Dorsal Motor Nucleus of the Vagus: A site for evoking simultaneous changes in crural diaphragm activity, lower esophageal sphincter pressure and fundus tone

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**Abbreviations:** DMV, dorsal motor nucleus of the vagus; NTS, nucleus tractus solitarius; cs, calamus scriptorius; NO nitric oxide; VIP, vasoactive intestinal polypeptide.

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2. **ABSTRACT**

The sphincter mechanism at the esophagogastric junction includes the smooth muscle of the lower esophagus and the skeletal muscle of the crural diaphragm (CD). The smooth muscle is known to be under the control of the dorsal motor nucleus of the vagus (DMV), while CNS control of the CD is unknown. The main purposes of our study were to determine the CNS site that controls the CD, and whether simultaneous changes in lower esophageal sphincter (LES) pressure and CD activity occur when this site is activated. Experiments were performed on anesthetized male ferrets whose LES pressure, CD activity and fundus tone were monitored. To activate DMV neurons, L-glutamate was microinjected unilaterally into the DMV at three areas: intermediate, rostral and caudal. Stimulation of the intermediate DMV decreased CD activity (-4.8±0.1 bursts/min and -0.3±0.01 mV), and LES pressure (-13.2±2.0 mmHg; n=9). Stimulation of this brain site also produced an increase in fundus tone. Stimulation of the rostral DMV elicited increases in the activity of all three target organs (n=5). Stimulation of the caudal DMV had no effect on the CD but did decrease both LES pressure and fundus tone (n=5). All changes in LES pressure, fundus tone, and some DMV-induced changes in CD activity (i.e. bursts/min) were prevented by ipsilateral vagotomy. Our data indicate that simultaneous changes in activity of the esophagogastric sphincters and the fundus tone occur from the rostral and intermediate areas of the DMV, and that these changes are largely mediated by efferent vagus nerves.
3. INTRODUCTION

The sphincter mechanism at the esophagogastric junction is comprised of both smooth muscle of the lower esophagus and the skeletal muscle of the crural diaphragm (CD) (1, 7, 25, 27, 28). Together, they contribute to the competency of the lower esophageal high pressure zone (26), which serves as an anti-reflux barrier. In reflux disease, the competency of this barrier is compromised by increased occurrence of simultaneous relaxation of the smooth muscle of the lower esophagus and inhibition of the CD (26). To understand this phenomenon, it is necessary to elucidate how these two muscles are controlled. In this regard, it is known that a major source of innervation of the smooth muscle comprising the lower esophagus is the dorsal motor nucleus of the vagus (DMV) (1, 4, 29, 35, 43). However, to our knowledge, no published data are available on the location of a brain site or sites that exert control over the other component of sphincter function, the CD.

In considering the likely site (or sites) in the hindbrain that provides control over the CD, we focused our attention on the DMV. This hindbrain nucleus is a key component of vago-vagal reflexes (42), and activation of a vago-vagal reflex has been shown to inhibit the CD (13). In support of this approach to finding the brain site or sites for CD control, Yates's group (46) reported that injections of the retrograde transneuronal tracer, pseudorabies virus (PRV) into the diaphragm of the ferret (which included both the CD and the costal diaphragm) labeled neurons in the DMV. They interpreted their finding as due to leakage of PRV from the injection site into the peritoneal cavity; however, they did not rule out the possibility that DMV neurons were labeled because of transneuronal retrograde transport from the CD.

Recently, we reported preliminary data that PRV injected into the CD of the ferret retrogradely labels neurons in the DMV (16). Control injections of PRV into the abdominal space around the CD failed to label cells in the DMV (20) suggesting that the projections to the CD are not due to leakage of tracer into the peritoneal cavity. Subsequent experiments with the retrograde monosynaptic tracer, cholera toxin B (CTB), have since confirmed the direct nature of this projection from the DMV to the CD (Sahibzada and colleagues, unpublished data). Blackshaw and colleagues (47, 48) have also presented preliminary data showing that the vagus nerve directly innervates
the CD in the ferret. Following injections of CTB into the CD, they reported retrogradely labeled neurons in the DMV that were immunopositive for choline acetyltransferase.

Based on the above observations, the first aim of our study was to test the hypothesis that excitation of neurons in the DMV would result in changes in the activity of the CD. Additionally, we sought evidence that DMV stimulation would elicit simultaneous changes in both sphincter muscles.

According to Hyland et al. (15), approximately 50% of the DMV neurons that project to the LES in the ferret contain a collateral projection that innervates the fundus. Hence it would be expected that DMV stimulation that affects the LES would also affect the fundus. The influence of the DMV on the fundus has been suggested to be mediated in the rat by changes in vagus efferent activity to the stomach consisting of parallel excitatory and inhibitory non-adrenergic, non-cholinergic (NANC) pathways (14, 23, 33, 34). This NANC pathway is described as comprising of preganglionic DMV neurons synapsing onto nitric oxide releasing enteric neurons (14, 23, 40). We and another group of investigators have not been able to obtain evidence of functional parallel excitatory and inhibitory DMV vagal pathways to the smooth muscle of the stomach in the rat (8, 20). Thus, a second aim of our study was to test the hypothesis that DMV stimulation in the ferret at a site that evokes changes in LES pressure would simultaneously affect fundus tone in at least 50% of the studies, and that the fundus tone effect would not involve nitric transmission as suggested by others in the rat (14, 23).
4. METHODS

All experimental procedures conformed to the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" and were approved by the Georgetown University Institutional Animal Care and Use Committee.

**Animals and Surgical Preparation:** Adult male ferrets (*Mustela putorius furo*) weighing 600-700 grams (Marshall Farms, NY) were housed in controlled conditions at room temperature (22°C) and light (12:12 hour light-dark cycle) with free access to food and water. Before each experiment, food was withheld overnight, whereas water was provided *ad libitum*. Each animal was initially sedated with an intramuscular (IM) injection of ketamine (25 mg/kg). Each animal was then exposed to isoflurane via a nose cone (4% induction; 1.5% maintenance) vaporized with 95% oxygen and 5% CO₂ to produce a surgical level of anesthesia (confirmed by a lack of response to a toe pinch and a lack of corneal reflex). After induction of anesthesia, the nose cone was then switched with an intubation tube subsequent to a tracheotomy.

After induction of anesthesia, the carotid artery and the jugular vein were cannulated for monitoring blood pressure (BP) and for systemic administration of drugs, respectively. The cervical vagus nerve trunks on both sides of the neck were carefully isolated and encircled with a saline soaked suture. Blood pressure was monitored by a pressure transducer that was connected via a bridge amplifier to a data acquisition system (PowerLab; ADI Instruments, Colorado Springs, CO). Body temperature was monitored by a rectal thermometer and maintained at 37 ± 1°C with a thermostatically controlled heating pad (Kent Scientific, Inc.).

A midline incision was made in the abdomen to provide access to the LES, the stomach and the CD. For recording intraluminal LES pressure, a 7-lumen miniaturized manometric sleeve catheter (Dentsleeve, Int. Ltd., Canada) was inserted via the mouth into the LES under visual guidance such that its drainage port extended ~1.5 cm into the proximal stomach. The catheter assembly was 70 cm long with a 3 cm sleeve located 1.5 cm from the tip. The proximal end of the catheter was secured via two small suture loops to the upper incisors to prevent dislodging the catheter due to axial movement (catheter location was also confirmed *post mortem*). The catheter was...
connected to a high pressure, low compliance Arndorfer system (3), which allowed the perfusion of distilled, bubble-free water at a rate of 0.36 ml/min. A "zero" pressure was obtained by measuring the pressure through the sleeve outside the animal with water flowing at an identical rate and subtracted from the final recorded pressure. Barrier pressure was calculated as sleeve pressure minus the gastric pressure measured from the distal port. To record optimum pressure, we adjust the placement of the sleeve in the LES at the beginning of each experiment via the pull through method (19). Thus the midpoint of the sleeve lies in the LES at a distance of ~18-20 cm from the upper incisors and the distal port lies in the fundus region of the stomach ~20.5-22.5 cm from the upper incisors.

Following placement of the catheter, a gastric drain was inserted through a purse-string suture via the duodenum. Next, to record gastric tone and motility, a calibrated 2x2 mm strain gauge force transducer (RBI Products, Wisconsin) was diagonally oriented and then sewn onto the fundus at a tension of ~5 g. In some experimental tracings, evidence of phasic activity was present in the fundus trace, presumably due to the inadvertent inclusion of the corpus muscle in the placement of the force transducer (e.g. Figure 1). However, since the focus of this study was on fundus tone, this was measured using the trough values of the strain gauge signal. To record electromyographic (EMG) activity from the CD, hooked bipolar platinum electrodes were attached in a manner similar to that described previously by us (36, 44). These electrodes were in turn coupled to a P511 AC amplifier (Grass Instruments, Astro-Med). The EMG signal was recorded using a low-pass cut-off frequency of 200 Hz and integrated off-line using a 100-ms time window. The abdominal cavity was then sutured closed and the animal placed in a stereotaxic frame (Kopf, Tujunga, CA).

**Stereotaxic Procedure:** The dorsal medulla was exposed by way of a small occipital craniotomy. The underlying muscles, dura and the cerebellum were retracted. Prior to stereotaxic surgery, we routinely administer dexamethasone 4.2-6.6 mg/kg, SC, as a preventive measure against brain edema. The stereotaxic coordinates were calculated to place the micropipette tip into 3 distinct areas of the DMV: Caudal DMV, AP = -0.5mm to CS, ML = 0.4 mm and DV= -0.6mm; Intermediate DMV, AP = 0.5-
0.7mm rostral to CS, ML = 0.5mm and DV = -0.6mm; **Rostral DMV**, AP = 1.5-2.1mm rostral to CS, ML = 0.6mm and DV = -0.7mm.

Microinjections into the DMV areas were performed via a double-barreled glass micropipette (tip Ø = 60-70µm; FHC, New Brunswick, ME) connected to a 5 ml syringe via a PE 50 tubing. Drugs were loaded and ejected from each barrel using negative or positive pressure, respectively. All drug solutions were dissolved in 0.9% saline (pH 7.2-7.4). Injections were administered manually within 5 to 10 sec. Blood pressure (BP), gastric tone and LES pressure, together with the EMG signal from the CD, were acquired and analyzed via a PowerLab data acquisition system (ADI Instruments, Inc.) connected to a Macintosh G4 computer (Apple, Inc.). Heart rate was calculated from the blood pressure trace.

**Histology:** Each animal was euthanized with an overdose of pentobarbital. The brain was removed and fixed in a solution of 4% paraformaldehyde and 20% sucrose for at least 24 hours. Subsequently, the brainstem was cut into 50µm-thick coronal serial slices and stained with 0.5% neutral red. Using both brightfield and darkfield microscopy, the location of the micropipette tip was determined. All microinjection sites were photographed and camera lucida drawings were made of the brainstem sections that contained the pipette tracks. Since a brain atlas of the ferret was not available to us, we used the rat atlas of Paxinos and Watson (31) as a guide to identify brainstem structures. Although the microinjection sites for all studies were documented, some are not shown because of space limitations. This information is available on request.

**General Experimental Protocol:** At least 10 min of stable baseline recording from LES, CD, BP, and fundus were acquired prior to any experimental manipulation. The micropipette was then inserted unilaterally into the medulla (at a 30 degree angle from the perpendicular) using our aforementioned empirically derived stereotaxic coordinates.

The animal was then allowed an additional 1 to 2 min to stabilize at which point L-glutamate (500 pmol/30nL) was microinjected. L-glutamate is known to cause an increase in fundus tone when microinjected into the DMV (8, 10, 11), and is prevented by ipsilateral vagotomy (10). In contrast, L-glutamate injected into the NTS has been shown to decrease fundus tone that is abolished only by bilateral vagotomy (8, 11).
Based on the effects on fundus tone, we were able to determine which nucleus we were activating with our L-glutamate microinjections and adjust our dorsoventral coordinates accordingly so that the elicited responses were entirely from the DMV. All successful L-glutamate microinjections were repeated at least once after a 10 min interval to demonstrate the reproducibility of the response at a time of full recovery. The dose of 500 pmol/30nL was taken from the dose-response data of Ferreira (9), who reports its location towards the top of the curve, but not lying in the plateau of the response. In addition, this dose is lower than the dose range (>5nmol) shown to exert a depolarization induced blockade of neurons in the immediate vicinity of the pipette tip (24). Furthermore, this dose is in the same range as two other studies that have examined the effects of L-glutamate-induced excitation in the CNS on gastric motility (9, 38). Finally, it is several fold less than the dose used by Abrahams, et al. (1) in the ferret.

After establishing the effects of L-glutamate microinjection into the DMV on GI responses, the effects of other experimental manipulations were studied on these responses.

Experimental Protocols: For studying the effect of ipsilateral vagotomy on the L-glutamate response, at least two responses to microinjection of L-glutamate (500 pmol/30nL) into the DMV were obtained. Next, the ipsilateral cervical vagus was severed. A stabilization period of 10 to 15 minutes ensued before L-glutamate microinjection was repeated into the DMV. The effects of ipsilateral vagotomy on DMV- and NTS-induced responses of the GI tract have been extensively studied by us (11). Ipsilateral vagotomy was performed at the end of each experiment in all protocols to ensure the location of the pipette and to demonstrate the vagal nature of our elicited responses.

For studying the effect of L-NAME on the L-glutamate response, two reproducible responses to L-glutamate were first obtained. Next, L-NAME (10mg/kg, IV), a nitric oxide synthase inhibitor, was administered to assess the contribution of nitric oxide (NO) on the target tissues vis-à-vis the non-adrenergic, non-cholinergic (NANC) vagal pathway. The dose used is one that has been used by others (14, 23, 40, 41) and us (8) to block vagal-induced relaxatory responses from the GI tract. After an interval of 5
to 10 min following L-NAME administration, the L-glutamate microinjection was repeated. Similar to the L-NAME protocol, the VIP receptor antagonist, (D-P-C1-Phe$^6$,Leu$^{17}$)-VIP (VIPa), was administered (0.35mg/kg, IV) 5 to 10 minutes prior to L-glutamate microinjection into the DMV. The dose used of VIPa is comparable with that shown to be effective for blocking VIP related GI effects (6).

For studying the effects of both L-NAME and VIPa on the L-glutamate response, two reproducible responses to L-glutamate were first obtained. Next, each animal received a sequential IV administration of either L-NAME then VIPa or VIPa then L-NAME. Five to 10 minutes later, L-glutamate microinjection was made into the DMV and thereon at intervals of 10 min until signs of recovery of the responses were observed (typically within 30-40 minutes).

Finally, for studying the effects of atropine methyl bromide on the L-glutamate response, the drug was administered IV in a dose of 0.1mg/kg. The dose of atropine was chosen based on its ability to fully block muscarinic receptors in the periphery (12). Atropine methyl bromide was selected because it is a permanently charged molecule and does not cross the blood brain barrier.

**Data Analysis:** Data were analyzed using Chart software (ADInstruments, Inc.). Values for each experimental endpoint were divided into 2 values, "baseline" and "during L-glut". "Baseline" values for the LES pressure were defined to be the average barrier pressure of the LES over 2 min recording period prior to microinjection of L-glutamate. The "during L-glut" value was defined to be the largest magnitude of change in the barrier LES pressure within the time course of action of L-glutamate. The time course of the response was divided into two values: the "time to peak" and the "duration". The "time to peak" was defined as the time (in sec) between the initiation of the L-glutamate-induced response (typically within the time during which L-glutamate was being administered) and the point where the "during L-glut" value was recorded. The "duration" was defined as the time (in sec) between the initiation of the L-glutamate response and the point where the LES pressure returned to equivalent "baseline" value. Since in all animals, L-glutamate was administered more than once into the DMV prior to any experimental manipulation, both "baseline" values were averaged.
Values for the CD response were divided into two endpoints: "amplitude" and "frequency", which were then analyzed in the same way as described for the LES pressure. Values for the fundus tone were recorded in a similar manner to the LES pressure.

Data were analyzed for significance via a one-sample t-test and a paired t-test where appropriate. Analysis of variance (ANOVA) was done when more than one experimental intervention was utilized (i.e., both L-NAME and VIPα given sequentially) followed by the post hoc analysis test of Newman-Keuls. All data are presented as mean ± SEM. In all cases, p<0.05 was the criterion used to determine statistical significance.

**Drugs:** Isoflurane was purchased from Fisher Scientific (Pittsburgh, PA) and ketamine was obtained from Bedford Labs. (Bedford, OH); dexamethasone sodium phosphate was procured from American Regent Laboratories (Shirley, NY); L-glutamate, L-NAME and atropine methyl bromide were obtained from Sigma-Aldrich Company (St. Louis, MO); and (D-p-Cl-Phe⁶, Leu¹⁷)-VIP was purchased from Tocris (Ellisville, MO).
5. RESULTS

**Effects produced by microinjection of L-glutamate into the intermediate area of the DMV:** L-glutamate (500 pmol/30 nl) was microinjected unilaterally into the intermediate area of the DMV of 9 ferrets. End points measured were crural diaphragm (CD) EMG (amplitude and frequency), intraluminal pressure of the lower esophageal sphincter (LES), fundus tone, mean arterial blood pressure and heart rate. L-glutamate produced a decrease in EMG activity of the CD (both amplitude and frequency decreased) as well as a decrease in LES pressure. In addition, L-glutamate microinjection produced an increase in the tone of the fundus. These results appear in Table 1. L-glutamate microinjection did not affect mean blood pressure or heart rate (data not shown).

An example of the effects of L-glutamate microinjection appears in Figure 1. The location of the microinjection site for this experiment is shown (part A) as well as microinjection sites for all 9 ferrets studied (part C). There are decreases in the EMG activity and LES pressure, and an increase in the tone of the fundus. The time to peak of all of these changes ranged from approximately 15 seconds (CD) to 40 seconds (LES) and these changes lasted about 30 seconds for the CD and 4 minutes for the LES and fundus.

In the 9 animals reported in Table 1, the effects of ipsilateral vagotomy on all the end-points measured were evaluated. The data indicate that ipsilateral vagotomy prevented unilateral microinjection of L-glutamate from decreasing EMG frequency of the CD and LES pressure. Ipsilateral vagotomy also prevented unilateral microinjection of L-glutamate from increasing the tone of the fundus. On the other hand, ipsilateral vagotomy did not prevent unilateral microinjection of L-glutamate from decreasing EMG amplitude of the CD. The experimental traces in the right panel of Figure 1C illustrate the effects of ipsilateral vagotomy.

In addition to the 9 experiments described above, we performed 2 types of control experiments. In 4 animals, we purposely microinjected L-glutamate unilaterally into medullary areas just outside the DMV. In an additional 3 animals, we microinjected saline vehicle (pH 7.2-7.4; equivalent to the pH of the L-glutamate solution) unilaterally into the DMV. In both control groups, no significant effects were observed on EMG
activity of the CD, on LES pressure, and on fundus tone following microinjection. The microinjection sites for the control experiments where we purposely microinjected L-glutamate just outside the DMV are shown in Figure 1B (open squares). As can be noted, two of the sites outside the DMV were in the tractus solitarius (NTS) whereas the other two were in the hypoglossal nucleus. We have previously reported that NTS stimulation effects the esophagogastric sphincters (37), however, to do so the micropipette needs to be in the medial NTS. Our present control injections into the NTS were in the dorsomedial part of this nucleus.

The peripheral neurotransmitter(s) responsible for relaxation of the LES sphincter was (were) sought by determining whether an inhibitor of nitric oxide synthase, L-NAME (10mg/kg, IV), would alter the response. The magnitude of the decrease in LES pressure evoked by L-glutamate microinjection was unchanged by L-NAME pretreatment (Figure 2A). L-NAME pretreatment did change the time course of the response. Both the time to reach the nadir of the response and the duration of the response were altered (Table 2). Prior to L-NAME, the time to reach the nadir was 16.4±1.9 sec. After L-NAME, the time to reach nadir was significantly increased (Table 2). L-NAME pretreatment had no effect on either the magnitude of changes in EMG activity of the CD (Figure 2A), increase in tone of the fundus (data not shown), or their time courses (data not shown). L-NAME pretreatment per se produced an initial increase in the baseline values for LES pressure (+4.3±0.9 mmHg; p<0.05); this increase was observed 2 minutes after L-NAME administration but was not maintained.

Due to the lack of effect of L-NAME on the magnitude of the decrease in LES pressure, we next determined the effect of VIPa on this response. Pretreatment with VIPa (0.35mg/kg, IV) did not reduce the magnitude of the LES relaxation in response to L-glutamate microinjection into the DMV (Figure 2B) but did significantly shorten the time it took the response to reach nadir (Table 2). After VIPa administration the duration of the response was significantly reduced (Table 2). VIPa pretreatment had no effect on either the magnitude of changes in EMG activity of the CD (Figure 2B), increase in the tone of the fundus (data not shown), or their time courses (data not shown). Finally, VIPa pretreatment per se produced no significant changes in the baseline values for LES pressure (Figure 2B).
Since neither L-NAME nor VIPa reduced the magnitude of the decrease in LES pressure evoked by microinjection of L-glutamate, we next tested the 2 drugs in combination on the response. These data are tabulated in Figure 2A and 2B, and indicate that pretreatment with the combination of L-NAME and VIPa prevents the decrease in LES pressure evoked by L-glutamate microinjection into the intermediate area of the DMV.

Similar to pretreatments with L-NAME and VIPa alone, the combination had no effect on either the magnitude of changes in EMG activity of the CD or tone of the fundus (data not shown) that were evoked by L-glutamate microinjection, or their time courses (data not shown). Additionally, the combination produced no significant changes in baseline values for LES pressure (Figures 2A and 2B).

We also evaluated the effects of atropine methyl bromide on L-glutamate-induced excitation of the intermediate DMV (Table 3). Atropine methyl bromide blocked the increase in fundus tone, but not the decreases in LES pressure and EMG activity (i.e. decrease in frequency of bursts) of the CD. However, atropine methyl bromide did appear to prevent the L-glutamate-induced decrease in the amplitude of the CD. Atropine methyl bromide per se produced an initial decrease in the baseline values for LES pressure (-3.5+0.9 mmHg; p<0.05); this decrease was noted 2 minutes after atropine methyl bromide was administered but was not maintained.

**Effects produced by microinjection of L-glutamate into the rostral area of the DMV:** L-glutamate was microinjected into the rostral area of the DMV of 5 ferrets, and produced an increase in frequency of the EMG activity of the CD, LES pressure and tone of the fundus (Table 1). L-glutamate microinjections did not affect mean blood pressure or heart rate (data not shown).

An example of the effects of L-glutamate microinjection appears in Figure 3C. There was an increase in the EMG burst frequency of the CD. There was also an increase in the LES pressure and in the fundus tone. The time to peak for the sphincter changes was approximately 10 to 25 seconds and lasted an additional 30 to 60 seconds. The time to peak effect for the fundus was about 20 seconds and the response duration was approximately 1 minute. The site of microinjection of L-glutamate for this particular experiment appears in Figure 3A. Microinjection sites for all
experiments are depicted in Figure 3B. In each case, the micropipette tip was located in the rostral area of the DMV. The filled squares represent sites where L-glutamate was administered and results similar to Figure 3C were obtained.

The effects of ipsilateral vagotomy on the L-glutamate-induced changes in the experimental end points were also studied. L-glutamate microinjection following ipsilateral vagotomy was unable to produce any effect on the EMG frequency of the CD, on the LES pressure, or on the fundus tone (Table 1; Figure 3C, right panel). Control experiments were also performed. Neither saline vehicle microinjected into the DMV (n=3) nor L-glutamate microinjected outside the DMV (n=4) had any significant effect. Microinjection sites for these experiments are depicted by open squares in Figure 3B.

We also evaluated the effects of atropine methyl bromide on L-glutamate-induced excitation of the rostral DMV (Table 3). Atropine methyl bromide blocked the effects of L-glutamate on both the LES pressure and the fundus tone. However, the increase in the EMG frequency of the CD induced by L-glutamate microinjection was not significantly affected. Atropine methyl bromide per se produced an initial decrease in the baseline values for LES pressure (-3.6±0.2 mmHg; p<0.05). This decrease was noted approximately 2 minutes after the drug was administered, but was not maintained.

**Effects produced by microinjection of L-glutamate into the caudal area of the DMV:** L-glutamate was microinjected into the caudal area of the DMV of 5 ferrets and produced a decrease in LES pressure and fundus tone. There was no significant effect on the EMG activity of the CD. These results appear in Table 1. In addition, L-glutamate microinjection did not affect mean blood pressure or heart rate (data not shown).

An example of the effects of L-glutamate microinjection appears in Figure 4B. Following L-glutamate microinjection into the caudal DMV, there were significant decreases in the LES pressure and fundus tone. The time to peak effect of the LES decrease was approximately 10 seconds and about 90 seconds for the fundus; the decrease lasted about 1.5 minutes for the LES and 2.5 minutes for the fundus. In all 5 animals, the effects of ipsilateral vagotomy on the L-glutamate-induced changes were also studied. As can be seen in Figure 4B (and Table 1), L-glutamate microinjection
following ipsilateral vagotomy was unable to produce any effect on LES pressure or on fundus tone. The location of the microinjection sites for all 5 animals are shown as Figure 4A (filled squares). In 2 animals, we microinjected L-glutamate into medullary areas just outside the DMV (Figure 4A open squares). No significant effects were found on LES pressure, EMG activity of the CD, and fundus tone.

The peripheral neurotransmitter(s) responsible for relaxation of the LES and fundus was (were) sought by determining whether L-NAME (10 mg/kg, IV) would alter the responses. L-glutamate-induced changes in LES pressure and fundus tone were not significantly (p>0.05) altered (Figure 5A). L-NAME per se produced a significant increase in LES pressure (+2.9±0.4 mmHg; p<0.05) but the increase was not maintained.

We next determined the effect of the VIPa on the DMV-evoked decrease in LES pressure (and decrease in fundus tone) in 3 ferrets pretreated with L-NAME. Treatment with VIPa (0.35mg/kg) following L-NAME administration did not alter the effect of L-glutamate microinjection on either LES pressure or fundus tone (Figure 5B).

The effects of IV administered atropine methyl bromide were also evaluated on L-glutamate-induced decreases in LES pressure and fundus tone evoked from the caudal DMV. Atropine methyl bromide treatment (n=2) had no effect on the L-glutamate-induced decrease in LES pressure (Table 3). Its effect on L-glutamate-induced decrease in fundus tone was difficult to interpret. The decrease in fundus tone appeared to be attenuated but this might have been due to the change in the baseline fundus tone. Fundus tone appeared to be decreased after atropine methyl bromide in these animals and this lower baseline may have affected the magnitude of the L-glutamate-induced decrease in this tone (Table 3). Finally, atropine methyl bromide per se appeared to decrease the baseline LES pressure (-3.4±0.9 mmHg) but was not statistically significant, which was most likely due to the small number of animals studied (n=2).

Our lack of success in counteracting L-glutamate-induced decrease in LES pressure and fundus tone with L-NAME, L-NAME plus VIPa, and atropine methyl bromide led us to test whether combinations of L-NAME plus atropine methyl bromide (n=3) and L-NAME plus VIPa plus atropine methyl bromide (n=2) would antagonize
these effects. None of the drug combinations used as pretreatments altered these effects elicited from the caudal DMV.
6. DISCUSSION

In this study, we showed that excitation of rostral and intermediate but not caudal DMV produces significant changes in the activity of the CD. These changes were accompanied with alterations in the LES tone. A schematic depicting the functional neurocircuitry associated with these changes is shown in Figure 6. Focusing on the intermediate DMV, L-glutamate-induced stimulation produced simultaneous decreases in CD activity and in LES pressure that was vagally mediated. Furthermore, our data indicate that after L-NAME treatment, the early drop in LES pressure seen following intermediate DMV stimulation is absent; instead, it now takes longer for LES pressure to reach its nadir. These data point to NO as primarily mediating the decrease in LES pressure that occurs over the first 10-30 seconds of the response. The later decrease in LES pressure appears to be mediated mainly by VIP. Neither L-NAME or VIPa treatment had any effect on DMV-induced changes in CD activity.

The novel effect of the vagus on CD burst frequency, which was abolished by ipsilateral vagotomy, was not mediated via muscarinic receptors since IV atropine methyl bromide pretreatment did not modify the response. Hence, by exclusion, we suggest that the response was mediated by nicotinic receptors located on the CD muscle. The ineffectiveness of atropine in modifying the DMV-induced change in the burst frequency of the CD also suggests that these medullary vagal motoneurons directly innervate the CD. Precedence for this exists for the ciliary muscle of the eye. The ciliary muscle receives a significant part of the parasympathetic innervation directly from midbrain neurons that bypass the ciliary ganglion (17, 45).

Using two recombinants of PRV (PRV-152 and PRV-BaBlu) that were injected into the LES and the CD of the ferret, we recently reported the presence of retrogradely labeled neurons in the DMV after 5 days, some of which were double-labeled (16). The direct nature of these projections to the CD and LES was confirmed with the retrograde monosynaptic tracer, CTB (unpublished data). Control injections of the neuronal tracers into the abdominal space around the CD or LES failed to label cells in the DMV after a comparable time-period, thus demonstrating that the projections to the CD were not due to leakage of the virus into the LES or the surrounding stomach tissue.
How DMV stimulation with L-glutamate results in changes in the frequency of CD bursts per minute is puzzling. We suggest that some DMV neurons are conditional pacemakers and that their excitation with L-glutamate could directly increase their rate of burst discharge or indirectly (via release of GABA from nerve terminals in the DMV) decrease their rate of burst discharge. There is evidence that a receptor for L-glutamate is present at GABAergic synapses and that its stimulation will facilitate GABA release (18). It should be noted that the DMV to CD pathway was not responsible for the EMG amplitude decrease observed with intermediate DMV stimulation due to the lack of effect of ipsilateral vagotomy on the response. Instead, we assume that the amplitude change was mediated via the phrenic motor nucleus.

Another new finding is drawn from data obtained with L-glutamate induced excitation of intermediate DMV and the control of the duration of LES relaxation. That control involves the release of VIP at the level of the LES smooth muscle. Prolongation of the duration of LES relaxation was apparent when we tested the effect of intermediate DMV stimulation after pretreating ferrets with the nitric oxide synthase inhibitor, L-NAME. Without NO release, LES relaxation (as reflected by the decrease in pressure) was longer. Conversely, without VIP acting at the LES smooth muscle the duration of LES relaxation was reduced.

Abrahams et al. (1) reported that L-glutamate microinjected into the intermediate and caudal areas of the DMV relaxed the LES while L-glutamate microinjected into the rostral DMV contracted the LES. However, Abrahams et al. (1) did not test vagotomy on the LES contraction elicited from the rostral site. Hence, whether or not the response was mediated by the vagus was not addressed. In addition, they used bilateral vagotomy instead of ipsilateral vagotomy to determine the role of the vagus in the responses evoked from the intermediate and caudal areas of the DMV. The problem with bilateral vagotomy vs. ipsilateral vagotomy, as mentioned above, is that it will not distinguish between effects elicited from the DMV and from the NTS (8). Furthermore, no clear distinction was made between the mechanisms for LES relaxation evoked from the caudal area versus the LES relaxation evoked from the intermediate area. Thus from their study, the profile of LES effects from both areas is similar. In our study, data obtained with L-NAME plus VIP receptor antagonist
separated the responses from the 2 DMV areas. LES relaxation produced from the intermediate DMV was mediated by both NO and VIP, while LES relaxation produced from the caudal DMV was not mediated by NO and VIP. Thus data from Abrahams et al. (1) suggest two separate populations of DMV neurons (caudal/intermediate population and rostral population) while our data suggest at least three separate populations (caudal, intermediate and rostral populations).

Most of the published information on extrinsic neural control of the LES relates to the effects of electrical stimulation of peripheral autonomic nerves such as the cervical vagus nerve (e.g. Blackshaw, et al. (5)). With the new information we now have about stimulating the cells of origin of these cervical vagal fibers, we conclude that stimulation of efferent fibers of the cervical vagus nerves should influence the LES in a way that would resemble the effects of simultaneous activation of all 3 divisions of the DMV. This appears to be the case based on results reported by Blackshaw and colleagues (5). Electrical stimulation of the peripheral cut end of either the right or left cervical vagus nerve of the ferret produced a triphasic response in LES pressure, namely, a brief decrease during stimulation, followed by a brief increase upon cessation of stimulation, and finally a prolonged decrease that followed the brief increase in pressure. The first phase of LES relaxation appeared to be mediated by NO release and based on the findings of the present study, is presumably due to activation of efferent vagal fibers originating from the intermediate division of the DMV. The second phase of LES contraction was blocked by atropine and may be due to activation of efferent vagal fibers originating from the rostral division of the DMV. The third phase (late inhibitory response) was not significantly altered by inhibiting nitric oxide synthase and therefore was considered not to be mediated by NO. This pharmacological profile of the third phase response fits with our data obtained by stimulating the caudal division of the DMV. Data obtained with L-glutamate-induced activation of each of the three divisions of the DMV and electrical stimulation of the cervical vagus nerve would differ of course because the former would evoke selective changes in LES pressure while the latter would evoke more complex changes in LES pressure. An interesting question posed by comparing DMV stimulation with cervical vagus stimulation is whether it (i.e., cervical vagus stimulation) would alter the EMG activity of the CD. When vagal
stimulation was tested, no effect on CD was observed (2). This is consistent with our data in that EMG amplitude of the CD is not mediated by the efferent vagus nerve, and that control of the frequency of EMG bursts of activity requires intervention at the level of the pacemaking neurons in the DMV.

One of the reasons for measuring fundus tone was to determine whether a site exists in the DMV that upon stimulation would simultaneously affect both the LES pressure and fundus tone. Since extensive co-innervation of both the LES and fundus has been reported in the ferret (15), our expectation was that a positive result would be obtained. Indeed, excitation of all three areas of the DMV produced simultaneous changes in both LES pressure and fundus tone (Table 4). The other reason for measuring fundus tone in the ferret was to assess whether the DMV pathway that influences the fundus contains nitric oxide-releasing enteric neurons as proposed by others in the rat (14, 23, 40). Excitation of DMV neurons in the intermediate and rostral areas of the nucleus in the ferret only increased fundus tone, which was always prevented by ipsilateral vagotomy (indicative of DMV stimulation) and IV atropine methyl bromide (indicative of enteric neurons releasing acetylcholine) (Table 4). We did not observe any evidence for activation of a NANC pathway to the fundus with stimulation of the intermediate DMV, even though the effects produced on the LES were always inhibitory through activation of a NANC pathway. These results obtained with strain gauge recordings from the fundus of the ferret are similar to our recently reported findings in the rat (8) using an intragastric balloon.

In the present study in the ferret, we did obtain evidence for activation of the NANC pathway to the fundus with stimulation of the caudal DMV. However, the decrease in fundus tone noted was not mediated by nitric oxide (or VIP), as blockade of nitric oxide synthesis and VIP receptors did not prevent this decrease. These results obtained in the ferret are similar to those obtained in the rat (8). In this species, stimulation of the caudal DMV results in a decrease in intragastric pressure, which is blocked by ipsilateral vagotomy but unaffected by IV L-NAME. These results from the rat indicate that the functional inhibitory pathway arising from the caudal DMV to the gastric smooth muscle does not employ nitric oxide as a neurotransmitter. This is contrary to some studies (14, 21, 23, 40) that report the existence of a functional DMV-
NANC pathway that utilizes nitric oxide. Details of these experiments and discussions relating to resolving differences in results can be found in the Cruz et al. (8) paper.
7. PERSPECTIVES AND SIGNIFICANCE

The DMV contains primarily vagal preganglionic neurons that exert control over the lower esophageal sphincter (35), gastric motility (30), gastric secretion (39), pancreatic secretion (22) and liver gluconeogenesis (32). Data of the present study indicate that the DMV may also exert control over the CD. Burst frequency of the CD was in part controlled by vagal efferent neurons that appeared to bypass the parasympathetic ganglia and directly innervate the CD. Since stimulation of a large portion of the DMV produces simultaneous changes in the two muscles that comprise the sphincter mechanism at the esophagogastric junction, we propose that activity in this hindbrain nucleus is important for maintaining an anti-reflux barrier.

While the CD was affected by stimulating 2 of the 3 areas of the DMV (intermediate and rostral areas), the LES was affected by stimulating all three areas of the nucleus. Studies of the intermediate DMV revealed that stimulation evoked a decrease in LES pressure in 2 phases: nitric oxide release at the level of the LES was responsible for the early phase, and VIP release was responsible for the later phase. The requirement for nitric oxide in the early phase (first 10 to 30 seconds of the response) of LES relaxation fits with the conclusion that the postsynaptic neurotransmitter for relaxation of the internal sphincter is NO (26). Thus the part of the DMV involved in physiological changes in LES tone is likely the intermediate area.

Changes in LES pressure produced by stimulation of the rostral and caudal areas of the DMV did not involve NO and VIP release. The rostral area induced effect was mediated by acetylcholine and the caudal area was mediated by a yet to be identified inhibitory neurotransmitter.

As mentioned above, while we were able to demonstrate a NANC mediated response from the intermediate DMV on the LES, an associated effect on the fundus could not be determined. Instead, an excitatory effect was apparent that was of cholinergic origin. This is in contrast to some studies in the rat that report of an inhibitory NANC pathway to the fundus from the intermediate DMV (34). Altogether, these observations suggest that a DMV-NANC pathway from the intermediate DMV is important for sphincter control but not for gastric smooth muscle control.
The larger question of why there are 3 separate pools of DMV neurons exerting control over the LES needs to be addressed. Recognition of these 3 populations may be important in trying to understand vago-vagal reflex control of the LES, possible pathophysiological processes leading to sphincter disorders such as acid reflux and achalasia, and a new basis for developing drugs for therapy of these sphincter disorders.


44. **Wasserman AM, Sahibzada N, Hernandez YM, and Gillis RA.** Specific subnuclei of the nucleus tractus solitarius play a role in determining the duration of inspiration in the rat. *Brain Res* 880: 118-130, 2000.


8. FIGURES AND FIGURE LEGENDS

**Figure 1:** The effects of microinjection of L-glutamate (GLU; 500 pmol/30nl) into the intermediate DMV.  (A) Photomicrograph of a representative pipette track in the DMV.  (B) Camera lucida drawings of pipette locations where GLU either elicited a response (filled squares) or had no effect (open squares).  (C) Chart recording showing GLU induced activity from the DMV (vertical dashed lines represent time of drug injection).  (1) GLU decreased CD activity, LES pressure and increased fundus tone.  Ipsilateral vagotomy (2) prevented most of the effects of GLU.  (The effect that remains, although difficult to observe in this experiment is that GLU after vagotomy still decreases the amplitude of the CD).  Distances in B are in reference to calamus scriptorius.  **Abbrev.** XII, hypoglossal nuc.; AP, area postrema; CC, central canal; DMV, dorsal motor nucleus; NTS, tractus solitarius nucleus.  CD traces at the bottom of the figure were taken at an expanded time scale to show the effect of DMV stimulation on burst frequency.

**Figure 2:** Pharmacology of the L-glutamate-induced decreases in CD activity and LES pressure evoked from the intermediate part of the DMV.  Part A (summarized data from 4 ferrets) shows that neither L-NAME (10mg/kg, IV) alone nor L-NAME followed by VIPa (0.35mg/kg, IV) exhibited a significant effect on the decreases in CD activity.  Part A does show that while L-NAME does not have a significant effect, the combination of L-NAME plus VIPa does prevent L-glutamate-induced decrease in LES pressure.  Part B (summarized data from an additional 4 ferrets) shows that neither VIPa alone nor VIPa followed by L-NAME exhibited a significant effect on the decreases in CD activity.  Part B does show that while VIPa alone does not have a significant effect on the decrease in LES pressure, the combination of VIPa plus L-NAME does prevent L-glutamate-induced decrease in LES pressure.  **Abbrev.** CD, crural diaphragm; LES, lower esophageal sphincter; and VIPa, (D-p-Cl-Phe6, Leu17)-VIP.  Data are expressed as mean±S.E.M.  The numbers at the base of each histogram indicate the baseline value for each measurement prior to the pharmacological tests.  *One-sample t-test; **ANOVA followed by the post hoc analysis test of Newman-Keuls (significance set at p<0.05, two-sided test).
Figure 3: The effects of microinjection of L-glutamate (GLU; 500 pmol/30nl) into the rostral DMV. (A) Photomicrograph of a representative pipette track in the DMV. (B) Camera lucida drawings of pipette locations where GLU either elicited a response (filled squares) or had no effect (open squares). (C) Chart recording showing GLU induced activity from the DMV (vertical dashed lines represent time of drug injection). (1) GLU increased the burst frequency of the CD, LES pressure and fundus tone. Ipsilateral vagotomy (2) prevented the effects of GLU. Distances in B are in reference to calamus scriptorius. **Abbrev.** XII, hypoglossal nuc.; AP, area postrema; CC, central canal; DMV, dorsal motor nucleus; NTS, tractus solitarius nucleus.

Figure 4: Microinjection of glutamate (GLU 500 pmol/30nl) into the caudal DMV. (A) Camera lucida drawings of pipette locations where GLU either elicited a response (filled squares) or did not (open squares). (B) A representative experiment showing the effect of microinjection of GLU into the DMV on fundus, LES and crural diaphragm (1). Effects noted on LES pressure and fundus tone were blocked by ipsilateral vagotomy (2). Distances in A are in reference to CS. **Abbrev.** XII, hypoglossal nuc.; AP, area postrema; CC, central canal; DMV, dorsal motor nucleus; NTS, tractus solitarius nuc.

Figure 5: Pharmacology of the L-glutamate-induced decreases in LES pressure and fundus tone evoked from the caudal part of the DMV. Part A (summarized data from 7 ferrets) shows that L-NAME (10mg/kg, IV) did not exhibit a significant effect on the decreases in LES pressure and fundus tone. Part B (summarized data from 3 ferrets) shows that the L-NAME followed by VIPa (0.35 mg/kg, IV) does not exhibit a significant effect on the decreases in LES pressure and fundus tone. **Abbrev.** LES, lower esophageal sphincter; VIPa, (D-p-Cl-Phe⁶, Leu¹⁷)-VIP. Data are expressed as mean±S.E.M. The numbers at the base of each histogram indicate the baseline values for each measure prior to the pharmacological test. *One sample t-test (significance set at p<0.05, two sided test).

Figure 6: A summary diagram of efferent neurocircuitry in the dorsal medulla that controls both the internal and the external sphincters. Activation of the intermediate DMV produces simultaneous inhibition of both sphincters. Initially, the relaxation of the
internal sphincter (i.e., LES) is largely due to the intermediate DMV preganglionic vagal fibers activating a NO releasing enteric neuron. Once LES relaxation is underway, the same vagal input can cause VIP release that can potentially extend the duration of the LES relaxation. Simultaneously, inhibition of the external sphincter occurs due in part to activation of a DMV pathway innervating the CD, and in part to activation of a DMV pathway involving the phrenic motor nucleus. Moreover, an increase in fundus tone occurs that is due to excitation of a cholinergic excitatory vagal pathway to this part of the stomach. In the caudal region of the DMV, activation of neurons result in decreases in LES pressure and fundus tone. However, the nature of the peripheral neurotransmitter(s) mediating these responses is not clear. Excitation of neurons in the rostral DMV only increases LES pressure, CD activity and fundus tone. **Legend:** +, excitatory; -, inhibitory; ?, mechanism or neurotransmitter unknown; ACh, acetylcholine; AP, area postrema; CS, calamus scriptorius; DMV, dorsal motor nucleus of the vagus; LES, lower esophageal sphincter; mAChR, muscarinic acetylcholine receptor; NE, norepinephrine; NO, nitric oxide; NTS, nucleus tractus solitarius; VIP, vasoactive intestinal polypeptide. R, I, C (white letters) refers to rostral DMV, intermediate DMV, and caudal DMV, respectively. R and C (black letters) refers to rostral and caudal, respectively. D and V refers to dorsal and ventral, respectively.
Figure 1: Niedringhaus et al.
Figure 2: Niedringhaus et al.
Figure 3: Niedringhaus et al.
Figure 4: Niedringhaus et al.
Figure 5: Niedringhaus et al.
Figure 6: Niedringhaus et al.
TABLE 1:

Effects of L-glutamate microinjected into 3 areas of the DMV (i.e., intermediate, rostral and caudal areas) on CD activity, LES pressure and Fundus tone before and after ipsilateral vagotomy (IpsiVx)

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>CD Bursts/min</th>
<th>Amplitude (mV)</th>
<th>LES Pressure (mmHg)</th>
<th>Fundus Force (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>During L-glut</td>
<td>Baseline</td>
<td>During L-glut</td>
</tr>
<tr>
<td>Control Intermediate DMV n=9</td>
<td>18.8 ± 1.4</td>
<td>-4.8 ± 0.1*</td>
<td>0.4 ± 0.01*</td>
<td>33.4 ± 3.1</td>
</tr>
<tr>
<td>After IpsiVx n=9</td>
<td>15.4 ± 1.1</td>
<td>-0.7 ± 0.9**</td>
<td>0.5 ± 0.04</td>
<td>32.5 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>21.5 ± 1.9</td>
<td>+6.5 ± 0.17</td>
<td>0.6 ± 0.04</td>
<td>31.0 ± 2.4</td>
</tr>
<tr>
<td>Rostral DMV Control n=5</td>
<td>16.6 ± 2.3</td>
<td>+0.34 ± 0.16</td>
<td>0.7 ± 0.06</td>
<td>26.9 ± 3.5</td>
</tr>
<tr>
<td>After IpsiVx n=5</td>
<td>15.7 ± 0.4</td>
<td>-0.2 ± 0.04</td>
<td>0.3 ± 0.04</td>
<td>26.0 ± 3.3</td>
</tr>
<tr>
<td>Caudal DMV Control n=5</td>
<td>11.5 ± 0.9</td>
<td>-0.4 ± 0.04</td>
<td>0.4 ± 0.04</td>
<td>26.0 ± 3.3</td>
</tr>
</tbody>
</table>

Data expressed as mean±S.E.M; *one-sample t-test and **paired t-test comparing magnitude of change before IpsiVx with change after IpsiVx (significant at p<0.05).
TABLE 2:
Effects of L-NAME (10mg/kg, i.v.) and L-NAME combined with VIPa (0.35mg/kg, i.v.) on the time course of the decrease in LES pressure produced by L-glutamate microinjected unilaterally into the intermediate part of the DMV.

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>Time to peak decrease in LES Pressure (sec)</th>
<th>Duration of decrease in LES Pressure (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control n=4</td>
<td>16.4±1.9</td>
<td>127±8</td>
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<tr>
<td>After L-NAME n=4</td>
<td>37.8±6.8*</td>
<td>221±34*</td>
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<tr>
<td>Control n=4</td>
<td>14.0±1.9</td>
<td>126±13</td>
</tr>
<tr>
<td>After VIPa n=4</td>
<td>4.0±0.6*</td>
<td>42±13*</td>
</tr>
</tbody>
</table>

Data expressed as mean±S.E.M; *paired t-test (significant at p<0.05).
TABLE 3:
Effects of L-glutamate microinjected into 3 areas of the DMV (i.e., intermediate, rostral and caudal areas) on CD activity, LES pressure and fundus tone before and after atropine methyl bromide.

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>CD Bursts/min</th>
<th>CD Amplitude (mV)</th>
<th>LES Pressure (mmHg)</th>
<th>Fundus Force (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>During L-glut</td>
<td>Baseline</td>
<td>During L-glut</td>
</tr>
<tr>
<td>Intermediate DMV</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control n=3</td>
<td>18.9</td>
<td>-6.4</td>
<td>0.5</td>
<td>-0.3</td>
</tr>
<tr>
<td></td>
<td>±1.4</td>
<td>± 2.3*</td>
<td>± 0.02</td>
<td>± 0.12*</td>
</tr>
<tr>
<td>Atropine n=3</td>
<td>17.9</td>
<td>-7.4</td>
<td>0.5</td>
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</tr>
<tr>
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<td>±1.1</td>
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<td>Rostral DMV</td>
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<td>Control n=3</td>
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<td>0.4</td>
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<tr>
<td></td>
<td>±3.2</td>
<td>± 0.2*</td>
<td>± 0.03</td>
<td>± 0.04</td>
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<tr>
<td>Atropine n=3</td>
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<td>+3.0</td>
<td>0.4</td>
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<tr>
<td></td>
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<td>Caudal DMV</td>
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<td>0.3</td>
<td>+0.01</td>
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<tr>
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<td>±2.3</td>
<td>± 0.2</td>
<td>± 0.03</td>
<td>± 0.01</td>
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<tr>
<td>After Atropine n=2</td>
<td>16.7</td>
<td>-0.1</td>
<td>0.4</td>
<td>+0.03</td>
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<tr>
<td></td>
<td>±2.8</td>
<td>± 0.2</td>
<td>± 0.03</td>
<td>± 0.01</td>
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Data expressed as mean±S.E.M; *one-sample t-test and **paired t-test comparing magnitude of change before Atropine with magnitude of change after Atropine (significant at p<0.05).
Table 4:

<table>
<thead>
<tr>
<th>Treatment</th>
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<th>post-Atropine</th>
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<th>post-L-NAME</th>
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Summary of data from DMV stimulation studies.

Niebruggeus et al.