinary divergence of input to the enteric plexus from single vagal efferent fibers. Thus, to antagonize the RRR, broad coverage of the DMN is required for unambiguous effects. In contrast, nearly complete agonist effects can be expected from point applications to the DMN [e.g., thyrotropin releasing hormone – (24); oxytocin – (29); tumor necrosis factor-α – (17)].

All stereotaxic and surgical preparations are as described above for the in vivo motility measurements, including the exposure of the brainstem surface. Once all surgical preparations were completed, basal fundic activity was monitored for one hour. The fundic balloon was then inflated to 2ml, producing a modest distension of the fundus [i.e., consistent 1g distension pre-load strain]. Within 5min of the onset of the gastric pre-load, 40nL of PBS were microinjected into the left DVC directly over the NSTc [coordinates relative to calamus scriptorum: AP = 0.3mm rostral; ML = 0.3mm off midline; DV = 500micron below surface of medulla]. The gastric balloon pre-load was relieved five minutes after the microinjection was delivered. This session constituted the control or basal motility response to microinjection; thus, each animal served as its own control for comparison purposes [N = 4]. One hour after this control response session, the protocol was repeated with the exception that 40nL of 50mM NE [total dose = 0.3ng] was microinjected into the same injection site. At the end of this gastric motility/tone recording session, a micropipette [tip diameter approximately 20µM, as above] filled with 1% Pontamine dye was lowered into the microinjection site and 40nL of dye was delivered to the site to mark the location for histological verification [Fig. 6].
**NE agonist: in vivo data analysis**

The change in fundic tone elicited by microinjection into the DVC was evaluated by averaging the strain gauge output for the minute before and after the microinjection. The difference in fundic tone following PBS versus NE microinjection represents the magnitude of the fictive RRR. The difference scores ["before" vs. "after" DVC microinjection] of the PBS injections were compared to the difference scores obtained after microinjection of NE using paired t-test analysis. As an additional measure of comparison, the average magnitude of the normal RRR that was elicited by esophageal distension in the preceding set of experiments was compared to the magnitude of the average change in fundic tone elicited by unilateral microinjection of NE or PBS into the DVC [Fig. 6]. These data were subjected to a one-way analysis of variance; Tukey post-hoc comparisons were made. Statistical significance was defined at P < 0.05.

**Results**

**Distribution of cFos labeled neurons in the NST following esophageal distension**

Periodic esophageal distension resulted in a significant increase in the numbers of cFos–positive neurons in the throughout the entire NST [Fig. 1; ANOVA F\(_{3,33} = 26.0\); P<0.0001; Dunnett’s post-test for comparisons against control: ** = p <0.001]. The distribution of cFos-positive neurons after esophageal distension was higher in the central regions of the NST than in the lateral or medial subregions at any given rostro-caudal plane [Fig. 2; ANOVA F\(_{8,135} = 11.21\); P<0.05, Bonferroni post-hoc comparisons, P<0.05].

In view of the observation that esophageal distension activated more cFos-positive neurons in the central division of the NST than in the other subregions, we concentrated our analysis of the neurochemical phenotype cFos-double immunostained neurons in this area. Figure 3A shows that a relatively large number of cFos–activated neurons are located just outside the confines of the NOS core of the central division of the NST. This stands in sharp contrast with the observation in the adjacent histological section where numerous cFos-TH positive NST neurons are seen to encircle the same core of presumably NOS-positive NSTc neurons [Fig. 3B-C]. Note that some NOS-ir neurons outside the dense core NOS-ir area of the NSTc did contain cFos-ir [Fig. 3D], but these were few in number. Although esophageal distension does not appear to activate many NOS-ir neurons in the NST, nevertheless a very dense plexus of presumptive NOS-ir terminal fibers are interwoven between DMN neurons. These fibers have a coarse appearance [Fig. 3E] compared with the equally dense, but fine filamentous TH fibers that surround practically all neurons in the DMN [Fig. 3F]. As Figure 3G
shows, single TH containing fibers in the NST send short filamentous projections into the immediately subjacent DMN [Fig. 3 G-H].

An attempt was made to test whether a different location [i.e., proximal esophageal distension] or mode of stimulation [i.e., acid infusion] could activate neurons in the dense core NOS region of the NSTc. In neither set of stimulation conditions were NOS-neurons in this region of the NSTc activated by these stimuli as demonstrated by cFos expression. Specifically, proximal esophageal distension produced a similar distribution pattern of cFos activation and the proportion of activated TH and NOS-positive NST neurons as was observed with distal esophageal distension. Therefore, these data were combined for analysis purposes. In contrast, repetitive infusion of small amounts of acid into the distal esophagus had no significant effect to elevate the number of cFos positive neurons above control values, nor did it activate a significant number of NOS-positive neurons in the NSTc.

The total numbers of NOS-ir or TH-ir neurons found in the NSTc in either the control or esophageal distension groups are not different [Fig. 4]. However, the number of activated TH-ir neurons [i.e., TH+cFos-positive] responding to esophageal distension was significantly greater than control group [ANOVA F_{3,40} = 51.0; P<0.0001; distension: 25 ± 2 vs. control: 11 ± 3; Bonferroni post-hoc comparison, P < 0.001]. There was no significant difference in the number of NOS-cFos double immunoreactive cells in the NSTc [Fig. 4; distension: 3±1 vs. control: 0±0; Bonferroni post-hoc comparison P > 0.05].

**Effects of noradrenergic and NOS antagonism on the RRR**

Similar to our previous studies on the antrum (30), we observed that low volume esophageal distensions produce brisk relaxations of the mildly distended fundus [Fig. 5]. Application of either yohimbine or prazosin, but not propranolol or L-NAME, to the floor for the fourth ventricle significantly reduced the magnitude of the fundic relaxation elicited by esophageal distension compared with control [yohimbine 56±6%, prazosin 55±18% of control, respectively]. The combination of yohimbine and prazosin produced a further reduction in the magnitude of the RRR [28±9% of control; overall ANOVA F_{4,43} = 12.0; p<0.0001; Dunnett’s post-test p<0.05].

**Effects of microinjection of noradrenergic agonist into DVC**

Microinjections [40nL] of PBS into the DVC did not change fundic tone. In contrast, unilateral microinjections of NE [40nL; 0.3ng total dose] into the same site of the DVC evoked a transient relaxation [Fig. 6]. Paired t-tests on these difference scores indicate that this drop in
gastric tone after NE is highly significant [df=3, t=15.3; p= 0.0006]. As a point of comparison, we re-analyzed the magnitude of the evoked relaxation after NE [or PBS] microinjection in the context of the magnitude of the “normal” RRR [i.e., mild esophageal distension as seen in the previous group of experiments] using a one-way ANOVA followed by Tukey’s post-tests. This re-analysis reveals that, although the relaxation in fundic tone evoked after unilateral NE microinjection is significantly different to that evoked by PBS microinjection [Fig. 6; ANOVA $F_{2,29} = 5.28$; $p = 0.011$; Tukey’s post-test * $p<0.05$], the magnitude of the drop in tone after NE microinjection it is not statistically different to the magnitude of the drop elicited by esophageal distension [i.e., NE vs. RRR].
Discussion

The principal findings of this report are: 1) repetitive esophageal distension activates NST neurons in the central subdivision of the anterior solitary nucleus; 2) the majority of these central NST neurons that were activated by esophageal distension are TH-positive; 3) the gastric relaxation induced by esophageal distension could be modulated [i.e., suppressed] by antagonism of both α−1 and α−2 adrenoreceptors; and 4) unilateral microinjection of NE into the DVC was sufficient to mimic the reflex. Remarkably, esophageal distension had no effect to activate the core of NOS-ir neurons in the NSTc; an area that anatomical studies show receive substantial esophageal afferent input. Given that we did observe scattered NOS-ir neurons in the NST that were also cFos-positive, it is not likely that the lack of cFos label was due to any inherent inability of NST NOS-ir neurons to produce cFos.

Our physiological results paralleled the immunohistochemical observations. Local brainstem application of α-1 or α-2 adrenoceptor [but not beta-adrenoceptor] blockade reduced the magnitude of the reflex. Combined α-1 and α-2 adrenoceptors blockade nearly eliminated the RRR, while blockade of the synthesis of nitric oxide with L-NAME had no effect on the RRR. Lastly, unilateral microinjections of NE into the DVC were sufficient to transiently mimic the reflex drop in fundic tone seen with mild esophageal distension.

The possibility that local noradrenergic inputs to the DMN can influence vagal parasympathetic functions has been appreciated for some time. Based on their observations of the density of TH and dopamine beta-hydroxylase [DβH] terminal fields in the DMN, Kahlia et al., (20) speculated that local adrenergic inputs from the A2 cell group [adjacent to the NSTc] should powerfully modulate vagal autonomic functions. Since there is a nearly 100% overlap of TH with DβH neurons in the rat dorsal vagal complex (37), it is very likely that these localized reflex inputs to the DMN are principally noradrenergic.

There have been relatively few investigations into the physiological impact of NE on DMN neurons and the functions they regulate. Fukuda and colleagues (14), performing “blind” DMN recordings in the in vitro slice preparation [i.e., the cells recorded from had not been previously identified or pre-labeled] showed that most DMN neurons [55%] are inhibited by NE and this primary NE effect on DMN excitability was blocked by the α-2 adrenoceptor antagonist, yohimbine. A smaller number of DMN neurons [30%] are activated by NE and this activation is suppressed by the α-1 adrenoceptor antagonist, prazosin. Fukuda et al also found that beta-adrenergic antagonists had no effect to block NE influences on DMN excitability. Bertolino and colleagues (2) demonstrated further that excitatory glutamatergic inputs to the DMN are inhibited by NE and that this presynaptic effect to inhibit DMN neuronal firing is also blocked by
yohimbine. These earlier neurophysiological results are consistent with the present report. That is, both \(\alpha\)-2 and \(\alpha\)-1 adrenoceptor antagonists [yohimbine and prazosin, respectively] reduced the magnitude of the RRR as compared with control observations in the same animal. \(\alpha\)-2 adrenoceptor antagonism may reduce RRR magnitude by blocking inhibitory effects of NE directly on DMN neurons (14), which normally provide excitatory cholinergic input to the stomach. Further, \(\alpha\)-2 adrenoceptor blockade may remove noradrenergic inhibition of glutamate inputs (2) to the same population of DMN neurons. This dual action of \(\alpha\)-2 adrenoceptor antagonists would disinhibit the activity of the excitatory cholinergic path to the stomach resulting in a further blockade of the RRR. \(\alpha\)-1 antagonism probably blocks the direct postsynaptic effects of NE to activate the NANC component of DMN projections to the stomach. Our present observation that the combination of \(\alpha\)-1 and \(\alpha\)-2 blockade further suppresses the RRR argues for the operation of two, independent, yet parallel, \(\alpha\)-adrenergic circuits in the DVC that are responsible for this reflex. Preliminary in vitro patch-clamp studies on identified gastric projecting neurons in the brainstem slice preparation suggest further that NE can selectively activate or inhibit different populations of DMN neurons subserving different autonomic functions [Travagli, personal communication].

The combined results from a number of reports strongly suggested a role for NSTc-NOS neurons in the parasympathetic control of digestion. Krowicki and colleagues (21) have shown that microinjection of L-arginine [precursor to nitric oxide] significantly decreases intragastric pressure, while microinjection of the NOS inhibitor [L-NAME] increases intragastric pressure. These effects are vagally mediated. Anatomical evidence has shown that NOS neurons in the NSTc receive esophageal afferent input (1). These NOS-ir NSTc neurons certainly project to the NA and are also likely to be a source of nitrergic input to the DMN (1;36). Indeed, our present results clearly show that the DMN is the potential recipient of a large volume of NOS terminal input [Fig. 3E]. Previous studies show that neurons in the NSTc area are activated by esophageal distension(19;23;30). Neurons in this area project throughout the DMN and, therefore, are clearly in position to regulate gastric function as a consequence of these projections. Recordings made from the DMN show that medially located neurons in this nucleus are largely inhibited after esophageal distension while laterally and posteriorly located DMN neurons are excited by esophageal distension. Given the anatomical literature, we had speculated that some of this reflex signal from NSTc to DMN might be carried by NOS-ir NSTc neurons (30).

However, none of these electrophysiological studies (19;23;30) could identify the phenotypes of NSTc neurons; only their location in the NSTc area. So, given the present
anatomical and physiological results, it is possible that neurons recorded in the NSTc area that were responsive to esophageal distension may have been the TH and not the NOS phenotype. To resolve this apparent paradox, it may be necessary to consider the possibility that the NSTc requires a specific pattern of visceral afferent input to activate the NOS components of this nucleus. In a recent review, Goyal and colleagues observed that modest electrical stimulation of the superior laryngeal nerve [SLN; a proximal branch of the afferent vagus] activates neurons in all subdivisions of the solitary nucleus except the core of nucleus centralis (15). This moderate level of afferent stimulation also caused localized gastro-esophageal relaxation, but not esophageal peristalsis. Localized activation of the DMN was observed coincident with these limited effects, however, the nucleus ambiguus [NA] was not activated. In contrast, higher frequency stimulation of the SLN elicited a complete swallowing reflex [including esophageal peristalsis and sphincter relaxation]; this response was accompanied by c-Fos activation of the core of NSTc and the NA.

In addition to patterned afferent modulation of the activity of the NSTc, as described above, it is likely that the NOS core of the NSTc is under the control of CNS inputs. That is, esophageal afferent input may modulate the activity of NSTc-NOS neurons, but a central pattern generator is required to excite them. Such a mechanism was suggested by the pioneering work of Jean [(19) - review]. Jean concluded that the act of swallowing [initiated by the NSTc] required programmed input from other sites in the CNS. However, the "swallowing program" is modified by vagal afferent feedback from the esophagus. These observations, in combination with those we present in this manuscript, suggest that the activation of esophageal afferent pathways sufficient to produce gastric relaxation do not require the activation of the nitrergic core of the NSTc. Noradrenergic neurons in and near the NSTc carry out this particular reflex task. Activation of this nitrergic core of the NSTc is required to generate voluntary swallowing. Though vagal afferents can modulate this motor program, these inputs are not essential for program initiation (15).

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Figure 1. Illustration of cFos-ir in control vs. esophageal distension cases. Rostral-caudal sections are described in terms of distance [in mm] with respect to calamus scriptorium. Scale bar = 500 um.

Bar graph: Overall average of cFos-ir labeled neurons throughout the NST in control vs. esophageal distension or acidification cases. ANOVA $F_{3,33} = 26.0$; $P<0.0001$; Dunnett’s post-test for comparisons against control: ** = $p <0.001$

Figure 2. Distribution of cFos-ir neurons in different subregions of the NST.
A-C. Line drawings of micrographs in Figure 1 showing the scheme used for the identification of different NST divisions for the bar graphs D and E.

D. cFos distribution [cFos-ir cells counted per section] in unstimulated controls. No significant between group differences.

E. cFos distribution in rats receiving esophageal distension stimuli. All subregions of the NST are activated by distension, as seen by the increase in cFos-positive neurons. Within any given rostral-caudal level, the central subregion expressed more cFos [$F_{8,135} = 11.21$, $p = 0.044$; Bonferroni selected comparisons ** = $p<0.001$].
Figure 3. Montage of cFos, TH and NOS immunohistochemical results.

A. Illustration of NOS-ir neurons in the core of the NSTc. Although this area receives esophageal afferent fibers, no NOS-ir neurons here were cFos-positive labeled after repeated esophageal distension. Blue-black = NOS-ir; red-brown = cFos-ir

B. This section was stained for TH and cFos immunoreactivity. Note the high density of TH-ir neurons [arrows] that have been cFos-activated in response to esophageal distension. These cells surround the NOS-ir core of the NSTc demonstrated in A, above. Although additional cFos-positive cells can be seen as brown nuclei in this core region, they did not react to NOS immunostaining. Blue-black = TH-ir; red-brown = cFos-ir

C. Higher magnification image of a cluster of TH–cFos ir neurons in the NSTc.

D. A small but non-significant number of cells in the medial NST demonstrate NOS- cFos ir double label following esophageal distension. Blue-black = NOS-ir; red-brown = cFos-ir

E. Immunohistochemical staining shows that DMN neurons [“vacant” regions in this DIC photomicrograph marked by red asterisks] are surrounded by NOS-ir terminal arborizations. However, our physiological studies did not suggest that NOS plays a role in the RRR [refer to Fig. 5]. Blue-black = NOS-ir fibers

F. TH-ir fibers coursing through the DMN denoted by red arrows. Blue-black = TH-ir fibers

G. NST TH-ir neuron [red] sending an axon [denoted by black arrowheads] to the DMN [methyl green].

H. Low power micrograph of the dorsal medulla showing the TH-ir [red-brown] neurons and the “haze” of fine TH-ir fibers in the DMN.

Scale bars: A, B = 100um; C, D = 20 um; E, F, G = 10um; H = 500um

Figure 4. Esophageal distension causes a significant increase in the number of TH-cFos double immunostained neurons in the central division of the NST. Upper panel: Plots of the average number of TH or NOS neurons per histological section in control vs. esophageal distension conditions. There was no difference in the number of either phenotype in control vs. distension groups. Lower panel: esophageal distension caused a significant increase in the number of TH containing neurons that also demonstrated cFos activation [F 3,40 = 42.0; P <0.0001; selected Bonferroni post-tests ** = p<0.001. There was no significant increase in the number of NOS-ir/cFos ir double labeled neurons.
Figure 5  Esophageal distension in food deprived animals result in a reflexive, receptive relaxation of the fundus [RRR]. **Upper panels:** Sample raw motility records show the effects of receptor blockers on the RRR. Top trace indicates timing and duration of esophageal distension. Amplitude of the control [i.e., pre-drug] RRR is represented in the black traces. The amplitude of the RRR after any specific drug condition [listed at left of motility trace] is represented in gray. **Lower panel:** Bar graph summarizing the effects of adrenoceptor antagonists or NOS synthesis inhibition on the strength of the RRR. Data were normalized to a pre-drug control reflex = to 100%. ANOVA F_{5,44} = 13.0, P < 0.0001; Dunnett’s post-test, *p<0.05, ** p<0.001.

Figure 6  Unilateral injections of NE [40nL volume; 2pmoles = 0.3ng] into the left DVC of food-deprived animals equipped with a gastric pre-load balloon elicits a transient drop in gastric tone not unlike that seen during a RRR induced by mild esophageal distension [refer to Fig.5]. **Upper panel:** demonstration of drop in fundic tone elicited by microinjection of either PBS or NE. Bar on far left of panel is an illustration of the average magnitude of drop in tone evoked during an RRR. [ANOVA F_{2,29} = 5.28; P = 0.011; Tukey post-test * p<0.05] Post-tests indicate that there is no significant difference between the RRR's evoked in the first part of these studies and the drop in tone elicited by unilateral microinjections of NE. **Lower panel:** Microinjection of 1% pontamine blue [40nL volume] verifies location of micropipette in DVC. Scale bar = 200micron. cc = central canal; DMN = dorsal motor nucleus of the vagus; NST = nucleus of the solitary tract.
Figure 1
Figure 2
Figure 4

phenotypes in NSTc

FOS activated phenotypes in NSTc
Figure 5

Esophageal distension
160uL/1 minute

Yohimbine + Prazosin

Propranolol

L-NAME

0.6 volts [fundic strain]

% reflex post-drug (gastric relaxation: post/pre-drug)

L-NAME propranolol yohimbine prazosin yohimbine + prazosin

*  **
Figure 6