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Differential Activation of Medullary Vagal Nuclei Caused by Stimulation of Different Esophageal Mechanoreceptors

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Abstract

Esophageal mechanoreceptors, i.e. muscular slowly adapting tension receptors and mucosal rapidly adapting touch receptors, mediate different sets of reflexes. The aim of this study was to determine the medullary vagal nuclei involved in the reflex responses to activation of these receptors. Thirty-three cats were anesthetized with alpha-chloralose and the esophagus was stimulated by slow balloon or rapid air distension. The physiological effects of the stimuli (N=4) were identified by recording responses from the pharyngeal, laryngeal, and hyoid muscles, esophagus, and the lower esophageal sphincter (LES). The effects on the medullary vagal nuclei of the stimuli: slow distension (N=10), rapid distension (N=9), and in control animals (N=10) were identified using the immunohistochemical analysis of *c-fos*. The experimental groups were stimulated 3 times per minute for 3 hours. After the experiment, the brains were removed and processed for *c-fos* immunoreactivity or thionin. We found that slow balloon distension activated the esophago-UES contractile reflex and esophago LES relaxation response, and rapid air injection activated the belch and its component reflexes. Slow balloon distension activated the NTSce, NTSdl, NTSvl, DMNc, DMNr and NAr; and rapid air injection primarily activated AP, NTScd, NTSim, NTSis, NTSdm, NTSvl, NAc and NAr. We concluded that different sets of medullary vagal nuclei mediate different reflexes of the esophagus activated from different sets of mechanoreceptors. The NTScd is the primary NTS subnucleus mediating reflexes from the mucosal rapidly adapting touch receptors, and the NTSce is the primary NTS subnucleus mediating reflexes from the muscular slowly adapting tension receptors. The AP may be involved in mediation of belching.

Keywords

nucleus tractus solitarius; dorsal motor nucleus; nucleus ambiguus; esophageal mechanoreceptors; pharynx; larynx; belching

1. Introduction

Three types of mechanoreceptors of the esophagus have been identified: 1) the muscular slowly adapting tension receptor (Andrew 1957; Harding and Titchen, 1975; Mei 1970; Page et al 2002; Satchell 1984; Sekizawa, et al 2004), 2) the mucosal rapidly adapting touch receptor (Harding and Titchen, 1975; Mei, 1970; Page et al 2002; Sekizawa et al 2004), and

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3) the mucosal slowly adapting tension receptor (Page and Blackshaw, 1998). The location, structure, and reflex responses of the muscular slowly adapting tension and mucosal rapidly adapting touch receptors, but not the mucosal slowly adapting tension receptor have been identified and characterized. The muscular receptor is located in myenteric ganglia and has been identified as intraganglionic laminar endings (Zagorodnik and Brookes, 2000; Rodrigo et al. 1975). Stimulation of this receptor by slow air injection or balloon inflation activates secondary peristalsis (Lang et al 2001; Creamer and Schlegel 1957; Janssens et al 1976), the esophago-UES contractile reflex (Lang et al 2001; Reynolds et al 1987), and the esophago-LES relaxation response (Ryan et al 1977; Jiang et al 2009; Paterson et al 1986). The mucosal rapidly adapting touch receptor is located in the mucosa (Mei 1970; Page and Blackshaw 1998), and may be the intraepithelial free nerve endings (Rodrigo et al 1975; Robles-Chillida et al 1981). Stimulation of this receptor by rapid air injection activates the belch response and its component reflexes: the esophago-UES relaxation reflex, the esophago-glottal closure reflex, and the esophago-hyoid reflex (Lang et al 2001). The location, structure and reflex responses of stimulation of the mucosal slowly adapting tension receptors are unknown, but it has been hypothesized (Page and Blackshaw, 1998) that this receptor is located in muscularis mucosa.

The afferent and efferent connections of the esophagus to the medulla have been identified by tract tracing techniques (Altschuler et al 1989; Barrett et al. 1994; Bieger and Hopkins, 1987; Frycsak et al. 1984; Kalia and Mesulum, 1980), but tract tracing techniques are limited. Tract tracing techniques cannot distinguish among various functions of the afferents and efferents, and they have only been used to trace the termination of afferents in the medulla (Altschuler et al. 1989; Bieger and Hopkins, 1987; Frycsak et al 1984; Kalia and Mesulum, 1980) or to locate the premotor neurons within two synapses of the esophagus (Barrett et al, 1994; Brousard et al, 1998). There are numerous reflex functions mediated by various esophageal receptors acting on different sets of muscles (Lang et al 2001) and some of these pathways are multisynaptic. Therefore, the medullary nuclei involved in the control of the specific functions mediated by the known mechanoreceptors of the esophagus are unknown.

The aim of this study was to identify the areas of the medulla which are involved in the mediation of the reflex effects of stimulation of the muscular slowly adapting tension receptors and the mucosal rapidly adapting touch receptors of the esophagus.

2. Results

We selectively stimulated the muscular slowly adapting esophageal tension receptors or the mucosal rapidly adapting esophageal touch receptors in a group (N=4) of anesthetized cats to determine the physiological responses of these stimuli. In a separate set (N= 29) of anesthetized cats we determined the areas of the medulla activated by these stimuli by quantifying the number of *c-fos* positive neurons.

2.1. Physiological Studies

We found that repeated slow distension of the mid esophagus caused an increase in EMG activity of the cricopharyngeus, CP, and a decrease in pressure of lower esophageal sphincter, LES, but no change in EMG activity of the geniohyoideus, GH, or thyroarytenoideus, TA, and no activation of secondary peristalsis (Fig 1). On the other hand, the rapid injection of air into the mid esophagus caused simultaneous transient increases in EMG activity of the thyrohyoideus, TH, thyroarytenoideus, TA, and cricothyroideus, CT, and decreases in EMG activity of the CP and thyropharyngeus, TP, but not activation of secondary peristalsis or alteration in LES pressure (Fig 2).

2.2 C-fos Studies

Slow distension of the esophagus was associated with an increase in the number of *c-fos* positive neurons (Figs 3-5) in the NTSce (Fig 6), NTSdl, NTSvl, DMNc (Fig 7), DMNr (Fig 6), and NAr.

Rapid air injection into the esophagus was associated with an increased number of *c-fos* positive neurons primarily in the NTSdl (Fig 7), NTSis (Fig 6), NTSim (Fig 6), NTSdm (Fig 6), NTSvl, NAr, NAc (Fig 8), and AP (Fig 9).

Slow distension, but not rapid air injection, of the esophagus not only activated more neurons in the DMNc and DMNr, but the neurons activated had larger (Figs 7 and 10) and more round nuclei (Fig 11).

Discussion

This study found that the medullary nuclei activated in response to stimulation of the reflexes mediated by slow balloon distension of the esophagus differed from those activated in response to stimulation of reflexes mediated by rapid air distension of the esophagus.

Slow distension of the esophagus

We found that slow distension of the esophagus activated the esophago-UES contractile reflex (EUCR) as well as the esophago-LES relaxation response (ELRR), but not secondary peristalsis. It is likely that these responses were mediated by the slowly adapting muscular tension receptors, because similar types of stimuli activate these mechanoreceptors (Harding and Titchen 1975; Sekizawa et al 2004; Page et al 2002; Pager and Blackshaw, 1998), and prior studies have found that removal or topical anesthesia of the esophageal mucosa does not eliminate the EUCR in response to the same stimulus (Lang et al 2001). The EUCR is integrated by the central nervous system through vagus nerves (Lang et al 2001), but the ELRR has been found to be integrated peripherally by the myenteric plexus (Ryan et al 1977, Jiang et al 2009).

Secondary peristalsis was probably not observed in the current study, because we used an anesthetic agent, and anesthesia has been found to inhibit the activation of secondary peristalsis in cats (Lang et al 2001). In addition, there is a refractory period associated with secondary peristalsis of the striated muscle portion of the esophagus due to central inhibition which prevents rapid repeated activation of secondary peristalsis (Sifrim and Janssens, 1996; Pandolfino et al 2005; Bardan et al 2000). We repeatedly stimulated the mid-esophagus, i.e. the striated muscle portion of the cat esophagus, therefore, the stimuli probably occurred during the refractory period thereby preventing the occurrence of secondary peristalsis.

We chose to use an anesthetic agent in the current studies, because we wanted to avoid activating a response, i.e. secondary peristalsis, that itself could stimulate esophageal afferents and thus activate brain areas not specifically activated by the applied stimulus, i.e. slow balloon or rapid air distension. The EUCR and ELRR were probably less inhibited by the anesthetic, because these reflexes are less complex, i.e. they involve fewer synapses, than secondary peristalsis, and the effectiveness of anesthetics in blocking neural pathways is related to the number of synapses involved (Richards 1983; De Jong et al 1968).

NTS subnuclei

The NTS subnuclei activated by slow distension of the esophagus included, in order of significance, the NTSce, NTSdm, NTSdl, and NTSvl. Slow distension of the esophagus activated twice as many neurons in the NTSce than any other subnucleus. This finding is

consistent with prior tract tracing studies which found that afferent fibers from the esophagus primarily terminated in the NTSce (Altschuler et al 1989). However, tract tracing studies cannot discern which reflex functions are mediated by the NTSce. In our prior study (Lang et al 2001) we concluded that it is the slowly adapting muscular tension receptors that mediate secondary peristalsis and the EUCR. Therefore, we conclude from the current study that the NTSce is the primary medullary nucleus that mediates the reflex responses generated by the activation of the slowly adapting muscular tension receptors of the esophagus.

The other NTS subnuclei activated by slow distension of the esophagus, in order of significance, were the NTSdl and NTSvl, which confirms our prior study that these NTS subnuclei are activated by esophageal distension. However, in our prior study we used decerebrate cats in which secondary peristalsis was also activated by esophageal distension, therefore, we were unsure of the source of the afferents that activated these nuclei. The current study eliminates this uncertainty and indicates that the NTSdl and NTSvl are activated by stimulation of the muscular slowly adapting tension receptors of the esophagus. However, the role of these nuclei in generating the reflex responses to stimulation of these receptors is unknown.

DMN

The slow distension of the esophagus caused a significant increase in *c-fos* positive neurons in the DMNc and DMNr. In our prior study (Lang et al 2004) of the medullary nuclei activated during the phases of swallowing in decerebrate cats, we found two sizes of DMN nuclei that were activated during phases of swallowing. The neurons with larger sized nuclei were associated with a motor response, e.g. secondary peristalsis, whereas the smaller nuclei were not. These small neurons not associated with a motor response were probably interneurons (Lang et al 2004; Rossiter et al 1990) and some may have been inhibitory (Lang et al 2004). In our current study, we found that slow balloon distension that activated the ELRR was associated with activation of neurons in the DMNc which had nuclei significantly larger than the control group, and in the DMNr which had nuclei the same size as with the control group. Thus, it is likely that slow esophageal distension activated motor neurons in the DMNc, but interneurons in the DMNr. The DMNc has been found to be the seat of motor neurons that cause LES relaxation (Rossiter et al 1990; Sang and Goyal 2000; Niedringhaus et al 2008), and the DMNr is associated with neurons that cause LES contraction (Rossiter et al 1990; Niedringhaus et al 2008). These findings are consistent with our physiological results that the only LES response observed during slow distension of the esophagus was a decrease in pressure.

In prior studies, the LES relaxation caused by slow distension of the esophagus was found to not be mediated centrally (Ryan et al 1977; Jiang et al 2009), which would preclude the necessity for central inhibition of the LES through activation of the DMNc. However, the prior studies differed from our current study in an important way. Prior studies distended the smooth muscle portion of the esophagus in opossum (Ryan et al 1977; Paterson et al 1986) or the abdominal portion of the esophagus in mice (Jiang et al 2009), whereas we distended the striated muscle portion of the esophagus in cats. Just as secondary peristalsis is mediated centrally when initiated from the striated muscle esophagus but peripherally when initiated from the smooth muscle esophagus (Goyal and Paterson, 1989), so too the mediation of LES relaxation may differ depending on the source of the stimulus. Unfortunately, we could not find in the literature any studies that examined the effects of vagotomy on LES relaxation due to distension of the striated muscle portion of the esophagus. However, even though in prior studies (Paterson et al. 1986) vagotomy did not block LES relaxation due to distension of the smooth muscle portion of the esophagus, vagotomy did decrease the sensitivity of this response. Therefore our studies, as well as that of others (Paterson et al. 1986), suggest a

role for the central nervous system in mediating relaxation of the LES caused by slow distension of the esophagus. This striated muscle esophagus may be a greater source of receptors for this effect than the smooth muscle esophagus.

NA

The slow distension of the esophagus significantly increased the number of *c-fos* positive neurons in the NAr, but not in the NAc. The NAr is the source of motor neurons for the cricopharyngeus muscle in cats (Yoshida et al 1981), and the striated muscle portion of the esophagus in most species (Collman et al 1993; Yashida, et al 1981; Won et al 1998; Holstege et al 1983; Lawn 1964; Bieger and Hopkins, 1987). In our prior study (Lang et al 2004) we had found that application of a similar stimulus, but in decerebrate cats, not only activated EUCR and *c-fos* positive neurons in the NAr, but also secondary peristalsis and *c-fos* positive neurons in the NAc. Although swallowing affects respiration (Paydarfar et al 1995; Feroah et al 2002), there is no reported data that suggests that secondary peristalsis affects respiration. Therefore, it is possible that the difference in activation of the NAc between our current and prior studies may be related to the difference between the anesthetized and decerebrate preparations.

We found in our current studies that slow distension of the esophagus causes activation of the esophago-UES contractile reflex, and in prior studies we (Lang et al 2001) found that this reflex response is mediated by vagal afferents. The primary muscle of the UES is the cricopharyngeus (Lang 2006). Therefore, it is likely that the reflex arc for activation of the EUCR comprises the slowly adapting muscular tension receptors of the esophagus, vagal afferents, the NTSce, NAr, pharyngo-esophageal branch of the vagus nerve (Lang et al 2001), and the cricopharyngeus muscle (Lang 2006).

Rapid air injection into the esophagus

The rapid injection of air into the esophagus activated a short burst of EMG activity of the following muscles at a short delay: TA, TH and CT; while causing a short lasting inhibition of EMG activity of the CP and TP. This stimulus caused a short lasting common cavity pressure response in the esophagus, but no active muscle contractions of the esophagus or LES and no relaxation of the LES. This unique set of responses activated by this unique stimulus defines the belch response as found in the cat (Lang et al 2001) and dog (Lang et al 1988) and many of these responses have also been found during belching in humans (Kharilas et al 1988; Shaker et al 1992; Cook et al 1989). However, in our anesthetized cat model the responses were of shorter duration and lower amplitude. It is likely that the blunted belch response observed in the anesthetized cats was due to central depression caused by anesthesia.

In prior studies (Lang et al 2001) we found that the belch response was mediated by activation of the mucosal rapidly adapting touch receptors, because topical anesthesia or removal of the mucosa eliminated this response while preserving the responses to stimulation of the muscular slowly adapting tension receptors. A slowly adapting receptor activated by mucosal tension, whose location is unknown, has been found in the ferret esophagus (Page and Blackshaw 1998), but its function has not been defined. Since the rapid nature of the injected air in these studies would not be appropriate or sufficient stimulus for activation of these slowly adapting tension receptors, it is unlikely that they participated in the responses observed in our current study.

NTS subnuclei

The NTS subnuclei activated by rapid distension of the esophagus include, in order of significance, the NTScd, NTSis, NTSim, NTSdm, and NTSvl. The primary NTS subnucleus

activated by rapid distension of the esophagus was the NTScd and as many as three times as many neurons were activated than the next highest subnucleus. Interestingly, while slow distension of the esophagus primarily activated the NTSce, rapid distension of the esophagus had no effect on the number of neurons activated in the NTSce. This activation pattern of NTS subnuclei does not match the results from tract tracing studies.

Prior tract tracing studies in rats found that afferents from the esophagus terminate primarily in the NTSce rostral to the obex (Altschuler et al 1989; Fryszak et al 1984) and that the primary premotor nucleus of the esophagus is the NTSce ((Broussard et al 1998; Barrett et al 1994). While our findings regarding the effects of stimulation of the slowly adapting receptors on NTS neurons closely match the results of tract tracing studies in rats, the pattern of activation due to stimulation of the rapidly adapting receptors has little if any similarity to the rat tract tracing studies. These results suggest that with regard to the functions activated by the slowly adapting esophageal receptors, e.g. secondary peristalsis and the EUCR, the cat and rat are very similar, but with regard to those functions mediated by the rapidly adapting receptors, e.g. belching, the rat and cat are different. This difference in function and anatomy between the cat and rat may be due to a difference in techniques, receptive mechanisms, or in brain processing of sensory information.

The *c-fos* technique, as used in this study, involves the immunohistochemical localization of the expression of the immediate-early gene, *c-fos*, in neurons (Dragunow and Faull, 1989) which have been stimulated for a sustained period using a physiological stimulus. Thus, neurons in the sensory as well as motor pathways, regardless of number of synapses involved, should have been affected, although neurons differ in their capacity to express *c-fos* (Dampney and Horiuchi 2003). On the other, tract tracing techniques (Kobbert et al 2000) can only follow a pathway unidirectionally, either orthograde or retrograde, and cannot cross synapses except for pseudorabies virus tracers, and these have only been used to track neurons within two synapses of the esophagus (Broussard et al 1998; Barrett et al 1994). However, it is not likely that these differences accounted for the difference we observed between techniques. The other difference, which may be the most significant, related to the issue discussed in this manuscript, is that the tract tracing studies depend on the agent being injected into the appropriate area of the esophagus, picked up by the appropriate neural structure, and transported by the appropriate neural pathways. It is possible that the tract tracing techniques employed were able to only trace afferents from the muscularis and not the mucosa of the esophagus due to one of these characteristics of the tract tracing technique.

Another possible source of the difference in results between our *c-fos* and prior tract tracing studies (Altschuler et al 1989; Fryszak et al 1984, Broussard et al 1998; Barrett et al 1994) was the species used. This species difference could account for the experimental difference in two ways: difference in sensory receptors or central processing. Studies have found that the cat esophagus contains the rapidly adapting touch receptors (Mei 1970; Harding and Titchen, 1975), but they have not been demonstrated in rats. However, mice (Page et al 2002) do have these mucosal rapidly adapting touch receptors and it is likely that rats do as well. On the other hand, there is a well documented difference in central processing between cats and rats. Cats, as well as humans, and unlike rats exhibit a number of reflexes, e.g. vomiting (Lang and Sarna, 1987), belching (Bredenoord AJ. Smout AJ. 2007), and regurgitation (Lang and Sarna, 1987), that function to cause gastroesophageal reflux of fluid or gas. Therefore, we suggest that the rat does not have the neural programs to govern gastroesophageal reflux, and therefore, afferents from esophagus of rats do not terminate in the same nuclei and have the same patterns of organization as do esophageal afferents from cats or humans. This probably explains the paucity esophageal afferents of rats that

terminate caudal to obex compared to the extensive activation of this region observed in cats when the rapidly adapting receptors of the esophagus are stimulated.

We propose that the NTS_{cd} is one of the NTS subnuclei that integrates functions related to digestive tract reflux events not only because we found that this is the primary NTS subnucleus activated by a stimulus that causes belching and its associated reflexes, but also because the NTS_{cd} is also one of the most strongly activated subnuclei of the NTS in response to vomiting in cats (Miller and Ruggiero 1994).

The NTS_{is} and NTS_{im} were activated to similar levels by rapid distension of the esophagus. These nuclei are the primary pharyngeal premotor nuclei in rats (Bao et al 1995; Barrett et al 1994) and afferents from the trachea and larynx terminate in these nuclei in cats (Kalia and Mesulum 1980). We also found that the NTS_{vl} is activated during by rapid esophageal distension and the NTS_{vl} is one of the major termination sites for laryngeal afferents (Kalia and Mesulum, 1980). Belching is associated with relaxation of the upper esophageal sphincter (Kharilas et al 1986) due to inhibition of the cricopharyngeus (Asoh and Goyal 1978; Lang et al 1991) and with closure of the glottis (Shaker et al 1992) due to contraction of the cricothyroideus and intraarytenoideus muscles (Lang 2006; Monges et al 1978), therefore, it is not surprising that stimulation of belching is associated with activation of the NTS_{is} and NTS_{im}.

The NTS_{dm} was activated by both slow and rapid distension of the esophagus, but only the response to rapid air distension was statistically significant. The NTS_{dm} has been found to be one of the NTS subnuclei most activated by stimulation of the carotid baroreceptors (Dean and Seagard, 1997). In addition, balloon distension of the esophagus at noxious levels has been found to increase arterial pressure and heart rate (Loomis et al 1997; Pickering et al 2001). In our current study we did not record cardiovascular measures, but our stimuli were not at noxious levels as used in prior studies, i.e. 100mmHg (Loomis et al 1997; Pickering et al 2001), therefore, it is unlikely that NTS_{dm} activation in our studies was due to stimulation of baroreceptors. However, the role of the NTS_{dm} in mediating the responses to rapid or slow distension of the esophagus is unknown.

DMN

Rapid distension of the esophagus did not cause any significant increase in the number of *c-fos* positive neurons in the DMN. Considering that the responses to activation of the mucosal rapidly adapting touch receptors of the esophagus (Lang et al 2001), e.g., esophago-UES relaxation response, esophago-glottal closure response, esophago-hyoid response, do not involve any muscle controlled by the DMN in cats (Kalia and Mesulum 1980), it is not surprising that the DMN was not activated.

NA

Rapid distension of the esophagus activated both the NAc and Nar. Tract tracing studies have found that the NAc contains motor neurons of the larynx (Kalia and Mesulum, 1980), and the Nar contains motor neurons of the pharyngeal (Collman et al 1993; Holstege et al 1983; Van Loveren et al, 1985; Yoshida, et al 1981), laryngeal (Pasaro, et al 1983; Yoshida, et al 1981; Kalia and Mesulum, 1980) and hyoid (Holstege, et al 1983) muscles in cats. Therefore, our finding of increased numbers of *c-fos* positive neurons in the NAc and Nar due to activation of the mucosal rapidly adapting touch receptors of the esophagus that activated laryngeal and hyoid muscles as part of the belch response and its component reflexes is consistent with tract tracing studies.

Area Postrema

Perhaps the most surprising result of this study was the strong activation of the AP due to rapid distension of the esophagus. Lesions of the AP alter chemical-induced vomiting (Borison et al 1984; McCarthy and Borison, 1984) and chemical stimulation of vomiting is associated with an increase in the number of *c-fos* positive neurons in the AP (Dejonghe and Horn 2009; Boissonade and Davison 1996; Boissonade et al 1994). However, the AP has not previously been associated with belching (Borison 1988) and the rapid distension of the esophagus has not been reported to cause vomiting (Kharilas et al 1986; Shaker et al 1992). Therefore, we conclude that the AP is not only an important structure for the generation of chemical-induced emesis, but also an important structure for the generation of belching and perhaps other reflux events like regurgitation.

We found in our current study that rapid distension of the esophagus causes activation of the belch response and its component reflexes as well as specific set of medullary sensory and motor nuclei. In prior studies we (Lang et al 2001) found that this set of reflex responses is mediated by vagal afferents, and the muscles of these reflexes are various pharyngeal and laryngeal muscles (Lang et al 2001). Therefore, it is likely that the reflex arc for activation of the belch and its component reflexes comprise the esophageal rapidly adapting mucosal touch receptors, vagal afferents, NTS_{cd}, NTS_{is}, NTS_{im}, NA, vagus nerves, and various laryngeal and pharyngeal muscles.

In summary, selective stimulation of the slowly adapting muscular tension receptors of the esophagus activates a specific set medullary vagal nuclei that differs from those activated by stimulation of the rapidly adapting mucosal touch receptors (Fig 12). Therefore, these studies have identified the reflex arc of some of the reflexes activated by these stimuli. These studies also provide evidence that like vomiting, belching may be mediated in part by neurons of the area postrema and that the subnuclear region of the NTS caudal to obex may contain neurons that integrate gastroesophageal reflux functions.

Methods

1. Animal Preparation

We studied 33 cats of either sex weighing from 2.2 to 4.5Kg. The surgical and experimental protocols were approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin. The cats were anesthetized because we wanted to limit the motor responses to esophageal stimulation. We found in a prior study (Lang et al 2004) that esophageal stimulation in unanesthetized (decerebrate) cats resulted in the activation of numerous motor responses and brainstem nuclei. Some of these nuclei were activated not by the original stimulus, but by the resulting motor response. For example, secondary peristalsis, that was activated by distension of muscular slowly adapting tension receptors, could itself stimulate mucosal receptors. In this study we were interested in identifying the primary medullary nuclei activated by each stimulus rather than nuclei activated secondarily by the stimulus, therefore, we anesthetized the cats. We used alpha-chloralose because this anesthetic preserves reflexes better than other anesthetics. The cats were anesthetized using alpha-chloralose and the dose was titrated to provide a surgical plane of anesthesia while minimizing the drug dose. The cats were first injected IP with 55mg/kg of alpha-chloralose and one hour later their anesthetic plane was assessed. Those animals requiring more anesthesia were administered IP an additional 25% of the original dose. We waited another hour and if the cats still required more anesthesia they were administered Nembutol at 3mg/kg IV. A gastric fistula was placed to allow removal of gastric contents by vacuum to prevent gastroesophageal reflux, and to allow escape of air injected into the esophagus. A 20cm long plastic sleeve (5mm OD) was inserted through the mouth and its distal end

ligated in place just distal to the cricopharyngeus. This sleeve allowed the insertion of devices into the esophagus without stimulating receptors in the mouth, pharynx, or larynx. The femoral vein was cannulated in all animals for intravenous administration of 0.9% NaCl and all cats were placed on a heating blanket to maintain body temperature.

Four cats were used to assess the physiological responses of the stimuli and 29 were used to identify the brainstem nuclei activated by the stimuli. We used a separate set of cats to define the responses to the stimuli, because the surgical preparation to implant devices or record responses may have activated receptors other than the esophageal mechanoreceptors we were investigating. The twenty-nine cats used for *c-fos* studies had no further surgical preparation. The four cats used for physiological studies had bipolar EMG electrodes sewn onto the cricopharyngeus (CP), thyropharyngeus (TP), thyroarytenoideus (TA), cricothyroideus (CT), and thyrohyoideus (TH). A solid state manometric pressure transducer was placed into the esophagus through oral sleeve to record esophageal pressure, and a perfused catheter with a Dent sleeve was placed in the LES through a gastric fistula to record LES pressure. These recording devices allowed us to identify and distinguish the motor responses to esophageal stimulation that include: esophago-UES contractile reflex (EUCR); secondary peristalsis (2P), esophago-LES relaxation response (ELRR), swallowing, and belching. The EUCR was identified as increased EMG of the CP (Lang et al 2001), the ELRR was identified as relaxation of the LES, 2P was identified as a wave of esophageal contraction that propagated caudally (Lang et al 2001), belching was identified as a transient increase in EMG activity of the TA and TH and decrease in EMG activity of the CP and TP (Lang et al 2001).

The esophageal mechanoreceptors were stimulated in two ways, and in each case the stimulating device was first inserted into the esophagus through the oropharyngeal sleeve. Whether for physiological or *c-fos* studies, each animal was stimulated using only one of these methods. While each esophageal mechanoreceptor has been found to be maximally stimulated by a specific type of stimulus in reduced preparations (Page and Blackshaw 1998; Page et al 2002), it was not possible to stimulate these receptors in this way for the current studies where the esophagus was left intact. We used stimuli that primarily activated one set of receptors, but probably did not activate these receptors exclusively. For stimulation of the esophageal muscular slowly adapting tension receptors, we distended a polyethylene balloon. The balloon was 2.5 cm in diameter, 4 cm long and was inflated to 40 mmHg in a sinusoidal fashion using a ventilator set at 3 cycles per minute. For stimulation of mucosal rapidly adapting touch receptors air was injected rapidly in the esophagus for 0.2s at 8 mmHg pressure three times per minute using WPI Pneumatic Picopump. The tubing was blocked at its end and holes placed in the side in order to create a local flow of air tangential to the esophagus. In prior studies (Lang et al 2001) we found that this type of stimulus primarily activated mucosal rapidly adapting touch receptors. For both types of esophageal stimulation the stimulus was applied to the mid esophagus 8-10 cm above the LES. This location is in the thoracic cavity and the esophagus at this location is composed of striated muscle. For the control *c-fos* group a 5mm diameter polyethylene tube was inserted through the oral sleeve into the esophagus, but no stimulus was applied.

Protocols

1. Physiological studies

After recording spontaneous activity for 30 minutes, the mechanoreceptors were stimulated for 30 minutes as described above.

2. C-fos studies

In each cat the esophageal rapidly adapting or slowly adapting muscular receptors were stimulated and for the control group no stimulus was applied for 3 hours to allow sufficient time for maximization of *c-fos* expression. We stimulated for three hours, because we found in a prior study (Lang et al 2004) that this time period was very successful in activating *c-fos* in cats using very similar physiological stimuli. At the end of the experiment, the brain was removed and processed for *c-fos* immunohistochemistry as described below.

Our prior studies using very similar physiological stimuli indicated that the responses observed at three hours were not different from those observed at 30 minutes (Lang et al. 2004). The only difference that occurred over three hours is that the number of responses per stimulus declined, perhaps because of desensitization. Therefore, the physiological responses observed at 30 minutes are representative of the responses observed over three hours.

Techniques

1. EMG Activity

Bipolar Teflon-coated stainless steel wires (AS 632, Cooner Wire, Chatsworth, CA) bared for 2-3mm were placed in each muscle, and the wires were fed into differential amplifiers (Grass P15 or A-M Systems 1800). The electrical activity was filtered (bandpass of 0.1-3.0KHz) and amplified (1000-10,000 times) before feeding into the computer (Lang et al 2001).

2. Manometry

The intraluminal pressures of the esophagus were recorded using a five channel solid state manometric catheter (Gaeltec, Medical Measurements Inc). The individual recording sites were situated 3 cm apart. A water perfused reverse flow Dent sleeve with flow rate at 0.1ml/min was used to record LES pressure. The reversed flow allowed the catheter to be inserted into the LES through the gastric fistula such that the infused water entered the stomach and flowed out the gastric fistula rather than entering and stimulating the esophagus. The pressure of the sleeve was recorded using a Statham pressure transducer. The pressure signals were recorded using Grass DC preamplifiers (P122) of a Grass polygraph (Model 7) and stored on computer using CODAS hardware and software (Lang et al 2001).

3. C-fos immunohistochemistry

At the end of the experiments the animals were administered Nembutol (30mg/kg) and 20ml of Heparin (1000U/ml) intravenously, the trachea was cannulated, and ties were placed around the jugular veins. The animals were then placed on a ventilator (20/min; 15ml/kg) and the chest opened. The jugular veins were ligated and cut toward the head, and the descending aorta clamped. A 14 gauge needle was inserted into the left ventricle and the heart infused with 0.1M PBS at about 50ml/min at a mean infusion pressure of 80-100mmHg. All vessels at the base of the heart except for the aorta were then ligated with a single tie. After infusion of 3L of 0.1M PBS, the infusion was changed to 4% paraformaldehyde fixative in 0.1 M PBS. The brainstem was removed and stored in 0.1M PBS containing 30% sucrose at 4°C for 24-36 hrs. Transverse sections through the medulla (40 μ m) were cut, floated in 0.1 M PBS (pH 7.4), and divided into three sequential groups. Two groups were processed immunohistochemically for *c-fos* immunoreactivity using an avidin-biotin technique with one set of sections incubated in *c-fos* antibody and a second set of control sections incubated in pre-immune sheep serum. A third set of sections were stained with thionin for histological comparison. Sections for immunohistochemical processing were rinsed thoroughly in 0.1M PBS (2×15 mins) and then pretreated for 30

mins in 10% (w/v) heat-inactivated normal rabbit serum (Vector Labs.) containing 0.1% Triton X-100. Sections were washed in 0.1M PBS (2×15 mins) and transferred to the appropriate treatment set containing either sheep anti-*c-fos* protein primary antisera (Genosys; 1:6000) or dilute pre-immune sheep serum (Sigma Immunochemicals; 71:6000) for 18-24 hrs at room temperature. After rinsing with 0.1M PBS (2×15 mins), sections were incubated with biotinylated anti-sheep IgG secondary antibody for 60 mins followed by ABC reagent (Vector Labs.). Sections were again washed in 0.1M PBS (2×15 mins) and reaction incubated with hydrogen peroxide and diaminobenzidine using a nickel intensification procedure. Sections were washed in two additional changes of PBS, floated onto gelatin-coated slides and air dried, after which sections were dehydrated and coverslipped with DPX mounting medium. Sections were examined under a light microscope (Olympus BH-2) to visualize staining for *c-fos* immunoreactivity and adjacent thionin stained sections were compared for histological localization of subnuclei of the nucleus tractus solitarius, dorsal motor nucleus, and nucleus ambiguus. Homologous absorption controls (10 μ M solutions) were performed to examine the specificity of the *c-fos* antibody with the *c-fos* antibody preabsorbed with *c-fos* peptide (Genosys).

4. Microscopic analysis

The *c-fos* is confined to the nucleus of activated cells, visualized in immunopositive cells as a dark, rounded or oval structure. Each subnucleus was identified from corresponding thionin stained sections by comparison with our prior study (Lang et al 2004). Nuclei staining positive for *c-fos* in each brainstem subnucleus were digitally recognized, counted and measured. The *c-fos* positive neurons were quantified in single sections at 0.5 mm intervals from 1.0 mm caudal to 5.0 mm rostral to obex for each animal using a Spot RT digital microscope camera on an Olympus BH-2 microscope. We chose the most representative slice within each 0.5 mm for each cat to accommodate differences in brainstem size and technical artifacts.

We examined neurons of the NTS, AP, DMN, and NA because these are the primary vagal nuclei that mediate reflex responses to esophageal stimulation. These animals were anesthetized to minimize activating motor responses, e.g. secondary peristalsis, that would have confused analysis of results, in order to get a better understanding of the underlying physiology. However, we understood and expected that these studies would underestimate the normal level of responses and nuclei activated. Anesthesia primarily affects multi-synaptic pathways which accounts for the suppression of peristalsis but not more simple reflex responses like the EUCR, therefore, it was expected that medullary motor nuclei might be affected to a greater extent than premotor nuclei. However, by recording the actual responses to the applied stimuli we were able to relate these specific responses to specific activated medullary nuclei.

5. Image analysis

Grayscale microscopic images were digitized at 1600 $\times$ 1200 pixels and stored on computer and analyzed. The contrast of the images was first maximized and the dark areas marked and an overlay created by setting the threshold sufficient to fill the nuclear profiles of the darkest nuclei. This level of detection closely approximated 1/3 of maximum intensity. The following variables of the neuronal nuclei were measured for each brainstem subnucleus using SigmaScan Pro 5.0 software: number, area, and shape factor ($(4 \times \pi \times \text{area}) / \text{perimeter}^2$). The brainstem images contained sections of spherical nuclei cut at various orientations such that the nuclei appeared round to various oval shapes and sizes. The images also contained darkened areas which were not neuronal nuclei, and may have been microglia or histological artifacts. The artifacts were usually elongated objects and the microglia were usually very small and round. We excluded these objects from the analysis

by setting the following exclusion criteria. Firstly, all objects with a shape factor greater than 1.0 or less than 0.2 were eliminated. A shape factor of 1 is a circle and 0 is a straight line, therefore anything above 1.0 was considered an artifact, and anything less than 0.2 was considered too long and narrow to represent a neuronal cell nucleus. A shape factor of less than 0.2 was rare. Objects with an area of less than $15\mu\text{m}^2$ were considered too small to be neuronal cell nuclei. This exclusion criteria probably eliminated some real cell nuclei and resulted in an undercount, but this error was applied consistently across brain sections and therefore was not likely to cause differences among groups. The total number of nuclei with areas of less than $15\mu\text{m}^2$ was less than 5% for any subnuclear region examined. Sometimes neuronal cell nuclei overlapped one another or the threshold procedure picked up only part of the cell nucleus. In both cases the problems were fixed by hand. The overlapping nuclei were separated and the incomplete nuclei were completed. These situations occurred at most twice per subnuclear region examined. After correction of the too large, small, and irregular objects the total count and mean measurements were taken for each subnuclear region of interest. Considering that the sections were $40\mu\text{m}$ thick and the nuclei less than $20\mu\text{m}$ in diameter and that we did not count nuclei in adjacent sections, there was no chance for counting the same nuclei twice in different sections. This automated analysis technique was used successfully in our prior study (Lang et al 2004).

6. Physiological analysis

The physiological recordings were digitized and stored on computer using CODAS hardware and software. The motor responses to the stimuli were inhibited by anesthesia and did not exhibit the same level of activation or sensitivity as in the non-anesthetized state (Lang et al 2001). The basic motor responses exhibited in the awake state are present in the anesthetized state, but in a depressed or blunted manner. The motor responses are less frequent, less strong and have a higher threshold for activation (Lang et al 2001). However, the motor responses were adequate for the purposes of this study which was to identify the set of responses activated by a particular stimulus and not to characterize the normal response to such a stimulus. We characterized these responses in non-anesthetized cats in previous studies (Lang et al 2001, 2004).

7. Statistics

The mean values of the number *c-fos* positive nuclei within each subnucleus were calculated. We also calculated the mean values of the area and shape factor for the DMN. We used ANOVA and Tukey's multiple comparison test for assessing differences among groups. A P value of less than 0.05 was considered statistically significant.

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Abbreviations

ANOVA	analysis of variance
AP	area postrema
CC	central canal
CP	cricopharyngeus
CT	cricothyroideus
DMN	dorsomedial nucleus
DMNc	caudal DMN
DMNr	rostral DMN
EMG	electromyography

ELRR	esophago-LES relaxation response
EUCR	esophago-UES contractile reflex
GH	geniohyoideus
LES	lower esophageal sphincter
LRN	lateral reticular nucleus
NA	nucleus ambiguus
NAc	caudal NA
NAr	rostral NA
NAd	dorsal NA
NaCl	sodium chloride
NTS	nucleus tractus solitarius
NTScd	caudal subnucleus of NTS
NTSce	central subnucleus of NTS
NTScom	commisural subnucleus of NTS
NTSdl	dorsolateral subnucleus of NTS
NTSdm	dorsomedial subnucleus of NTS
NTSim	intermediate subnucleus of NTS
NTSis	interstitial subnucleus of NTS
NTSmed	medial subnucleus of NTS
NTSv	ventral subnucleus of NTS
NTSvm	ventromedial subnucleus of NTS
NTSvl	ventrolateral subnucleus of NTS
PBS	phosphate buffered saline
sRFN	subretrofacial nucleus
sV	subtrigeminal nucleus
TA	thyroarytenoideus
TH	thyrohyoideus
TP	thyropharyngeus
TS	tractus solitarius
XII	hypoglossal nucleus
UES	upper esophageal sphincter
VN	trigeminal nucleus

Figure 1. Effects of slow distension of the esophagus on the larynx, pharynx, hyoid muscle, and esophagus

The sinusoidal inflation of a balloon in the mid esophagus was associated with an increase in CP EMG and decrease in LES pressure. No effect was observed on hyoid or laryngeal muscles. GH, geniohyoid; TA, thyroarytenoid; CP, cricopharyngeus; ESO#, esophageal pressure at #cm from the UES; BAL#, position of esophageal balloon in #cm from the UES.

Figure 2. The effects of rapid distension of the esophagus on larynx, pharynx, hyoid muscle, and esophagus

The rapid injection of air into the mid esophagus caused in increase in EMG activity in the TA, TH, and CT, decrease in EMG activity of the CP and TP, and. TA, and no effect of the esophagus and LES. TA, thyroarytenoideus; TH, thyrohyoideus; TP, thyropharyngeus; CP, cricopharyngeus; CT, cricothyroideus; ESO-#, esophageal pressure #cm from UES; LES, lower esophageal sphincter; IN, inspiration; EX, expiration. A and B are two examples of effects of rapid air injection. Note the common cavity pressure pulse in the esophagus (A) and LES (B).

Figure 3. The effects of slow or rapid distension of the esophagus on the area postrema (AP) and some NTS subnuclei

Note that rapid air distension significantly increased the number of *c-fos* positive neurons in the AP, NTS_{cd}, NTS_{im}, and NTS_{is} compared to control and slow distension. *, $P < 0.05$ for a difference from control; #, $P < 0.05$ for a difference from slow distension.

Figure 4. The effects of slow or rapid distension of the esophagus on some NTS subnuclei
 Note that slow distension of the esophagus significantly increased the number of *c-fos* positive neurons in the NTSce, NTSdl, and NTSvl compared to control, and rapid air distension increased the number of *c-fos* positive neurons in the NTSvl compared to control. The rapid and slow distension responses of the NTSce differed significantly. *, $P < 0.05$ for a difference from control; #, $P < 0.05$ for a difference from slow distension.

Figure 5. The effects of slow or rapid distension of the esophagus on vagal motor nuclei
 The slow distension of the esophagus increased the number of *c-fos* positive neurons in the DMNc, DMNr, and NAr, whereas the rapid air distension of the esophagus increased the number of *c-fos* positive neurons in the NAc and NAr. The rapid and slow distension responses of the DMNc and DMNr differed significantly. *, $P < 0.05$ for a difference from control; #, $P < 0.05$ for a difference from slow distension.

Control

Slow

Rapid

Figure 6. The effects of slow or rapid distension of the esophagus on the *c-fos* immunohistochemistry of the dorsal medulla rostral to obex

Slow distension activated *c-fos* positive neurons in the NTSce and DMNr, whereas rapid air distension activated *c-fos* positive neurons in the NTSis and NTSim. TS, tractus solitarius; NTS, nucleus tractus solitarius; im, intermediate subnucleus; is, interstitial subnucleus; DMN, dorsal motor nucleus; r, rostral to obex. Note that the size of the nuclei of the activated neurons of the DMNr are not much different from those of activated neurons of the NTS.

Control

Slow

Rapid

Figure 7. The effect of slow or rapid distension of the esophagus on the *c-fos* immunohistochemistry of the dorsal medulla caudal to obex

Slow distension of the esophagus activated neurons in the DMNc and rapid distension activated neurons in the NTScd. Note that the nuclei of the activated neurons of the DMNc are much larger than the neurons of the activated NTS neurons and larger than the activated neurons of the DMNr of Figure 6.

Control **Slow** **Rapid**

Figure 8. The effect of slow or rapid distension of the esophagus on the *c-fos* immunohistochemistry of the NAc

Rapid, but not slow, distension of the esophagus significantly increased the number of *c-fos* positive neurons in the NAc.

Figure 9. The effect of slow or rapid distension of the esophagus on the *c-fos* immunohistochemistry of the area postrema (AP)

Rapid distension of the esophagus activated neurons in the AP, whereas neither rapid or slow distension had any effect on NTSm.

Figure 10. The effects of slow or rapid distension of the esophagus on the area of the nuclei of the activated neurons of the DMN

The slow distension of the esophagus activated neurons with significantly larger nuclei in the DMNc and rapid distension of the esophagus activated neuron with significantly larger nuclei in the DMNr than those of the control animals. Rapid and slow distension of the esophagus cause significantly different effects on the size of c-fos nuclei in the DMNc activated. *, $P < 0.05$ for a difference from control; #, $P < 0.05$ for a difference from slow distension.

Figure 11. The effects of slow or rapid distension of the esophagus on the shape factor of the nuclei of the activated neurons of the DMN

The slow distension of the esophagus activated neurons with significantly greater shape factor than those of the control animals in the DMNc. . Therefore, these DMNc neurons had more rounded nuclei. Rapid and slow distension of the esophagus cause significantly different effects on the shape factor of DMNc neurons. *, $P < 0.05$ for a difference from control; #, $P < 0.05$ for a difference from slow distension.

Figure 12. Summary map of the location of vagal nuclei activated by slow or rapid distension of the esophagus

Note that slow distension of the esophagus activated the central, dorsolateral, and ventrolateral subnuclei of the NTS, the caudal and rostral DMN, and the rostral NA. Rapid distension of the esophagus activated the caudal, interstitial, intermediate, dorsomedial, and ventrolateral subnuclei of the NTS, the caudal and rostral NA, and the AP. TS, Tractus solitarius; NTS, nucleus tractus solitarius; cd, caudal subnucleus of the NTS; ce, central subnucleus of the NTS; dl, dorsolateral subnucleus of the NTS; vl, ventrolateral subnucleus of the NTS; vm, ventromedial subnucleus of the NTS; v, ventral subnucleus of the NTS; dm, dorsomedial subnucleus of the NTS; m, medial subnucleus of the NTS; is, interstitial

subnucleus of the NTS; im, intermediate subnucleus of the NTS; DMN, dorsal motor nucleus; DMNc, caudal DMN; DMNr, rostral DMN; NA, nucleus ambiguous; NAc caudal NA; NAr, rostral NA; NAd, dorsal NA; CC, central canal; XII, hypoglossal nucleus; AP, area postrema; LRN, lateral reticular nucleus; VN, trigeminal nucleus; sV, subtrigeminal nucleus; sRFN, subretrofacial nucleus.