Lower Esophageal Sphincter Relaxation and Activation of Medullary Neurons by Subdiaphragmatic Vagal Stimulation in the Mouse

Q. SANG and RAJ K. GOYAL

Center for Swallowing and Motility Disorders, Department of Veterans Affairs Medical Center, West Roxbury, Massachusetts, and Harvard Medical School, Boston, Massachusetts

Background & Aims: **Isolated lower esophageal sphincter (LES) relaxation associated with belching and vomiting and the transient LES relaxation associated with gastroesophageal reflux are gastric afferent-mediated vagovagal reflexes. We aimed to identify the brain stem vagal subnuclei involved in these reflexes.** ***Methods:*** **In anesthetized mice, LES pressures were recorded using a manometric technique and response to electrical stimulation of the ventral trunk of subdiaphragmatic vagus was investigated. Anatomy of the vagal subnuclei was defined, and activated subnuclei with ventral subdiaphragmatic vagus stimulation were detected by c-fos immunohistochemical staining.** ***Results:*** **Ventral subdiaphragmatic vagal stimulation elicited frequency-dependent LES relaxation without evoking esophageal contractions and induced c-fos expression in interneurons in medial, dorsomedial, and commissural subnuclei along with outer shell of area postrema and motoneurons in the caudal dorsal motor nucleus of vagus. Brain stem subnuclei including interstitial, intermediate, and central subnuclei, and nucleus ambiguus, which have been reported to be involved in the response to swallowing, were not activated.** ***Conclusions:*** **Stimulation of the ventral subdiaphragmatic vagus causes isolated LES relaxation and activates neurons in select vagal subnuclei that may represent the brain stem circuit involved in the abdominal vagal-afferent–evoked isolated LES relaxation. These observations suggest that different brain stem circuits are involved in swallow-induced and gastric afferent-mediated isolated LES relaxations.**

The lower esophageal sphincter (LES) remains tonically contracted at rest. It relaxes during a variety of reflex activities including swallowing allowing antegrade flow and belching and gastroesophageal reflux allowing retrograde flow of gastric contents.[1–3] Swallow-induced LES relaxation (LESR) is accompanied by esophageal peristaltic contraction, whereas isolated LESR is not.[1,4] The isolated LESR that is associated with gastroesophageal reflux, also known as the transient LESR, is accompanied by the relaxation of the crural diaphragm.[4]

The efferent limb of both swallow-associated and isolated LESR lies in the preganglionic vagal inhibitory pathway to the postganglionic nitrergic neurons in the LES.[5] Both LESRs can be blocked by bilateral cervical vagotomy, cervical vagal cooling, or chemical blockers of neuronal nitric oxide synthase (nNOS).[1,3,4] The afferent arm of swallow-evoked LESR lies in pharyngeal and superior laryngeal nerves. Electrical stimulation of the superior laryngeal afferent nerve (SLN) has been used to study the swallowing reflex.[6] Studies using localized medullary electrical and chemical stimulations and lesions have defined that the central neural circuit for swallowing reflex is present in the medullary subnuclei.[7,8] More recent studies using a variety of electrophysiologic and tracer studies in the rat and larger animal species and c-fos expression in the mouse have further localized the brain stem circuit in the swallowing reflex to include interneurons in interstitial (SolI), intermediate (SolIM), and central subnuclei (SolCe) and motor neurons in nucleus ambiguus (NA) and dorsal motor nucleus of vagus (DMV).[9–12] On the other hand, isolated LESR is thought to be mediated by gastric afferents that are carried in the gastric afferents in the subdiaphragmatic vagus, particularly in its ventral branch (vSDV).[4,13,14] It is not known whether subnuclei for isolated LESR and swallow-induced LESR use the same vagal subnuclei.[4]

Although no information is available in the mouse, studies in other animal species have identified the brain stem projections of subdiaphragmatic vagal afferents us-

Abbreviations used in this paper: AP, area postrema; aAP, anterior (rostral) border of AP; ChAT, choline acetyltransferase; DMV, dorsal motor nucleus of vagus; LESR, lower esophageal sphincter relaxation; NA, nucleus ambiguus; NAc, compact formation of NA; NAsc, semicompact formation of NA; nNOS, neuronal nitric oxide synthase; pAP, posterior (caudal) border of AP; SLN, superior laryngeal afferent nerve; Sol, solitary nucleus; vSDV, ventral trunk of subdiaphragmatic vagus.

0016-5085/00/$10.00
doi:10.1053/gast.2000.20234

ing tracer and electrophysiologic techniques.[13–19] The aim of the present study was to examine whether electrical stimulation of vSDV would elicit isolated LESR, and further by examining c-fos expression to identify the brain stem neurons that might be involved in this reflex.[16,20,21] Our results show that in the mouse, electrical stimulation of vSDV evokes frequency-dependent isolated LESR and induces c-fos in interneurons in medial (SolM), dorsomedial (SolDM), and commissural (SolC) solitary subnuclei and motor neurons in the caudal part of DMV. These results suggest that the brain stem circuit involved in isolated LESR is different than that involved in swallowing-associated LESR.[12]

Materials and Methods

Twenty-three adult male Swiss Webster mice (Taconic, Germantown, NY) weighing 20–40g were used. The procedures were approved by the animal committee of Brockton/West Roxbury Veterans Affairs Medical Center.

Esophageal Manometry

Esophageal manometry was performed in 9 animals using a custom-designed catheter assembly (Dentsleeve Ltd., Parkside SA, Australia). The catheter assembly consisted of 3 silicon catheters (ID, 0.3 mm; OD, 0.6 mm) that had been glued together. The catheter assembly was 6.5 cm long with an OD of 1.2 mm. Each catheter had a 0.3-mm side opening, and they were spaced 2 mm apart. The catheters were continuously perfused with bubble-free preboiled water at a rate of 7 μL/min from a reservoir that was pressurized with nitrogen (10 psi) to provide a low-compliance pressure monitoring system. The catheters were connected to ETH 400 Bridge amplifiers through pressure transducers (CB Science, Dover, NH). The output of pressure signals was displayed and recorded on a MacLab software (AD Instruments Pty Ltd., Castle Hills, NSW, Australia).

Mice were anesthetized with a subcutaneous injection of sodium pentobarbital (60 mg/kg) and placed on a heating pad. The catheter assembly was introduced into the esophagus through a gastrostomy incision. The catheters were then withdrawn gradually in 0.5–1-mm increments to identify the high-pressure zone of the LES. The middle catheter opening was then positioned in the LES. LES pressure changes in response to a spontaneous swallow and electrical stimulation of vSDV were recorded. The pressure changes were expressed as the percentage change in the basal LES pressure, and statistical analysis was performed using a paired *t* test.

The vSDV was stimulated with a bipolar (silver/platinum) hook electrode using a Grass stimulator (model S11; Grass Medical Instruments, Quincy, MA). The stimulus consisted of square wave pulses of 0.5 milliseconds at 8 V. Stimulations were performed for a period of 5 seconds at frequencies of 1, 5, and 10 Hz. In some studies, the vagal trunk distal to the stimulating site was sectioned before the stimulation. Left cervical vagotomy was performed in 3 animals, in which the left vagus was severed at 2 mm below the nodose ganglion, and the LES responses to vSDV stimulation were then recorded.

Anatomy of Dorsal Vagal Complex and c-fos Expression

Anatomy of the Sol and its subnuclei were examined with cresyl violet staining of serial sections of the medulla oblongata. The subnuclei were further defined by immunostaining with choline acetyltransferase (ChAT) and nNOS. Vagal motor nuclei were identified with retrograde labeling studies using fast blue injected into the abdominal esophagus in anesthetized mice. This was done by injection of 2 μL of 2% fast blue (dissolved in dimethylsulfoxide; Sigma Chemical Co., St. Louis, MO) with a glass micropipette connected to a Hamilton syringe (Reno, NV). The animals were then recovered and left in their cages for 3–6 days to allow the dye to transport. After 3–6 days, the animals were killed and medullary subnuclei identified as described below.

c-fos expression was induced by electrical stimulation of the vSDV using stimulus parameters that were used for manometric studies except that the 5-second stimuli were repeated every 5 seconds for a total period of 30 minutes. Repeated stimuli were used to facilitate c-fos expression in the brain stem neurons.[16,20,21] Groups of 4 separate animals received vSDV stimulation at 1, 5, and 10 Hz, respectively. Some animals used for c-fos expression with vSDV stimulation had received fast blue injection in the abdominal esophagus 3–6 days before the studies.

On completion of the vSDV stimulation protocol, the mice were killed with a subcutaneous injection of sodium pentobarbital (100 mg/kg) and perfused transcardially with 60 mL phosphate-buffered saline (PBS; 0.9% NaCl in 0.01 mol/L sodium phosphate buffer, pH 7.0) followed by 60 mL 4% formaldehyde (4% formaldehyde in 0.1 mol/L sodium phosphate buffer, pH 7.0). The brain stem with the upper cervical spinal cord attached was removed and postfixed in the same fixative at 4°C overnight. After the fixative was removed with 3 washes in PBS, the brain stem was stored in PBS containing 1% sodium azide plus 30% sucrose for 24 hours at 4°C before being sectioned. Frozen coronal serial sections of 25-μm thickness of the entire medulla were cut and processed for histochemical and immunohistochemical stains. The orientation and sequence of serial sections were maintained.

The first of every 3 sections was stained with cresyl violet. The section was washed in water and stained with 1% cresyl violet (Fisher Scientific, Pittsburgh, PA) containing 0.25% acetic acid for 5 minutes. After rinsing in water, the section was differentiated in 50% ethanol for 5 minutes and mounted with mounting medium (Sigma).

The second of every 3 sections was immunostained for ChAT and c-fos. The section was incubated in a mixture of primary antisera that contained a rabbit anti–c-fos (1:3000; Oncogene Research, Cambridge, MA) and a goat anti-ChAT (1:50; Chemicon International, Temecula, CA) for 16–24 hours. The unbound antibodies were removed by 3 washes in

PBS. The section was then incubated in a biotinylated donkey anti-rabbit immunoglobulin (Ig) G (1:200; Jackson ImmunoResearch, West Grove, PA) for 2 hours. After another 3 washes in PBS, the immunoreactivity of c-fos was revealed by streptavidin Texas red (1:100; Amersham Life Science, Inc., Arlington Heights, IL) and the immunoreactivity of ChAT was revealed by incubating the section in a fluorescein isothiocyanate–conjugated donkey anti-sheep IgG (1:50; Jackson ImmunoResearch) for 90 minutes. ChAT immunoreactivity can be revealed by a fluorescein isothiocyanate–conjugated donkey anti-sheep IgG, although the primary antibody is a goat anti-ChAT.[22] After another 3 washes in PBS, the section was then mounted with the mounting medium.

The third of every 3 sections was immunostained for c-fos and nNOS. The section was incubated in a mixture of a rabbit anti–c-fos and a rabbit anti-nNOS (1:400; Santa Cruz Biotechnology, Santa Cruz, CA). After unbound antibodies were removed, the section was incubated in biotinylated donkey anti-rabbit IgG for 2 hours, then vectastain ABC kit (Vector Laboratories, Burlingame, CA) for another 2 hours, after which the immunoreactivity was revealed using a peroxidase substrate kit (DAB; Vector Laboratories). c-fos and nNOS immunoreactivity can be distinguished by the location of immunoreactive products. c-fos staining was nuclear, whereas nNOS staining was cytoplasmic in location.

All the preparations were examined on a Zeiss Axioplan microscopy, and images were captured on a Hamamatsu camera with Openlab software. Some sections were also examined with a Bio-Rad multiphoton laser microscope (MRC 1024; Hercules, CA). Examination of all the serial sections allowed us to calculate the craniocaudal extent of the vagal subnuclei and the distribution of c-fos–activated neurons in these subnuclei. c-fos–positive neurons in the subnuclei were quantitated semiquantitatively in coronal sections that showed a maximum number of activated neurons.

Results

The mouse esophagus is approximately 35-mm long with about a 6-mm subdiaphragmatic segment. It is composed mainly of striated muscle except its lowermost part, which is composed of smooth muscle. The vagus nerve below the diaphragm has 2 distinct trunks: ventral and dorsal. The ventral vagal trunk has also been called the anterior or right branch and the dorsal vagal trunk as the posterior or left branch of abdominal vagus (Figure 1). As in the rat, the ventral vagal trunk in the mouse has 3 branches: hepatic, accessory celiac, and right gastric.[15] The overwhelming majority of afferent fibers in the ventral branch in the rat are from the stomach.[15] Efferent fibers to the LES are not present in the vagus at this level because they leave the vagal plexus in the thorax.[23] The ventral (right) subdiaphragmatic vagal trunk is a continuation of the left cervical vagus originating from the left side of the medulla. During the development, the left vagus rotates with the stomach in its abdominal part and comes to lie ventrally and to the right.

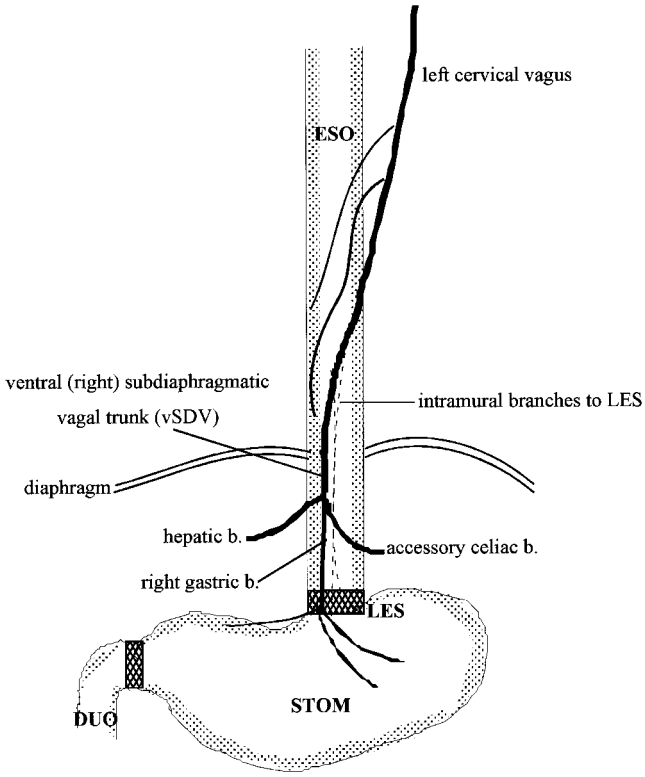

Figure 1. Anatomy of vagus nerve and the gastroesophageal area in the mouse. Note that the left cervical vagus came to lie anterior to the esophagus as it passed through the diaphragmatic hiatus. Below the diaphragmatic hiatus, it constituted the right or the ventral vagal trunk. vSDV gave off hepatic and accessory celiac branches and continued as the right gastric branch. b., branch; ESO, esophagus; DUO, duodenum.

Motor Response of the LES to vSDV Stimulation

Manometrically, the mouse LES is revealed as a high-pressure zone between the esophagus and stomach in anesthetized animals. As shown in Figure 2*A*, spontaneous swallowing is characterized by a peristaltic contraction of the esophageal body and a relaxation of the LES. In contrast, electrical stimulation of vSDV only induced isolated LESR, without evoking esophageal body contraction. In addition, the degree of pressure drop in the LES increased as the stimulation frequency increased (Figure 2*B*). Sectioning of the vagal trunk distal to the stimulation site did not modify the LES responses. However, left cervical vagotomy eliminated the LES response, indicating that vagal afferents were involved in vSDV stimulation–mediated LESR.

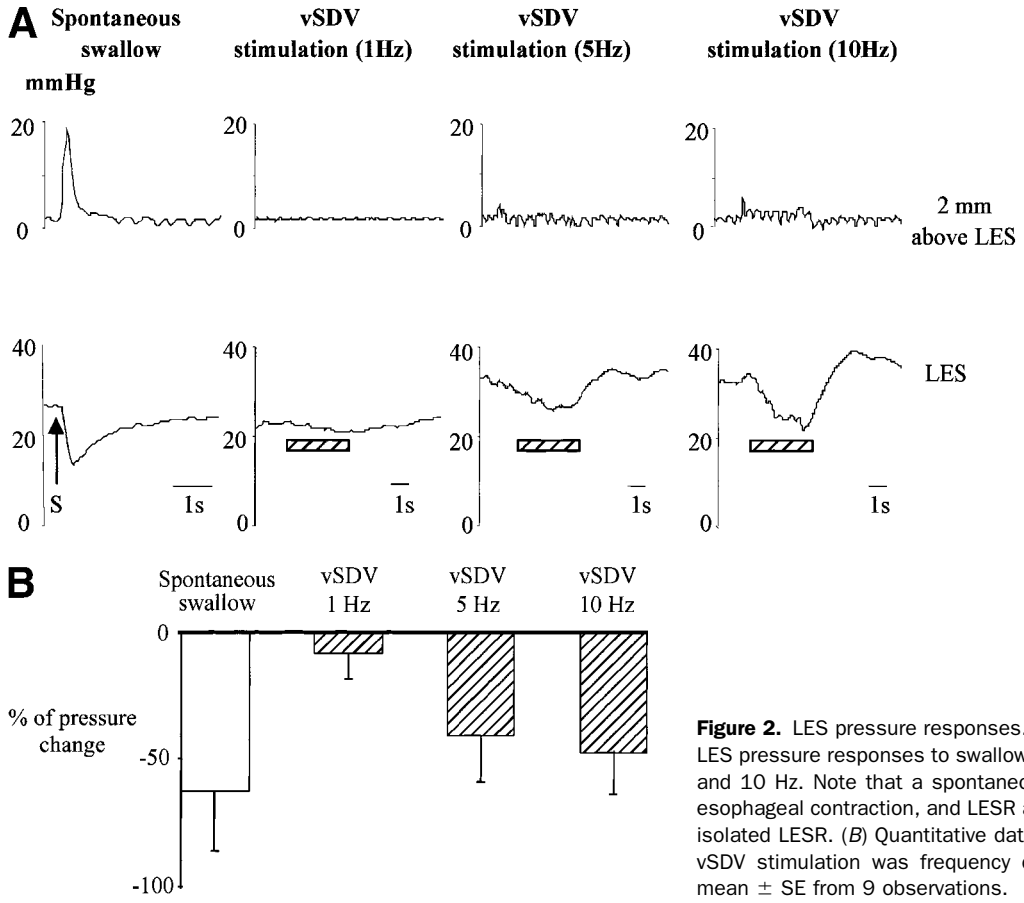

Figure 2. LES pressure responses. (*A*) Examples of esophageal and LES pressure responses to swallowing and vSDV stimulation at 1, 5, and 10 Hz. Note that a spontaneous swallow was associated with esophageal contraction, and LESR and vSDV stimulation elicited only isolated LESR. (*B*) Quantitative data on LESRs. Note that LESR with vSDV stimulation was frequency dependent. Each bar represents mean ± SE from 9 observations.

Anatomy of the Vagal Subnuclei in Mice

The anatomic organization of the solitary subnuclei and the vagal motor nuclei in mice is generally similar to that described in rats.[15,24,25] In the present study, we used area postrema (AP as the reference point for describing levels in craniocaudal orientation. AP stood out as a compact group of small cresyl violet–stained neurons with a subpopulation of ChAT-positive neurons. The craniocaudal extent of the AP in the mouse is approximately 500 μm. This and all other measurements described below are rough average measurements in all the animals studied.

The subnuclei of Sol have been classified and named differently by different investigators based on cytoarchitectural and histologic criteria in different animal species.[17,24,25] We used the nomenclature used by Franklin and Paxinos.[25] The anatomic sites were defined by anatomic landmarks such as the fourth ventricle (IV), AP, SolT, and DMV. The anatomic regions of the subnuclei were correlated with cytoarchitectonic subdivisions defined by cresyl violet staining and supplemented by nNOS staining.[24]

The Sol is located dorsal to DMV, ventral and lateral to AP, and medial, lateral, ventral, and dorsal to the ST depending on the level. The Sol is broadly divided into a smaller lateral and a larger medial subdivision based on their position in relation to the SolT (Figures 3–5).[25] The lateral subdivision in the mouse is subdivided into 4 subnuclei: dorsolateral (SolDL), ventrolateral (SolVL), ventral (SolV), and interstitial (SolI). In the rat, SolV is included in SolVL. The interstitial subnucleus (SolI) in the mouse lies in the lateral, ventral, and medial parts of SolT in its rostral part.[25] This is different from the rat, in which SolI spans the entire extent of the nucleus.[11,24] The craniocaudal extent is described in reference to posterior (caudal) border of AP (pAP). SolV was seen at +750 μm through +575 μm, SolVL at +750 μm through +50 μm, and SolDL at +475 μm through +250 μm levels. The medial subdivision is subdivided into 7 subnuclei, including the intermediate (SolIM), central (SolCe), medial (SolM), commissural (SolC), dorsomedial (SolDM), gelatinosus (SolG), and retrocentral (SolRCe). SolRCe is not recognized separately in the rat but is included in the SolM. SolCe is a distinct compact subnucleus that is formed by a group of neurons that are easily distinguished by their intense nNOS immunoreactivity.[14,24] SolG is recognized by its acellular gelatinous appearance because of the preponderance of the unmyelinated fibers.

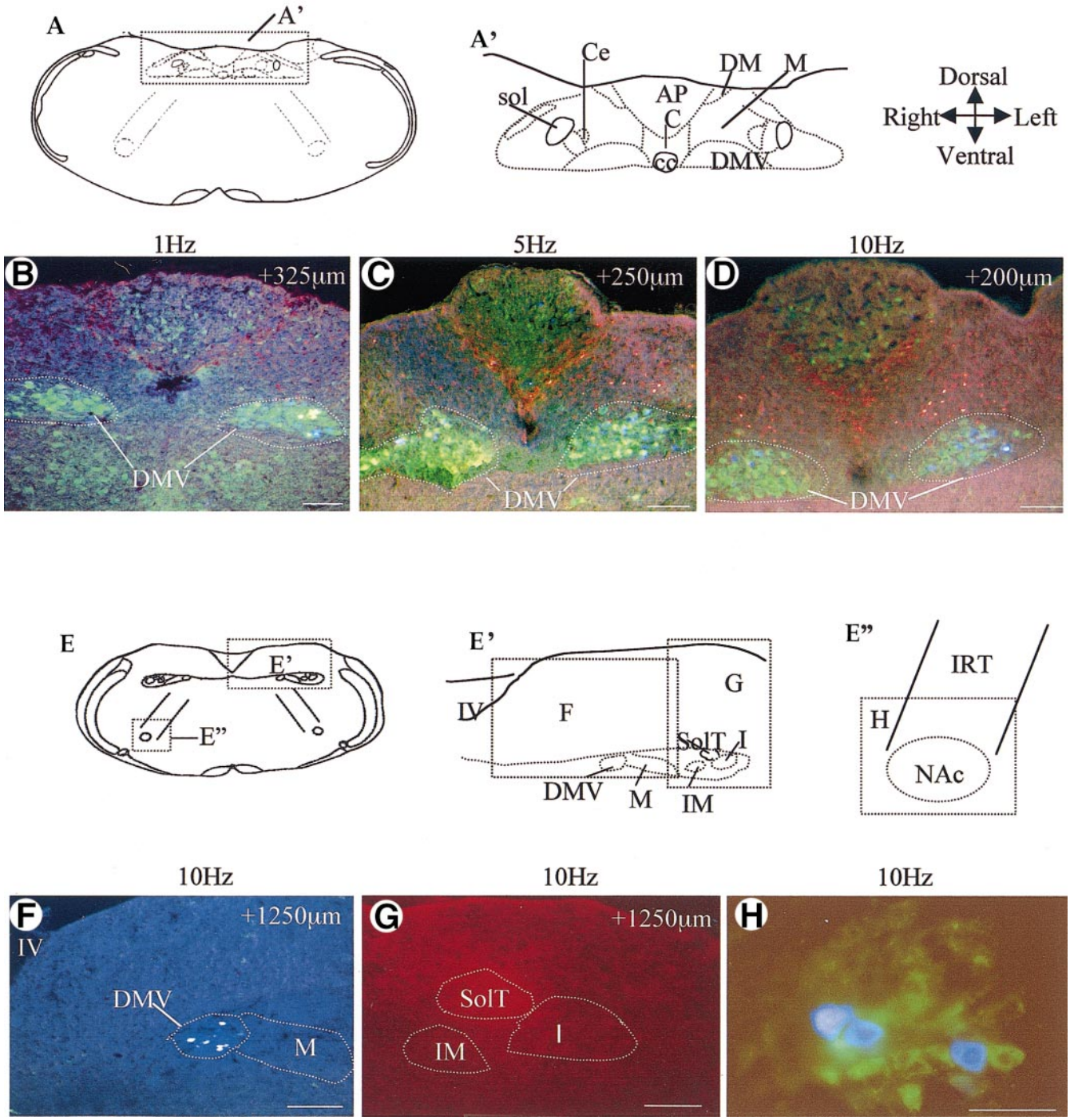

Figure 3. c-fos expression in the subnuclei of the dorsal motor complex. (*A* and *A*') Schematic representation of vagal subnuclei in the coronal section at mid level of AP. (*B*, *C*, and *D*) c-fos expression in the dorsal vagal complex after vSDV stimulation at 1, 5, and 10 Hz, respectively, in 3 different animals. Note c-fos–reactive neurons (red) in SolM, SolDM, SolC, and outer shell of AP (but not the core of AP). A few c-fos–expressing neurons are also found in DMV. c-fos–expressing neurons are seen bilaterally but are more numerous ipsilaterally (left side). Also note that the number of c-fos–reactive neurons increased with higher stimulus frequencies. In these animals, DMV was positively identified by neurons (blue) that are retrogradely labeled with fast blue. Simultaneous ChAT immunostaining (green) also identified DMV motoneurons and the AP neurons. (*E*, *E*', and *E*") Schematic representation of dorsal vagal complex 1250 μm rostral to pAP vSDV stimulation at 10 Hz. (*F*) A section from the medial region in which DMV is located lateral to SolM. DMV is positively identified by retrogradely fast blue–labeled cells (blue). Note the absence of c-fos–positive neurons in SolM with 10 Hz vSDV stimulation. (*G*) A section from the lateral area around the SolT. There are no c-fos–positive cells in SolI or SolIM at 10 Hz vSDV stimulation. (*H*) A section from the ventral part of the medulla in the region of the NAc. NAc is positively identified as a group of ChAT-positive neurons (green) including a few retrogradely fast blue–labeled neurons (blue). One neuron showing c-fos expression at 10 Hz vSDV stimulation in this section was an exception. *Bar* = 100 μm in *B*, *D*, *F*, and *G* and 50 μm in *H*.

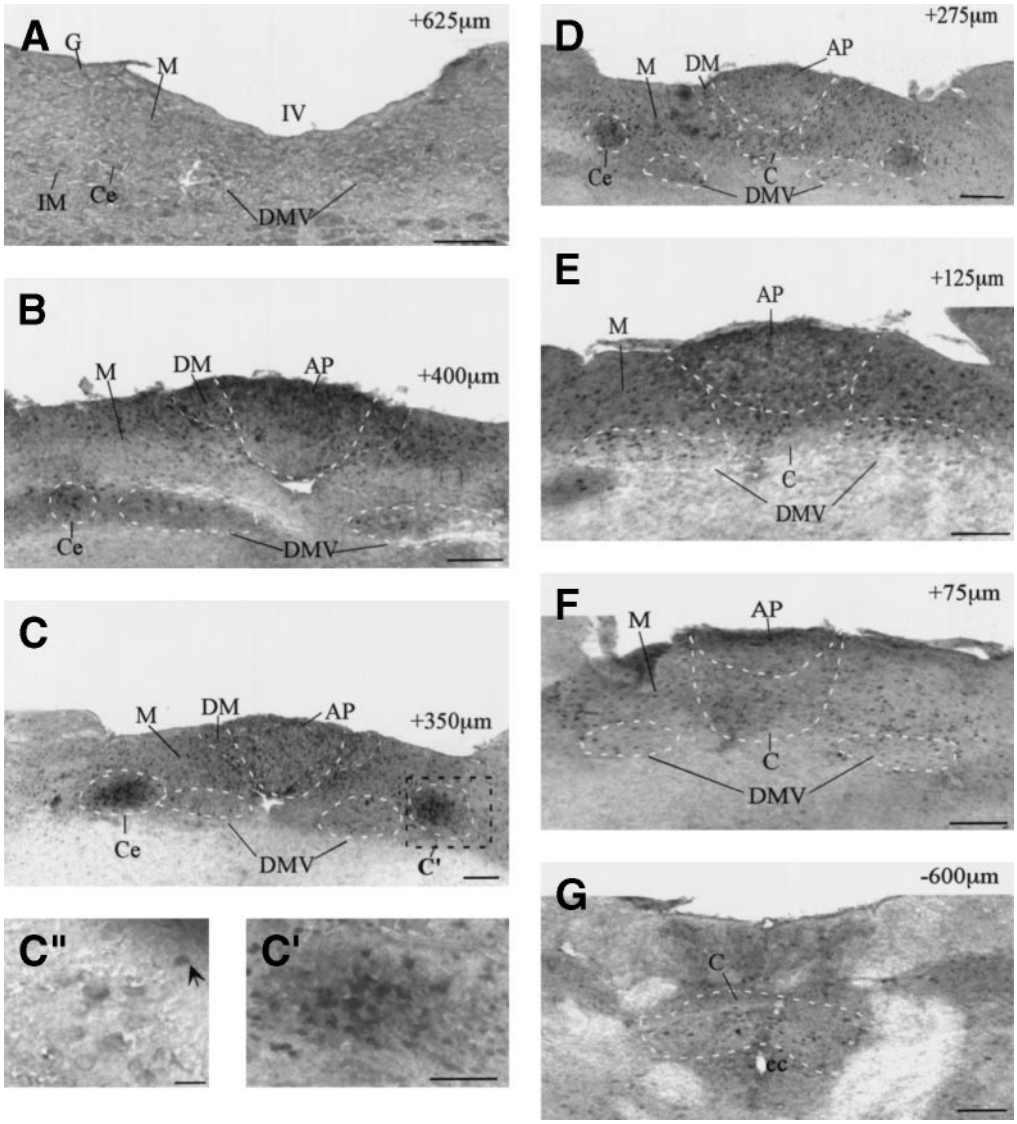

Figure 4. c-fos expression pattern in the medial subnuclei of Sol at various craniocaudal levels around AP in 1 animal. In this animal, vSDV was stimulated at 10 Hz and the coronal sections were immunoreacted for c-fos and nNOS. (*A*) A section rostral to aAP (+625 μm rostral to pAP) showing a lack of c-fos in SolG and DMV at this level. (*B–E*) Sections at +400 μm through +125 μm rostral to aAP. They show c-fos–positive cells (nuclear staining) in SolM, SolDM, and SolC and DMV but not in SolIM and SolCe. In *C* (a section at +350 μm), SolCe is clearly identified as a population of densely packed nNOS reactive neurons. (*C′* and *C″*) Higher-power multiphoton microscopic examination of SolCe to more clearly identify c-fos– and nNOS-reactive neurons. Note nNOS (cytoplasmic)-staining neurons that lacked c-fos (nuclear) staining in the SolCe. In *C″*, a c-fos–staining neuron is seen in SolM, dorsal to SolCe. (*D*) A section at the level of +275 that clearly identifies SolCe. No c-fos staining is found in SolIM, which is present between SolT and SolCe. (*E* and *F*) Sections through caudal parts of AP. (*G*) A section at −600 μm caudal to pAP. Note extensive c-fos–reactive neurons throughout the extent of SolC. *Bar* = 100 μm in all except *C″*, in which *bar* = 50 μm. All levels are in reference to pAP.

Among the medial subnuclei, SolIM, SolRce, and SolM were seen at levels rostral to AP. SolG was seen at +575 μm and +475 μm. Sections +475 μm through +50 μm spanned the region of AP and showed SolIM just medial to SolT, whereas SolCe is placed between SolIM and SolM. SolDM was dorsal to the SolM. SolC was identified ventral to AP and extended caudal to the pAP.

The DMV is located just ventral to the Sol. Injection of fast blue into the anterior wall of the distal esophagus labeled cells in both rostral and caudal regions of the DMV. The labeling was bilateral but more prominent on the left side. Immunostaining with ChAT showed that a subpopulation of DMV neurons are cholinergic. DMV extends throughout the extent of the Sol. NA is located in the ventral part of the medulla and is broadly divided into the rostrally located part consisting of compactly packed cells (NAc) and the loosely packed cells present around the NAc and extending caudally, called the semicompact formation (NAsc).[26] All the NA motoneurons are cholinergic. A few retrogradely labeled cells were observed in the NAc.

c-fos Expression in the Vagal Nuclei With vSDV Stimulation

c-fos is an immediate-early gene whose expression has been used widely to identify neurons involved in reflex activities.[20,21] c-fos is easily expressed in transsynaptically activated second-order neurons. It may also be expressed in third-order, or subsequent order, neurons upon sufficient stimulation of the afferent fibers. However, its expression decreased as the order of neurons in a multisynaptic pathway increased.[20,21] Therefore, c-fos expression has the potential to identify both premotor and motor neurons in a neural reflex.

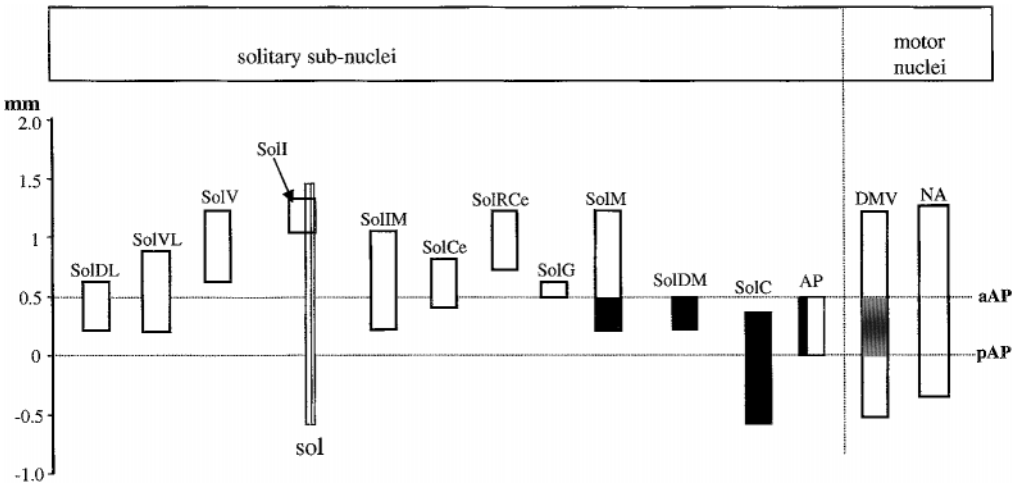

Figure 5. Diagrammatic representation of subnuclei of dorsal vagal complex in craniocaudal orientation showing c-fos–active neurons (*shaded area*) with electrical stimulation of vSDV. The presentation of the subnuclei in the mice is based on our anatomic studies. Level 0 represents the pAP. c-fos–active neurons were seen in SolM, SolDM, SolC, outer shell of AP, and DMV. The darkness of the shade represents the density of c-fos–positive neurons, the darkest being the densest.

Electrical stimulation of vSDV induced c-fos expression in neurons that were located in the craniocaudal extent spanned by AP. c-fos expression evoked by vSDV stimulation with all the stimulus frequencies was seen in the same vagal subnuclei. However, the number of c-fos–positive neurons increased as the stimulation frequency increased. Figure 3 illustrates c-fos–positive neurons at the middle level of AP, in SolM, SolDM, and SolC subnuclei. c-fos expression was also found in neurons in the outer shell of the AP, but not in the core of AP. c-fos reactivity was also observed in DMV neurons at this level. c-fos reactive neurons were lacking in SolCe and SolIM. Table 1 summarizes the semiquantative data. With electrical stimulation of vSDV at 1 Hz, c-fos immunoreactivity was only observed in none (−) or less than 5 neurons (+) per section in most of the subnuclei except SolM and SolC. SolM showed from 5 to 10 (++) up to 10 to 50 (+++) c-fos–positive neurons in a section. The outer shell of AP showed between 5 and 10 (++) c-fos–positive neurons per section. The number of c-fos–reactive neurons increased as the stimulation increased to 5-Hz and to 10-Hz stimulation. c-fos–reactive neurons were not seen in SolI, SolIM, SolCe, core of the AP, rostral part of DMV, and NAsc at any of the stimulus frequencies. Only one c-fos–activated neuron was found in NAc in one of the sections at 10-Hz stimulation. This was not considered to be significant. The activated neurons were present in the vagal subnuclei bilaterally but were more numerous ipsilaterally (Figure 3).

Table 1. c-fos Expression in Brain Stem Nuclei With vSDV Stimulation at Different Stimulus Frequencies

Main nuclei	Subnuclei	Frequency of vSDV stimulation		
		1 Hz	5 Hz	10 Hz
Sol	SolI	−	−	−
	SolIM	−	−	−
	SolM	++ to +++	+++ to ++++	++++
	SolCe	−	−	−
	SolDM	+	+ to ++	++
	SolG	−	−	−
	SolC	+	++	+++
AP	Outer shell	++	+++	+++
	Core	−	−	−
DMV	Rostral	−	−	−
	Caudal	+	+ to ++	++ to +++
NA	NAsc	−	−	−
	NAc	−	−	−

−, Not detected; +, highest number of positive cells on a single section <5; ++, highest number of positive cells on a single section between 5 and 10; +++, highest number of positive cells on a single section between 10 and 50; ++++, highest number of positive cells on a single section >50.

The number of neurons that expressed c-fos was also different in rostrocaudal orientation in different Sol subnuclei (Figure 4). In the SolM, the maximum number of c-fos–expressing neurons was observed in a region from 100 μm rostral to the pAP and extended 200 μm further rostrally. The number of the activated neurons decreased both rostrally and caudally into this region. In SolDM, the maximal number of activated neurons was found in the most caudal 100 μm of this subnucleus. The number of activated neurons in SolDM was smaller than that in SolM. In the SolC, c-fos–positive neurons were observed throughout its craniocaudal extent, but the largest number was present in the caudal AP region (300 μm rostral to pAP). No c-fos reactivity was seen in SolG. This may reflect the scarcity of neurons in this subnucleus.[13,18] A small number of c-fos–expressing neurons was observed in DMV in an area extending caudally up to about 500 μm from the level of anterior (rostral) border of AP (aAP). c-fos–active neurons were not observed in rostral DMV. No c-fos reactivity was found in SolI, SolIM, SolCe, core of AP, or NAsc. One activated neuron was

found in NAc; this was not considered to be significant. The c-fos expression pattern in vagal subnuclei with vSDV stimulation is summarized in Figure 5 and Table 1. After ipsilateral cervical vagotomy, no c-fos immunoreactivity was found in any of the vagal subnuclei.

Discussion

This study shows that electrical stimulation of the vSDV in the mouse causes isolated LESR and activates brain stem interneurons in the SolM, SolDM, SolC, and preganglionic motor neurons in the caudal DMV. This pattern of activated Sol neurons with vSDV stimulation is quite different from that evoked by electrical stimulation of SLN that elicits a swallowing response and activates neurons in SolI, SolIM, SolCe, SolC, as well as motoneurons in NA and both caudal and rostral regions of DMV.[12]

Isolated LESR, in response to gastric distention, is a vagovagal reflex that has been shown to occur during various reflex activities, including gastric belching[2] and the transient LESR.[3,4] Our study now shows that electrical stimulation of the gastric afferent in the abdominal vagus of the mouse elicits isolated LESR, and that the mouse can serve as a suitable model to investigate the brain stem circuit in this reflex.

Electrical stimulation of the mouse vSDV induced c-fos expression in Sol neurons that were localized primarily over a 500-μm area at the level of AP in the SolM, SolDM, and SolC. Activated neurons were also found around the outer shell of AP and extended caudally in the SolC. The distribution of activated neurons in the Sol subnuclei correlates well with the reports of projection of afferents from vSDV and gastric afferents in the rat with one exception.[13–15,18,19] No c-fos expression was found in SolG, which had been shown to be the site of dense projection of gastric afferents.[15,18] However, the lack of c-fos expressing neurons in SolG with sSDV stimulation can be explained by the scarcity of neurons in SolG.[15,27]

It is reasonable to assume that some of the Sol subnuclei activated by vSDV stimulation constitute the brain stem interneurons responsible for the isolated LESR. The Sol subnuclei expressing c-fos with vSDV stimulation are similar to those described for gastric distention in rats.[16] Activated subnuclei expressing c-fos most likely also represent brain stem circuits for other reflexes mediated by afferents in the vSDV because they represent all those that receive input from all afferents in vSDV, including those arising from the gastroesophageal junctional area and the upper part of intestinal and hepatobiliary areas.[15] From our study, it is not possible to draw inferences regarding the organotropic representation of the activated neurons. However, studies have shown that the projection of afferents into the celiac and hepatic branches of vSDV are most prominent in the region around AP, suggesting that activated neurons around the outer shell of AP may represent neurons that are involved in hepatic and celiac reflexes.[15]

vSDV stimulation did not induce c-fos expression in the Sol subnuclei (SolI, SolIM, and SolCe), which have been shown to be activated by SLN stimulation and to play a key role in swallowing responses in oropharyngeal and esophageal striated muscle.[9–11,28] These observations correlate well with the fact that vSDV stimulation does not evoke swallowing or esophageal peristalsis. Although not fully established, SolCe is also believed to integrate swallowing responses in the smooth muscle portion of the esophagus, including the LES. Roger et al.[29] have suggested that SolCe serves as a coordinating site for gastric inhibition because of esophageal distention. Therefore, the lack of c-fos expression in SolCe by vSDV stimulation was striking. Our data suggest that unlike swallow-induced LESR, gastric-induced isolated LESR may be coordinated in SolM and SolDM. A large number of neurons in SolC were activated by vSDV stimulation, which is similar observations with SLN stimulation.[12] SolC neurons may play an important role in bilateral activation of the medullary nuclei with unilateral afferent stimulation. It is also possible that the swallowing and abdominal afferent pathways converge on SolC in the processing of LESR.

Stimulation of vSDV also induced c-fos expression in motoneurons in the caudal part of DMV. These neurons presumably represent third-order neurons activated via the Sol neurons. c-fos may be expressed in third- or subsequent-order neurons upon sufficient stimulation of the afferent fibers. However, c-fos expression decreases as the order of neurons in a multisynaptic pathway increases.[16,20] The activated neurons in DMV were localized at the level of AP and extended caudally. Previous studies in other animal species have shown that DMV contains preganglionic vagal motoneurons that supply most of the gastrointestinal tract including LES.[13–15] Furthermore, there is a viscerotropic efferent organization in this nucleus, so that gastric and colonic vagal preganglionic neurons are located in medial and lateral columns, respectively.[13–15] Preganglionic vagal motor neurons to the LES are also located throughout the rostral and caudal parts of the DMV in the mouse, which is similar to findings in rats and ferrets, but unlike those in cats in which the LES motor neurons are distributed in the most rostral and caudal DMV.[15,30–32] It has been

shown that the neurons in the rostral and caudal DMV provide excitatory and inhibitory projections, respectively, to the LES and gastric fundus in some species.[29–31] In the present study, we show that with vSDV stimulation, c-fos expression occurred in neurons in the caudal DMV only, which is consistent with the role of caudal DMV neurons providing inhibitory inputs to the LES.[29–31] Neurons in the rostral DMV that are thought to provide excitatory input to the LES were not activated by vSDV stimulation.[29–31] It is possible that the Sol neurons that receive subdiaphragmatic vagal afferent inputs do not send any neural inputs to the rostral DMV neurons. It is also possible that during activation of the caudal inhibitory pathway neurons, the rostrally located excitatory pathway neurons are reciprocally inhibited. If so, the inhibited neurons would not be expected to express c-fos in the rostral DMV.[20,21] Further studies are needed to resolve this issue. In contrast to the findings with vSDV stimulation, stimulation of SLN that elicits primary esophageal peristalsis has been shown to evoke c-fos expression in both caudal and rostral regions of DMV.[12] It is possible that isolated LESR caused by vSDV stimulation and swallow-associated LESR caused by SLN stimulation use the same motoneurons in the caudal part of the DMV. However, the rostral part of the DMV coordinates inhibition and contraction of the smooth muscle portion of the esophagus during swallowing.

The communication between the SolM and SolDM interneurons and motor neurons in the DMV that are involved in isolated LESR may be via dendrites of the motoneurons in the DMV that have been shown to penetrate into certain Sol subnuclei, including the caudal parts of the SolM and SolC, but not into SolCe.[13,14] In addition, SolG may provide an additional site that allows a direct monosynaptic communication between vagal afferents and dendrites of the DMV preganglionic vagal motoneurons.[27] There may also be direct projections of the vagal afferents onto DMV neurons.[33]

NAsc and NAc subnuclei were not activated by vSDV stimulation. NAsc and NAc contain motoneurons to striated muscle of the oropharynx and the esophagus, respectively,[11,26,34] and are activated by SLN stimulation and swallowing.[7–11,26,34] The lack of c-fos–activated neurons with vSDV stimulation correlates well with the fact that vSDV stimulation does not evoke swallowing response.

In summary, these studies in the mouse show that electrical stimulation of vSDV elicits a vagovagal inhibitory LES reflex that activates neurons in SolM, SolDM, and SolC subnuclei and vagal motoneurons in the caudal DMV. These neurons may provide the neural circuit that is responsible for abdominal vagal afferent–mediated isolated LESR during belching and transient LESR, during gastroesophageal reflux, and more generalized ascending inhibition during vomiting. These studies also show that the mouse can serve as a useful model for the investigations of central control of LES function.

References

1. Goyal RK, Sivarao DV. Functional anatomy and physiology of swallowing and esophageal motility. In: Castell DO, Richter JE, eds. The esophagus. Philadelphia: Lippincott Williams and Wilkins, 1999:1–31.
2. McNally EF, Kelly JE, Ingelfinger FJ. Mechanism of belching: effects of gastric distension with air. Gastroenterology 1964;46: 254–259.
3. Martin CJ, Patrikios J, Dent J. Abolition of gas reflux and transient lower esophageal sphincter relaxation by vagal blockade in the dog. Gastroenterology 1986;91:890–896.
4. Mittal RK, Holloway RH, Penagini R, Blackshaw LA, Dent J. Transient lower esophageal sphincter relaxation. Gastroenterology 1995;109:601–610.
5. Boulant J, Fioramonti J, Dapoigny M, Bommelaer G, Bueno L. Cholecystokinin and nitric oxide in transient lower esophageal sphincter relaxation to gastric distention in dogs. Gastroenterology 1994;107:1059–1066.
6. Paterson WG, Rattan S, Goyal RK. Experimental induction of isolated lower esophageal sphincter relaxation in anesthetized opossums. J Clin Invest 1986;77:1187–1193.
7. Jean A. Brain stem control of swallowing. In: Taylor A, ed. Neurophysiology of the jaws and teeth. London: McMillan, 1990: 294–321.
8. Kessler JP, Jean A. Identification of the medullary swallowing region in the rat. Exp Brain Res 1985;57:256–263.
9. Bieger D. The brain stem esophageal motor network pattern generator: rodent model. Dysphagia 1993;8:203–208.
10. Lu WY, Bieger D. Vagal afferent transmission in the NTS mediating reflex responses in the rat esophagus. Am J Physiol 198;274: R1436–R1445.
11. Broussard DL, Lynn RB, Wiedner EB, Altschuler SM. Solitarial premotor neuron projections to the rat esophagus and pharynx: implications for control of swallowing. Gastroenterology 1998; 114:1268–1275.
12. Sang Q, Goyal RK. Pattern of activation of vagal subnuclei by superior laryngeal nerve stimulation (abstr). Gastroenterology 1999;116:A1074.
13. Shapiro RE, Miselis RR. The central organization of the vagus nerve innervating the stomach of the rat. J Comp Neurol 1985; 238:473–488.
14. Altschuler SM, Bao X, Bieger D, Hopkins DA, Miselis RR. Viscerotopic representation of the upper alimentary tracts in the rat: sensory ganglia and nuclei of the solitary and spinal trigeminal tract. J Comp Neurol 1989;283:248–268.
15. Norgren R, Smith GP. Central distribution of subdiaphragmatic vagal branches in the rat. J Comp Neurol 1988;273:207–223.
16. Willing AQ, Berthold H-R. Gastric distension-induced c-fos expression in catecholaminergic neurons of rat dorsal vagal complex. Am J Physiol 1997;272:R59–R67.
17. Kalia M, Mesulam M-M. Brain stem projection of sensory and motor components of vagus complex in the cat. II. Laryngeal, tracheobronchial, pulmonary, cardiac, and gastrointestinal tract. J Comp Neurol 1980;193:467–508.
18. Leslie RA, Gwyn DG, Hopkins DA. The central distribution of the

cervical vagus nerve and gastric afferent and efferent projections in the rat. Brain Res Bull 1982;8:37-43.
19. Paton JF, Li YW, Deuchars J, Kasparov S. Properties of solitary tract neurons receiving inputs from the subdiaphragmatic vagus nerve. Neuroscience 2000;95:141–153.
20. Fields RD, Eshete F, Stevens B, Itoh K. Action potential–dependent regulation of gene expression: temporal specificity in Ca^{2+}, cAMP-responsive element binding proteins, and mitogen-activated protein kinase signaling. J Neurosci 1997;17:7252–7266.
21. Morgan JI, Curran T. Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes fos and jun. Annu Rev Neurosci 1991;14:421–451.
22. Sang Q, Young HM. The origin and development of the vagal and spinal innervation of the external muscle of the mouse esophagus. Brain Res 1998;809:253-268.
23. Blackman AH, Rakatansky H, Nasrullah M, Thayer WRJ. Transabdominal vagectomy and lower esophageal function. Arch Surg 1971;102:6–8.
24. Herbert H, Moga MM, Saper CB. Connections of the parabrachial nucleus with the nucleus of the solitary tract and the medullary reticular formation in the rat. J Comp Neurol 1990;293:540–580.
25. Franklin KBJ, Paxinos G, Franklin KBJ. The mouse brain in stereotaxic coordinates. San Diego, CA: Academic, 1997.
26. Bieger D, Hopkins DA. Viscerotopic representation of the upper alimentary tract in medulla oblongata in the rat: the nucleus ambiguus. J Comp Neurol 1987;262:546–562.
27. Rinaman L, Card JP, Schwaber JS, Miselis RR. Ultrastructural demonstration of a gastric monosynaptic vagal circuit in the nucleus of the solitary tract in rat. J Neurosci 1989;9:1985–1996.
28. Cunningham ET Jr, Sawchenko PE. A circumscribed projection from the nucleus of solitary tract to the nucleus ambiguus in the rat: anatomical evidence for somatostatin-28–immunoreactive interneurons subserving reflex control of esophageal motility. J Neurosci 1989;9:1668–1682.
29. Rogers RC, Hermann GE, Travagli RA. Brain stem pathways responsible for esophageal control of gastric motility and tone in the rat. J Physiol 1999;514:369–383.
30. Rossiter CD, Norman WP, Jain M, Hornby PJ, Benjamin S, Gillis RA. Control of lower esophageal sphincter pressure by two sites in dorsal motor nucleus of the vagus. Am J Physiol 1990;259:G899–G906.
31. Abrahams TP, Hornby PJ. Lower esophageal sphincter relaxation evoked by stimulation of the dorsal motor nucleus of the vagus in ferrets (abstr). Gastroenterology 1999;116:A589.
32. Collman PI, Tremblay L, Diamant NE. The central vagal efferent supply to the esophagus and lower esophageal sphincter of the cat. Gastroenterology 1993;104:1430–1438.
33. Neuhuber WL, Sandoz PA. Vagal primary afferent terminals in the dorsal motor nucleus of the rat: are they making monosynaptic contacts on preganglionic efferent neurons? Neurosci Lett 1986;69:126–130.
34. Zoungrana OR, Amri M, Car A, Roman C. Intracellular activity of motorneurons of the rostral nucleus ambiguus during swallowing in sheep. J Neurophysiol 1997;77:909–922.

Received January 8, 2000. Accepted July 19, 2000.

Address requests for reprints to: Raj K. Goyal, M.D., Research & Development (151), VA Boston Healthcare System, 1400 VFW Parkway, West Roxbury, Massachusetts 02132. e-mail: Raj_Goyal@hms.harvard.edu; fax: (617) 363-5591.

Supported in part by research grant DK 31092 from the National Institute of Diabetes and Digestive and Kidney Diseases and a VA Merit Review Award from the Office of Research and Development, Medical Research Service, Department of Veterans Affairs.

The authors thank Priyattam Shiromani, Ph.D., Heather Young, Ph.D., D. V. Sivarao, Ph.D., and Hemant Thatte, Ph.D., Director, Multiphoton Imaging Core, for technical advice and Donna Kantarges for editorial assistance.