THE FUNCTIONAL ORGANIZATION OF AFFERENT VAGAL MECHANISMS CONTROLLING SPECIAL AND GENERAL VISCERAL REFLEX RESPONSES OF THE RAT ESOPHAGUS

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THE FUNCTIONAL ORGANIZATION OF AFFERENT VAGAL MECHANISMS CONTROLLING SPECIAL AND GENERAL VISCERAL REFLEX RESPONSES OF THE RAT ESOPHAGUS

By

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ABSTRACT

The functional organization of esophageal afferent mechanisms controlling special (esophageal motility) and general (cardiovascular) visceral reflex responses was investigated in urethane-anesthetized rats. Techniques utilized included esophageal manometry, vagal nerve cooling, single nerve fiber recording, extracellular recording, and pharmacological receptor blockade and stimulation.

Distal esophageal distension elicits esophageal reflex contractions, and both excitatory and inhibitory cardiovascular reflexes. Based upon the effects of vagal cooling, it is inferred that separate subpopulations of A_δ vagal mechanosensory afferent fibers mediate these reflexes.

Single fiber recording experiments demonstrate that vagal mechanosensory afferent fibers innervating the distal esophagus respond to intraluminal pressure increases over a wide dynamic range and show little adaptation.

The pattern and strength of vagal motor output to the distal esophagus depend on the intensity of vagal afferent input to interneurons at the level of *nucleus tractus solitarii* (NTS). These interneurons respond to esophageal distension with distinct firing patterns. Increasing strength of stimulation changes the firing pattern or intensifies the responses of these interneurons. Load-dependent changes in esophageal reflex motor activities persist after spinal afferent input is eliminated.

In the striated muscle tunica muscularis propria of the rat esophagus, distal inhibition is an inhibitory motor reflex evoked by esophageal mechanosensory afferent input from

the proximal esophagus. The chief underlying process is the activation of $GABA_A$ and/or glycine receptors associated with NTS *submucleus centralis* (NTSc) esophageal premotoneurons. In contrast, deglutitive inhibition does not involve inhibitory amino acid mediated neurotransmission in this region.

Vagal mechanosensory afferent fibers mediating the excitatory component of the esophageal cardiovascular reflex (ECVR) terminate in the immediate vicinity of esophageal premotor neurons comprising the NTSc and activate second-order neurons via glutamate receptors of both the NMDA and non-NMDA subtype. Glutamatergic synapses at the level of the rostral ventrolateral medulla are involved in the mediation of the vasomotor component of the ECVR.

Taken together, the results of this thesis research lead to a more detailed understanding of the mechanisms by which vagal mechanosensory afferents innervating the rat esophagus evoke special and general visceral reflexes. Distension-evoked esophageal reflex contractions and the two components of the ECVR involve functionally distinct vagal mechanosensory afferent fibers and affect separate central pathways originating from NTS interneurons.

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ABBREVIATIONS

3-APPA	3-aminopropylphosphonic acid
γ-DGG	γ-D-glutamyl-glycine
ACh	acetylcholine
AMB	nucleus ambiguus
AMBc	compact formation of AMB
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP	area postrema
AP-5	D, L-2-amino-5-phosphonovaleric acid
CGRP	calcitonin-gene related peptide
ChAT	cholineacetyl transferase
DMV	dorsal motor nucleus of the vagus
DNQX	6,7-dinitroquinoxaline-2,3(1H,4H)-dione
EAA	excitatory amino acid
ECVR	esophageal cardiovascular reflex
ENK	enkephalin
EPSP	excitatory postsynaptic potential
GABA	gamma-aminobutyric acid
GVA	general visceral afferent
GVE	general visceral efferent
HR	heart rate

IGLEs	intraganglionic laminar endings
IPSP	inhibitory postsynaptic potential
LES	lower esophageal sphincter
mAChR	muscarinic cholinoceptor
MAP	mean arterial pressure
MSCP	methscopolamine
NADPH	nicotinamide adenine dinucleotide phosphate
NANC	nonadrenergic, noncholinergic
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
NO	nitric oxide
NPY	neuropeptide Y
NTS	nucleus tractus solitarii
NTSc	subnucleus centralis of NTS
RVLM	rostral ventrolateral medulla
SEM	standard error of means
SLN	superior laryngeal nerve
SP	substance P
SVE	special visceral efferent
TMM	tunica muscularis mucosae
TMP	tunica muscularis propria
TTX	tetrodotoxin

UES	**upper esophageal sphincter**
VIP	**vasoactive intestinal peptide**
VPL	**ventral posterolateral nucleus of the thalamus**
WDR	**wide dynamic range**
WGA-HRP	**wheat germ agglutinin-horseradish peroxidase conjugate**

Chapter One

Introduction

Vagal afferents provide the central nervous system with information required to initiate visceral regulatory reflexes through appropriate efferent pathways. Visceral efferents are of two types: those classified as general are spinal or cranial efferents controlling the activity of smooth muscles, blood vessels and glands; those classified as special are cranial efferents controlling the activity of striated muscles that have a branchiomeric origin. In this dissertation, visceral reflexes are referred to as general or special according to the efferent pathway utilized.

Vagal afferent inputs from the esophagus are known to evoke cardiovascular and motility reflexes (Miller et al., 1997; Loomis et al., 1997). The former is a general visceral reflex, because it is mediated by general visceral efferents. As the special visceral motor column of the brainstem innervates the striated muscle of esophagus (Bieger and Hopkins, 1987), afferent-driven motor control of the activity of these striated muscles is a special visceral reflex.

In keeping with the main thrust of this dissertation, the following overview will address neural systems controlling sensory–motor function of the esophageal body, with the emphasis on esophageal vagal afferents in the rat.

1.1 Structure and function of the esophageal body

The esophagus can be divided into three zones comprising the upper esophageal sphincter (UES), the esophageal body, and the lower esophageal sphincter (LES). The

mammalian esophageal body begins at the caudal edge of the cricopharyngeus, and extends to the rostral limit of the LES, a ring of thickened muscle with elevated intraluminal pressure at the gastroesophageal junction (Conklin and Christensen, 1994; Christensen, 1987; Diamant, 1989a,b). The musculature of the esophageal body executes the esophageal stage of swallowing and clearance of gastric contents from the esophagus when reflux occurs. It consists of three layers: the outer and inner layers of the main muscle coat (the tunica muscularis propria, TMP), and the muscle layer of the mucosa (the tunica muscularis mucosae, TMM) (Perlman and Christensen, 1997). In the rat, muscle fibers in both TMP layers have a spiral orientation (Marsh and Bieger, 1986), whereas in the human they are arranged in an outer longitudinal and an inner circular layer (Perlman and Christensen, 1997). The two layers of TMP contain both striated and smooth muscle, however, the proportion of these two muscle types varies widely between species. In the rat as well as the cow, sheep, dog, rabbit, and guinea pig, nearly the entire TMP is made up of striated muscle fibers, and in amphibians and birds entirely of smooth muscle, but in other species such as cat, opossum, pig and human, striated muscle predominates only in the proximal esophagus and gives way to smooth muscle at different levels (Diamant, 1989a; Christensen, 1987; Miller, 1982; Roman, 1982; Gruber, 1978). The transition from striated to smooth muscle usually lies a little more rostral in the inner circular layer than in the outer longitudinal layer of the TMP. The TMM is composed of smooth muscle throughout the whole organ and is much thicker than it is in the other gastrointestinal viscera (Perlman and Christensen, 1997). In the rat, although the TMM is capable of generating longitudinal and transverse tension (Bieger and Triggle, 1985) and may therefore maintain intraluminal pressure *in vivo*, phasic pressure waves

recorded from the esophagus lumen during swallowing or reflex peristalsis involve only the striated muscle coats (Bieger, 1993a; Lu and Bieger, 1998a).

1.2 Esophageal innervation

The esophagus is innervated by extrinsic vagal and spinal nerves and an intrinsic plexus. General visceral efferent innervation is provided through vagal and spinal sympathetic nerves. It controls the activity of smooth muscle, mucous glands and blood vessels of the esophagus. Special visceral efferent fibers run in the vagal pathway and control the activity of striated muscle in the esophagus. The sensory innervation consists of general visceral afferent (GVA) pathways, including the vagal (parasympathetic) and spinal (sympathetic) systems. Significantly more vagal than spinal afferents have been shown to project to the cat esophagus (Collman et al., 1992). The isolectin I-B4 binding study suggest that the majority of vagal afferent fibers are unmyelinated (Li et al., 1997). Intrinsic innervation provides peripheral neural control of esophageal motor and secretory function. (Diamant and El-Sharkawy, 1977; Diamant, 1989a, 1989b; Cunningham and Sawchenko, 1990; Perlman et al., 1997).

1.2.1 General visceral efferents (GVE)

Vagal GVE fibers innervate the smooth muscle of the esophagus. Preganglionic cells are located within the dorsal motor nucleus of the vagus (DMV) (Weisbrodt, 1976; Niel et al., 1980; Hudson and Cummings, 1985). Traveling to the esophagus through the vagal trunk, these efferents are relayed by neurons in the myenteric plexuses (Gidda and Goyal, 1984; Diamant, 1989; Christensen, 1987). Postganglionic fibers from these plexuses in

turn innervate the smooth muscle layers. Therefore, the activity of the smooth muscle esophagus is regulated by an integrated central and peripheral control mechanism (Gidda and Goyal, 1984; Jacobowitz and Nemir, 1969; Diamant, 1989; Roman, 1982). In the rat, vagal GVE innervation is sparse, because of the nature of striated muscle TMP (Neuhuber et al., 1998).

Spinal GVE fibers to the esophagus arise from postganglionic neurons in the cervical sympathetic ganglia, ganglia of the paravertebral chains, and the celiac ganglion (Niel et al., 1980; Hudson and Cummings, 1985; Cunningham and Sawchenko, 1990). Through the vascular supply and, to a lesser extent, through connections to the vagus nerves, these postganglionic sympathetic fibers terminate in the myenteric and submucous plexuses and in relation to blood vessels. Only a few fibers are confined to the smooth muscle of the distal esophagus (Conklin and Christensen, 1994; Christensen, 1987; Roman, 1982; Jacobowitz and Nemir, 1969). The sympathetic postganglionic efferents use norepinephrine as neurotransmitter, and the majority of these noradrenergic fibers in the esophagus also contain neuropeptide Y (NPY) and/or vasoactive intestinal peptide (VIP) (Uddman et.al., 1995; Cunningham and Sawchenko, 1990). Norepinephrine appears to inhibit contraction of striated and smooth muscle esophagus, most probably via β-receptors (Lyrenäs and Abrahamsson, 1986). The preganglionic neurons of esophageal sympathetic efferents are located in the thoracic spinal segments T_5 and T_6 (Diamant, 1989; Weisbrodt, 1976), and use acetylcholine (ACh) as neurotransmitter. Some neurons also contain enkephalin (ENK) (Schultzberg et al., 1979; Cunningham and Sawchenko,

1990), which has been reported to presynaptically inhibit cholinergic transmission in sympathetic ganglion cells (Konishi et al, 1979).

1.2.2 Special visceral efferents (SVE)

The SVE fibers arise from the nucleus ambiguus (AMB) in the brain stem (Lawn, 1966; Weisbrodt, 1976; Hudson and Cummings, 1985). In the rat, these efferent projections come from a distinct region, termed the compact formation of the AMB (AMBc), and show a crude rostrocaudal organotopy (Bieger and Hopkins, 1987; Altschuler et al., 1991; Barrett, 1994). Axons of AMB esophagomotor neurons exit through the vagal trunk and two of its branches, the superior laryngeal nerve (SLN) and recurrent nerve, and terminate directly on striated muscle cells forming a nicotinic cholinergic synapse, similar to the endplate of skeletal muscle fibers (Miller, 1982; Roman, 1982; Marsh and Bieger, 1987; Diamant, 1989a, 1989b; Bieger, 1993a). These motoneurons contain calcitonin-gene related peptide (CGRP) (Lee et al., 1992; Sang and Young, 1998).

1.2.3 Vagal afferents

1.2.3.1 Anatomy

Esophageal vagal afferent fibers arise from primary sensory neurons located in the nodose ganglion (Diamant, 1989; Christensen, 1984; Roman, 1982). Tracer injection into esophagus in the rat shows that labeled esophageal sensory neurons innervating the cervical and distal segments are mixed. These neurons are concentrated in two regions: the proximal posteromedial glossopharyngeal-vagal cuff and throughout the medial aspect of the nodose ganglion (Altschuler et al., 1989). Vagal afferent neurons

innervating the distal esophagus have relatively smaller cell bodies than those innervating the cervical esophagus (Dütsch et al., 1998). In the cat, these neurons show some topographical separation. Those innervating the cervical esophagus are concentrated in the rostral portion of the nodose ganglion, and those innervating the thoracic esophagus and LES are distributed diffusely throughout the ganglion but with a relative paucity at the rostral end (Collman et al., 1992).

In the medulla, esophageal vagal afferent fibers project via the tractus solitarius to the nucleus tractus solitarius (NTS) (Diamant, 1989; Roman, 1982). In the rat, the central termination is restricted to the central subnucleus of the NTS (NTSc), which extends from the obex to 800 μm rostrad (Altschuler et al., 1989). The esophageal afferents within the NTSc have a crude organotopic distribution, in that fibers arising from more proximal levels of the esophagus terminate at more rostral levels of the subnucleus (Altschuler et al., 1989). Results from an earlier tracing study suggesting that diaphragmatic esophagus and stomach have an overlapping central projection in the medial subnuclei of the NTS (Fryscak et al., 1984) may have resulted partly from tracer spread to the cardia.

In the periphery, vagal sensory fibers from the upper part of the cervical esophagus pass through the SLN; those from the lower cervical and upper thoracic esophagus traverse the recurrent laryngeal nerve, and those from the remainder of the esophagus travel through the esophageal branches of the vagus (Andrew, 1956b; 1956c; Christensen, 1984; Roman, 1982). Vagal sensory fibers have been described as endings primarily between the longitudinal and circular muscle layers, in and around the myenteric ganglia, with only scattered fibers innervating the submucosal and mucosal layer. (Neuhuber et al.,

1998; Cunningham and Sawchenko, 1990; Clerc and Condamin, 1987; Neuhuber, 1987; Rodrigo et al., 1982; Rodrigo et al., 1975). The following terminal structures have been found:

Intraganglionic laminar endings (IGLEs): IGLEs are complex neural formations consisting of a number of fibers distributed throughout a considerable number of intramural ganglia. They have been described as a characteristic neural formation found within the intramural ganglia all along the esophagus in both smooth and striated muscle segments. IGLEs are the most prominent terminal structures of nerves in the esophagus that arise from cells of the nodose ganglion (Rodrigo et al., 1975; Rodrigo et al., 1882). In the rat, nodose ganglion injection of anterograde tracer WGA-HRP, [^{3}H] Leucine, or DiI resulted in heavy labeling corresponding to IGLEs on almost every myenteric ganglion in all portions of the esophagus. In most cases, myenteric neurons in the esophagus are almost completely covered by IGLEs (Neuhuber, 1987; Neuhuber et al., 1998). Besides the esophagus, vagal afferent IGLEs also have been found in the stomach and throughout the small and large intestines; however, the density of such structures gradually decreases from oral to aboral. Furthermore, there are few structural differences in different gut segments, suggesting that they may serve a common function (Berthoud et al., 1997). The function of IGLEs is not well understood; however, it has been postulated that in the esophagus they serve as the structure for tension perception (Christensen, 1984), mediating parasympathetic reflexes and perhaps some sensation (Neuhuber, 1987). This hypothesis has been corroborated recently in an *in vitro* study in the guinea pig, where esophageal vagal tension-sensitive units have been recorded in the vagal trunk. The same nerve fibers were then anterogradely labeled, and the receptive field was consistently

shown to be associated with IGLEs (Zagorodnyuk and Brooks, 2000). It has been also suggested that IGLEs might have local effector function (Neuhuber, 1987), but evidence is lacking (Zheng et al., 1997).

Muscle spindles: Muscle spindles have been found in both layers of the TMP and in the intermuscular space in the striated muscle esophagus in the dog (Asaad et al., 1983). As the classic mechanoreceptive sensor, muscle spindles are believed to serve that function in the striated muscle of esophagus (Christensen, 1984). However, studies in the rat have shown that afferent fibers can rarely be traced to the striated muscle part of esophagus, and muscle spindles have not been found (Neuhuber et al., 1998; Neuhuber, 1987).

Intraepithelial endings: It is believed that fibers in the submucosal and mucosal layers may act as mechano-, thermo-, or chemoreceptors (Cunningham and Sawchenko, 1990). Intraepithelial endings have been found in the esophagus and described as a sensory structure in the cat and monkey (Rodrigo et al., 1975b). These endings could be the morphological substrate of these receptors and presumably be important in the modulation of the normal behavior of this organ (Christensen, 1984). However, in the rat, submucosal and mucosal fibers have been found in significant numbers only in the most rostral part of the esophagus, and some penetrate into epithelium. In the rest of the esophagus vagal fibers have been seen scarcely (Kressel, 1998; Neuhuber et al., 1998; Neuhuber, 1987), suggesting that intraepithelial endings are not well developed except in the cervical esophagus in the rat.

1.2.3.2 Neurochemistry

Although information about the neurochemistry of nodose ganglion neurons is extensive, experimental data about the sensory neurons innervating the esophagus are still limited. The following overview addresses information gathered about esophageal vagal afferents in recent studies.

Calcitonin-gene related peptide (CGRP) and substance P (SP):

The existence of CGRP in vagal esophageal afferents is uncertain. In the rat, CGRP has been described in a small percentage of thin-caliber fibers of esophageal vagal afferent origin (Dütsch et al., 1998; Green and Dockray, 1987). However, in recent work with combined anterograde tracing from nodose ganglion and immunohistochemistry, colocalization of CGRP within vagal afferents has not been observed (Kressel and Radespiel-Tröger, 1999). The majority of esophageal vagal afferents do not contain CGRP in the mouse (Sang and Young, 1998), or the guinea pig (Zagorodnyuk and Brookes, 2000).

SP has been detected in a small number of esophageal vagal afferents in the rat (Green and Dockray, 1987; Kressel and Radespiel-Tröger, 1999). SP immunoreactivity has been detected in some myenteric ganglia (27%) and some fibers coursing through the submucosa (Kressel and Radespiel-Tröger, 1999). The peripheral projections of esophageal vagal afferents have been implicated in the release of SP from their terminals. SP released from vagal terminals could subsequently modulate neurotransmitter release of local myenteric neurons (Kerr et al., 1995), and could execute an effector role (Kressel and Radespiel-Tröger, 1999).

Calcium-binding proteins:

Calbindin and calretinin: Most esophageal vagal afferents contain the calcium-binding proteins, calbindin and calretinin. In the rat, calbindin- and calretinin- containing laminar nerve endings have been demonstrated in the myenteric ganglia of the esophagus, which were concentrated on the upper esophagus, and declined in the middle and lower segments (Kuramoto and Kuwano, 1994; Dütsch et al., 1998; Kressel and Radespiel-Tröger, 1999). In the nodose ganglion, most neurons (87% and 79% in two different studies) projecting to the cervical esophagus are calbindin-immunoreactive, compared with only 40% of neurons projecting to the subdiaphragmatic esophagus. About 80% of perikarya labeled after tracer injection into the cervical esophagus immunostained for calretinin, and only 5% of those labeled after injecting tracer into the subdiaphragmatic esophagus (Dütsch et al., 1998; Kutamoto and Kuwano, 1995). For comparison, it also has been demonstrated that about 18% of neurons in the dorsal root ganglia are immunoreactive for calbindin; however, calretinin immunoreactivity is absent from spinal afferent neurons innervating the esophagus. Thus, calretinin is believed to be a more specific marker for vagal afferent structures in the esophagus than calbindin, which is expressed by both vagal and spinal sensory neurons (Dütsch et al., 1998; Kutamoto and Kuwano, 1995). Functionally, the presence of these calcium-binding proteins in esophageal vagal terminal structures may indicate a low threshold sensor, similar to cutaneous and muscular rapidly adapting mechanoreceptors (Duc et al., 1994).

Neurocalcin: Neurocalcin is a newly identified neuronal calcium-binding protein. In the rat, it has been found in laminar terminals in esophagus and in nodose neurons projecting to the esophagus (in 69.4% neurons projecting to cervical and 29.6% neurons

projecting to the abdominal esophagus). The immunoreactivity of this protein is also shown in the motor endplates of striated muscle and spinal afferents of the esophagus. The function of this protein is not clear (Iino et al., 1998).

Tyrosine hydroxylase: The marker enzyme for catecholaminergic neurons, tyrosine hydroxylase, has been detected in the rat (Kummer et al., 1993) and mouse (Sang and Young, 1998) vagal afferent neurons in the nodose ganglion innervating the esophagus. The functional significance of this finding is not clear (Sang and Young, 1998), although it has been suggested that dopamine is a transmitter of rat primary afferent neurons (Kummer et al., 1993).

Nitric Oxide (NO): Although a high percentage of nodose ganglion neurons are shown to be NADPH-diaphorase positive (Zhuo et al., 1997), vagal afferent neurons innervating the esophagus are negative for NADPH-diaphorase in the rat. Since NADPH-diaphorase activity is considered a marker for NO-synthase, this finding presumably indicates that vagal afferents from the esophagus are non-nitrergic (Dütsch et al., 1998). In the mouse, very few nodose neurons (about 1.5%) innervating the esophagus contain NO synthase (Sang and Young, 1998).

Capsaicin: Work in rats has shown that up to 90% of the IGLEs in the myenteric plexus of the esophagus survived high capsaicin treatment, suggesting that a capsaicin-resistant population primarily innervates the esophagus (Berthoud et al., 1997b). In the ferret, only less than one-third of esophageal vagal afferents are activated by acute capsaicin treatment (Blackshaw et al., 2000).

1.2.3.3 Characterization of vagal afferent receptors

Using single unit recording techniques, studies have reported the functional properties of vagal afferent fibers from the esophagus in the rat (Andrew, 1956a; 1956b; 1956c; Clarke and Davison, 1975) and other species (ferret, Andrews and Lang, 1982; Page and Blackshaw, 1998; Blackshaw et al., 2000; rabbit, Falempin and Rousseau, 1981; dog, Satchell, 1983; sheep, Falempin et al., 1978; cat, Mei, 1970; Harding and Titchen, 1975; opossum, Sengupta et al, 1989; guinea pig, Zagorodnyuk and Brookes, 2000). As information obtained in the rat is very limited, the following discussion is mainly based on the results obtained in other species. Two basic types of vagal sensory receptor have been described, namely the muscle tension receptor and the mucosal receptor (Sengupta, 2000; Cervero, 1994; Andrews, 1986).

Tension receptors:

Esophageal vagal tension receptors are believed to be in series with the muscle layers of the esophagus. These receptors are not uniformly distributed, but appear to be concentrated at the upper and lower ends of the thoracic esophagus. Most studies in recording esophageal vagal afferents show that these fibers have a low level of spontaneous activity and discharge in a slowly adapting manner in phase with esophageal peristaltic movements and with the pressure changes imposed by respiratory movements (Andrews and Lang, 1982; Falempin et al., 1978; Harding and Titchen, 1975; Clarke and Davison, 1975; Andrew, 1956a; 1956c). Studies based on analyzing conduction velocity have demonstrated that the majority of these afferent fibers are thinly myelinated fibers

(Aδ-fibers), and the rest are unmyelinated fibers (C-fibers) (Sengupta et al., 1989; Satchell, 1983; Andrews and Lang, 1982; Falempin and Rousseau, 1981; Mei, 1970).

Two quantitative studies of esophageal vagal afferent fibers have been done in the dog and opossum, and both demonstrate low threshold and saturation pressure. In the dog, the afferent discharge increases linearly in relation to the increase of the distension pressure in a relatively narrow range (from 3 to 8 mmHg), with the maximum discharge rate of 35 Hz (Satchell, 1984). The threshold and saturation pressures in the opossum are 10 mmHg and 70mmHg, much higher than reported in the dog. Moreover, the maximum discharge rate in the opossum is 60 Hz, also much higher than reported in the dog (Sengupta et al., 1989). These differences between the two species may be related to the differences in the physiology of tension receptors in the striated (dog) and the smooth (opossum) muscle regions of the esophagus (Sengupta et al., 1989).

In the rat, the esophageal vagal tension receptors were shown to fire spontaneously, to adapt slowly to distension, and to be modulated by respiration. On releasing the distension a period of silence was observed, before the spontaneous activity was restored (Andrew, 1956c; Clarke and Davison, 1975). Unit activities recorded from SLN fibers innervating the cervical esophagus in the rat (striated muscle) were shown to reach firing frequencies as high as 300 Hz when the bolus was pushed into the innervation zone (Andrew, 1956c). When a propulsive wave was elicited, muscle activity occurred after the beginning of the rise in the firing, and corresponding to the phase of maximal firing of the afferent fiber (Andrew, 1956c). This suggests that the muscle is reflex activated and the sensory ending is in series with the contractile elements.

The anatomic location and arrangement of these esophageal muscle tension receptors still need to be determined. The best-described structures for tension perception by vagal afferent tracer studies are IGLEs, which just have been shown to be associated with the receptive field of vagal afferents in the guinea pig esophagus (Zagorodnyuk and Brookes, 2000).

Mucosal receptors

Information about physiological properties of vagal afferents innervating the rat mucosa is still limited. It has been suggested that afferent fibers recorded from the SLN innervating the rat cervical esophagus are from mechanosensitive mucosal receptors (Andrew, 1956b; Sengupta and Gebhart, 1994). However, in the rat, as this type of innervation is only concentrated in the cervical esophagus and very sparse in the rest segments (Neuhuber, 1987; Kressel and Radespiel-Tröger, 1999), and the epithelium of esophagus is moderately to extensively keratinized (Hebel and Stromberg, 1986), we can surmise that mucosal receptors are not as well developed in the esophageal body as in some other species, such as the cat. The following discussion is based on experiments done mainly in the cat, except when indicated otherwise.

Mucosal receptors have been described as rapidly adapting mechanoreceptors that characteristically discharged briefly on esophageal distension and again on deflation, with a low threshold and high firing frequency. Some of them, especially in the caudal esophagus, also respond to chemical stimulation, such as acid, base and hypertonic NaCl. The conduction velocity of these afferents is in the range of thinly myelinated fibers (Andrews, 1986; Mei, 1970; Harding and Titchen, 1975). These receptors would be ideally suited for detecting the presence of boli that produce very low levels of distension

insufficient to activate the muscle receptors, as well as refluxed gastric contents (Andrews, 1986). A recent work has shown that 28 percent of mucosal mechanoreceptors also respond to chemical stimulation (Page and Blackshaw, 1998). Thus, some of the mucosal receptors have the polymodal character.

In addition to mucosal mechanoreceptors, another study (El-Ouazzani and Mei, 1982) has identified three types of slow adapting receptors responding to temperature change: warm receptors discharged between 39 and 50 °C; cold receptors between 10 and 35 °C; and mixed receptors responded in both temperature ranges. They were silent in the normal temperature range, discharged in relation to the stimulus temperature, and did not respond to mechanical or chemical stimulation. These thermoreceptors were shown to be connected to unmyelinated fibers with a conduction velocity around 1 m/s. Since stimulation of these receptors produced changes both in esophageal motility and in respiratory frequency, they were thought to be involved in the coordination of digestive activity as well as in thermoregulation.

1.2.4 Spinal afferents

Esophageal spinal afferent fibers arise from primary sensory neurons located in the lower cervical and upper thoracic dorsal root ganglia. Centrally, these fibers project to the dorsal horn of the spinal cord (Hudson and Cummings, 1985; Khurana and Petras, 1991; for review see Cervero, 1994; Cunningham and Sawchenko, 1990; Christensen, 1984). In the rat, single unit activity evoked by esophageal distension has been recorded from the neurons in the upper thoracic spinal cord. Most units show a wide dynamic range and

encode all intensities throughout the non-noxious and noxious range (Euchner-Wamser et al., 1993). In the opossum, recordings from afferent fibers in the paravertebral sympathetic chain and splanchnic nerves have shown that these spinal afferents are composed of small myelinated and unmyelinated afferent fibers connected to two types of mechanoreceptors, wide dynamic range (60% of the sample) and high-threshold (40% of the sample) mechanoreceptors (Sengupta et al., 1990). Both types of mechanoreceptor are activated by systemic bradykinin through a B_2-receptor subtype on the fiber ending (Sengupta et al., 1992).

In the rat, neurochemical studies have shown that the major proportion of esophageal spinal afferents is CGRP-immunoreactive (54-99%) and SP-immunoreactive (17-46%) (Uddman et al., 1995; Green and Dockray, 1987). Generally, it is believed that the esophageal spinal afferent pathway is involved in the transmission of esophageal nociceptive information (Lynn, 1992; Cervero, 1994).

1.2.5 Intrinsic innervation

The enteric nerves give rise to two networks, one named myenteric plexus and forming a sheet between the two main muscle layers, and the other called submucosal plexus and lying within the substance of the submucosa (Perlman and Christensen, 1997; Diamant, 1989; Christensen, 1987). Nerve cells in the plexuses are grouped together in ganglia (Van Driel and Drukker, 1973). In general, there are three kinds of nerve cells in the myenteric plexus: sensory neurons, interneurons, and motor neurons.

In the striated muscle, the plexuses are traditionally believed to serve mainly a sensory role, because the striated muscle is innervated by vagal motor fibers directly

through nicotinic receptors. (Diamant, 1989; Christensen, 1987; Marsh and Bieger, 1987). However, a direct nitrergic innervation of the motor endplate from the myenteric plexus neurons has been reported in the rat esophagus (Neuhuber et al., 1994; Wörl et al., 1997; 1994; Kuramono et al., 1999). Nitric oxide has been shown to modulate contractile properties of the striated muscle (Kobzik et al., 1994). However, in rat *in vitro* vagus nerve-esophagus preparations, nitric oxide donor drugs are ineffective in altering nerve-evoked twitch or tetanic responses (D. Bieger, unpublished observations). Recent work has shown that this innervation also contains galanin and vasoactive intestinal polypeptide (Kuramono et al., 1999). Therefore, possibly the local enteric nervous system of the esophagus has the ability to integrate the activity of striated muscle in the periphery.

The motor neurons in the myenteric plexus innervating smooth muscle represent at least two types, one mediating cholinergic excitation, and the other mediating nonadrenergic, noncholinergic (NANC) inhibition of the smooth muscle (Miller et al., 1997; Diamant, 1989). The former releases acetylcholine (ACh) acting on the muscarinic receptors (mAChR), and the latter probably uses nitric oxide (NO) as a neurotransmitter (Perlman and Christensen, 1997; Miller et al., 1997; Bult et al., 1990). The cholinergic innervation determines the amplitude of peristaltic contraction, and the nitritergic innervation determines the timing of peristalsis (Miller et al., 1997).

Some other neurotransmitters present in the esophageal myenteric plexus, such as CGRP and substance P, probably characterize sensory nerves, and others like vasoactive intestinal peptide (VIP), galanin, NPY and bombesin, seem to endow nerves with more than one function (Perlman and Christensen, 1997; Uddman et al., 1995).

1.3 Special visceral reflexes evoked by esophageal vagal inputs

At rest, the esophageal body is quiet and without motor activity. Contractions can be evoked by swallowing or events such as gastroesophageal reflux. The basic pattern of esophageal movement is characterized by a propulsive contraction of the esophageal musculature propagating in the aboral direction. This pattern is generally described as peristalsis, which has three principal modes of initiation (Diamant, 1989; Miller, 1982; Roman, 1982; Diamant and El-Sharkawy, 1977; Ingelfinger, 1958).

1.3.1 Esophagomotor patterns

Primary peristalsis: The esophageal contraction that is preceded by a voluntary or reflex swallow is called primary peristalsis (Meltzer, 1907; Ingelfinger, 1958; Fleshler et al., 1959; Diamant, 1997). Although it represents the esophageal component of swallowing, primary peristalsis does not necessarily follow buccopharyngeal deglutition in a one to one ratio during repetitive swallowing. When swallows are elicited in quick succession, esophageal contraction will only occur after the last swallow is completed (Meltzer, 1899). This phenomenon is called deglutitive inhibition (Hellemans et al., 1974; Roman, 1982). In the striated muscle esophagus, a second swallow causes rapid and complete inhibition of ongoing peristalsis evoked by the previous swallow. Inhibition of esophagus peristalsis in response to repetitive swallows, however, is much more pronounced in the striated muscle esophagus, and may be programmed centrally (Vanek and Diamant, 1987; and for review see Cunningham and Sawchenko, 1990).

Secondary peristalsis: This is the response of the esophagus to local stimulation, such as the distension of the esophageal wall. It is not directly related to swallowing. Under physiological conditions, this type of rhythmic peristalsis is elicited by material either refluxing from stomach, or left behind in the esophagus after a primary peristaltic activity. Experimentally, it can be evoked by inflating a balloon that has been placed in the esophagus (Roman, 1982; Fleshler et al., 1959; Ingelfinger, 1958; Meltzer, 1907).

Tertiary peristalsis: This type of esophageal peristalsis is also called autonomous peristalsis, and occurs only in the smooth muscle portion of the esophagus. As the propulsive contraction can be generated *in vitro* in response to local mechanical or electrical stimulation, this type of activity is not dependent on a central mechanism (Miller et al., 1997; Diamant, 1989; Roman, 1982). The tertiary peristalsis may not exist in the rat esophagus because of the nature of striated muscle TMP. Although intrinsic innervation of striated muscle tunica has been found (Wörl et al., 1994; 1997; Kuramono et al., 1999), to date there is no evidence that tertiary peristalsis can be organized by this innervation.

1.3.2 Central control of esophageal motility

In the striated muscle esophagus, both primary and secondary peristalses are centrally organized (Diamant, 1989; Bieger, 1993a). Concerning the smooth muscle esophagus, two hypotheses have been proposed for the control of the peristalsis. One is that the peristalsis is programmed centrally and modulated peripherally by the intramural mechanism; the other is that a central mechanism triggers an intramural system that

coordinates the peristalsis (Diamant, 1989; Christensen, 1987). The following discussion will address the central motility control of the striated muscle esophagus.

As a brainstem vago-vagal reflex arc, the central esophagomotor pathway consists of an afferent limb, central interneurons and an efferent limb. Two regions of the brainstem are essential for the central control of esophageal motility: One is in the dorsal brainstem around the NTS, containing neurons located in the NTS and the adjacent reticular formation. This is the central afferent system and a vital part of the central neural control for swallowing. The other region is in a more ventral site around the AMB, which probably includes neurons in the lateral reticular formation near the AMB. As an efferent system, motoneurons here innervate the pharynx, larynx, and esophagus (Miller et al., 1997; Roman, 1982).

In the rat, the esophageal striated muscle appears to be controlled by a distinct medullary neural structure, specifically the NTSc-AMBc pathway. This pathway consists of primary afferent neurons in nodose ganglion, esophageal premotoneurons in NTSc, and esophageal motoneurons in AMBc (Broussard et al., 1998; Barrett et al., 1994; Bieger, 1993a; Altschuler et al., 1989; Bieger and Hopkins, 1987). According to their connectivity, NTSc interneurons fit to the esophageal secondary sensory neurons or esophageal premotor neurons. These neurons receive esophageal primary afferent input, and project directly to the AMBc esophageal motoneurons controlling esophageal motility (Lu and Bieger, 1998a, 1998b; Barrett et al., 1994; Bieger, 1993a; Altschuler et al., 1989; Bieger and Hopkins, 1987).

1.3.3 Neurotransmitters and modulators operating in central esophageal motor control

Excitatory amino acids (EAAs): EAAs such as glutamate and aspartate are major excitatory neurotransmitters in the central nervous system and can activate most mammalian neurons (Ozawa et al., 1998). As regards esophageal motor control, an EAA appears to be the most likely transmitter candidate at the both esophageal premotoneuronal and motoneuronal levels (Miller et al., 1997; Bieger, 1991).

Studies reported to date suggest that a glutamate-like substance is the neurotransmitter released from esophageal vagal afferents. In the rat nodose ganglion, immunodetectable glutamate is present in a large population of vagal afferent neurons (Lawrence, 1995; Schaffar et al., 1997). During local blockade of solitarial EAA receptors of N-methyl-D-aspartate (NMDA) or non-NMDA subtype in the NTSc esophageal premotoneuronal level, both neuronal discharges and reflex responses to esophageal distension were eliminated or strongly inhibited, suggesting that both NMDA and non-NMDA receptors are involved in the neurotransmission from esophageal vagal afferents to premotoneurons (Lu and Bieger, 1998b). When agonists were ejected in the NTSc, NMDA receptor stimulation was more potent in eliciting an esophageal response than kainate and/or AMPA receptor stimulation (Bieger, 1984; Hashim and Bieger, 1989; Bieger, 1993b), indicating a predominant role of the NMDA receptor. Indeed, NMDAR1 mRNA is expressed in NTSc esophageal premotoneurons (Broussard et al., 1994).

EAA receptor-mediated transmission also plays an important role in information transfer from NTSc esophageal premotor neurons to motoneurons (Wang et al., 1991). In

the rat AMBc, local pulse ejection of glutamate evoked esophageal contraction at short latency (Bieger, 1984). Local pulse ejection of an NMDA antagonist blocked fictive esophageal peristalsis evoked by topical application of muscarine to the NTS surface. In an *in vitro* brainstem slice study, excitatory postsynaptic potentials (EPSP) were evoked by stimulation of presumptive solitario-ambigual pathway (Wang et al., 1991). NMDA receptor antagonists blocked the fast component, while non-NMDA receptor suppressed both the fast and slow components (Wang et al., 1991). This suggests that both NMDA and non-NMDA receptors are involved in neurotransmission at solitario-ambigual synapses, but activation of NMDA receptors is necessary for fast information transfer. By means of situ hybridization, NMDAR1 mRNA have been shown to exist in the AMBc esophageal motoneurons (Broussard et al., 1994).

Acetylcholine (ACh): ACh is thought to be an important neuromodulator at both the esophageal premotoneuronal and motoneuronal levels in the rat.

The esophageal premotoneurons in the NTSc require a muscarinic cholinoceptor-mediated input to generate the premotor drive that engages motoneurons in the firing pattern appropriate for peristalsis (Miller et al., 1997; Bieger, 1993b). Activation of these muscarinic cholinoceptors gives rise to rhythmic patterned esophagomotor output that resembles secondary peristalsis, and, conversely, blockade of these receptors abolishes swallow-induced esophageal peristalsis (Miller et al., 1997; Lu et al., 1997; Bieger, 1984). Information about the source of cholinergic afferents to the NTSc is still incomplete. Although the existence of cholinoacetyl transferase (ChAT)-immunoreactive neurons has been reported in the rat nodose ganglion (Palouzier et al., 1987), it is unlikely that esophageal vagal afferents themselves utilize ACh as their transmitter, because electrical

stimulation of the solitary tract only evoked fast synaptic responses that are resistant to the antagonists of muscarinic cholinoceptors (Lu and Bieger, 1998b).

Central cholinergic mechanisms are also involved in neurotransmission at esophageal motoneurons in the AMBc. When locally ejected into the AMBc, ACh evokes various types of short-latency, non-rhythmic esophageal contractions (Wang, 1991a; Bieger, 1984). Both mAChR and nicotinic cholinoceptors have been found within the AMB (Arneric et al., 1990; Swanson et al., 1987); however, studies suggest that nicotinic cholinoceptors mediate a fast inward current in AMBc neurons, leading to burst discharges and contraction of striated muscle esophagus (Zhang et al., 1993; Wang et al, 1991a). The nicotinic receptor-mediated excitation of esophageal motoneurons is subject to modulation by somatostatin, a putative transmitter in the solitario-ambigual pathway (Wang et al., 1993). Since the majority of NTSc esophageal premotoneurons are not ChAT positive (Cassell and Talman, 2000; Ruggiero et al., 1990), ACh is unlikely to be the neurotransmitter used by NTSc premotoneurons. A study combining retrograde tracing with choline acetyltransferase immunocytochemistry demonstrates that the AMBc receives a projection from a subpopulation of cholinergic neurons in the zona intermedialis reticularis parvicellularis (Zhang et al., 1993). Electrical stimulation of this region in slices evoked fast excitatory postsynaptic potentials (EPSPs) in AMBc neurons that were inhibited by nicotinic cholinoceptor antagonists (Zhang et al., 1993).

Gamma-aminobutyric acid (GABA): GABA is considered the principal inhibitory neurotransmitter at supraspinal levels of the mammalian central nervous system. In the rat, $GABA_A$ receptor $\alpha 1$ mRNA has been found in NTSc esophageal premotoneurons

(Broussard et al., 1996). Activation of $GABA_A$ receptor with agonist applied to the NTS surface strongly inhibited both the buccopharyngeal and esophageal stage of swallowing (Wang and Bieger, 1991). In the NTS ventralis and intermedialis, local ejection of $GABA_A$ receptor antagonist evoked pure pharyngeal or repetitive complete swallows, whereas in the NTSc, the antagonist produced peristaltic-like esophageal contractions (Wang and Bieger, 1991; Bieger, 1991). These results suggest that GABA neurons provide tonic inhibitory input that maintains buccopharyngeal and esophageal NTS premotoneurons in a quiescent state (Miller et al., 1997; Bieger, 1993b). GABA immunoreactivity has been found in a population of the neuronal cell bodies in the nodose ganglion (Szabat et al., 1992; Broussard and Altschuler, 2000); however, it is doubtful that GABA is used as the neurotransmitter by esophageal vagal afferents. In brainstem slice preparations, electric stimulation of the solitary tract reportedly evokes only EPSPs in NTSc region neurons (Lu and Bieger, 1998b). A recent study using an *in vitro* brainstem-cranial nerve preparation has shown that glutamatergic-mediated excitation is the only response to vagal stimulation and suggested that the evoked inhibitory postsynaptic potentials (IPSPs) to the stimulation of the solitary tract in other earlier investigations were due to direct activation of intrinsic inhibitory neurons or fibers near the stimulating electrode (Smith et al., 1998).

1.3.4 Role of esophageal vagal afferents in motility control

Esophageal vagal afferents are important in esophageal motility control. Sensory input required for the reflex control of esophageal motility is conveyed by vagal afferents. Under normal circumstances, the esophageal central network pattern generator is

modified by esophageal vagal afferents that adjust the force and progression velocity of the peristaltic contraction to the esophageal contents (for reviews see Miller et al., 1997; Cunningham and Sawchenko, 1990; Christensen, 1987; Jean, 1984b, Roman, 1982). Generally, esophageal distension may evoke both excitatory and inhibitory motility responses at different levels of the organ.

At or above the level of distension, secondary esophageal peristalsis is elicited in response to distension. Bilateral proximal vagotomy abolishes this response in the striated muscle esophagus (Lu and Bieger, 1998a; and see Miller et al., 1997; Cunningham and Sawchenko; 1990; Christensen, 1987; Jean, 1984b, Roman, 1982; Ingelfinger, 1958 for reviews). In the rat esophagus, different types of responses are evoked, depending on the level stimulated. Distension of the cervical and thoracic portion causes a single pressure wave, whereas the diaphragmatic segment responds with rhythmic contractions (Lu and Bieger, 1998a)

Distension of an esophageal balloon inhibits either primary or secondary peristalsis below the level of the balloon, a response termed "distal inhibition" (Sifrim and Janssens, 1996; for reviews see Jean, 1984b; Roman, 1982; Diamant and El-Sharkawy, 1977). In the striated muscle portion of the esophagus, a vago-vagal reflex organizes distal inhibition. In the smooth muscle esophagus, although it is suggested that an intramural peripheral control mechanism is responsible for distal inhibition, afferent sensory input from the esophagus plays an important role in initiating this inhibition. (Diamant and El-Sharkawy, 1977). Distal inhibition has not been studied in the rat esophagus.

1.4 General visceral reflexes evoked by esophageal vagal inputs

Esophageal distension is known to evoke changes in blood pressure and heart rate. Several studies have measured these cardiovascular responses evoked by balloon distension; however, both the magnitude and direction of the cardiovascular changes reported differ among preparations, species and laboratories. It likely that the location of the distension balloon and the depth or type of anesthesia may be factors that can alter the direction of blood pressure changes (Satpathy and Al-Sattar, 1984; Sengupta et al., 1990; Meller and Gebhart, 1991; Euchner-Wamser et al., 1993; Loomis et al., 1997). Recently, a detailed study in the urethane-anesthetized rat (Loomis et al., 1997) has shown that both arterial pressor and tachycardia responses are evoked by esophageal distension. This esophageal cardiovascular reflex (ECVR) can be attenuated by unilateral, and blocked by bilateral, cervical vagotomy, suggesting that this is a vagally mediated reflex. During distension of the esophagus, the rise in arterial blood pressure and heart rate starts at a very low inflation pressure, increases logarithmically with inflation pressure, and is more effectively elicited in the distal esophagus. Morphine, when applied intravenously or on the NTS surface, but not intrathecally at T4-T5 segments, inhibited the ECVR in a dose-dependent fashion, suggesting the spinal afferents are not involved in ECVR. The detailed mechanism of ECVR remains to be determined.

1.5 Esophageal nociception

1.5.1 General aspects

Recurring substernal chest pain is an important clinical problem because of its association with cardiac disease. However, not all recurrent chest pain is of cardiac origin.

A survey in the United States estimated that approximately 600,000 new patients per year have cardiac catheterization, and in the 30% patients who have normal coronary arteries, 50% have a demonstrable esophageal abnormality (Richter et al., 1989). The specific role of the esophagus in this condition has been extensively studied, but the results have been controversial and have raised further questions. The specific mechanisms by which esophageal abnormality produces chest pain are not well understood.

Recent clinic studies suggest that a dorsal column pathway is essential for visceral pain transmission. Neurosurgical interruption of the midline posterior column has provided significant pain relief in cancer patients in whom visceral pain had been refractory to other therapies (Willis et al., 1999; Nauta et al., 2000). In animal experiments, a lesion of the rat dorsal column or nucleus gracilis dramatically reduced the responses of neurons in the ventral posterolateral nucleus of the thalamus (VPL) to noxious colorectal distension. In contrast, a lesion of the ventrolateral column greatly reduced the responses of VPL cells to noxious cutaneous stimuli, but not noxious colorectal distension (Al-Chaer et al., 1996; Al-Chaer et al., 1997). In a behavioral study on rats, cervical dorsal column lesions significantly reduced pain responses evoked by acute duodenal distension (Feng et al., 1998). As regards esophageal pain transmission, the connection between the spinal or vagal afferents and dorsal column nuclei need to be established; in other words, the involvement of a dorsal column pathway remains to be demonstrated. Previous tracing studies have failed to reveal connections between dorsal column nuclei and esophageal vagal afferent system (Altschuler et al, 1989; Broussard et al., 1998).

1.5.2 Afferent mechanism

It is generally believed that esophageal pain is mediated by the spinal afferents of the esophagus, while vagal afferents are thought to contribute to other nonpainful esophageal sensations as well as physiologic reflexes and homeostatic mechanisms (Cervero, 1994; Lynn, 1992). Manometric studies in the opossum suggest that esophageal vagal tension receptors have a low mechanical threshold and a low saturation pressure, while spinal mechanoreceptors have either a high-threshold for activation or a wide dynamic range, and are suitable for nociception processing (Sengupta et al., 1990; Sengupta et al., 1989). However, some indirect evidence for an involvement of vagal afferents in the genesis of esophageal pain in patients with high spinal cord lesions or sympathetic chain removal has been noticed (Andrews and Lawes, 1992). On the other hand, it has been proposed that some vagal afferents may have synaptic input to dorsal horn neurons at the level of C1-C2 (Chandler et al., 1996). Therefore, the contribution of vagal afferents to esophageal pain awaits further study.

Besides evoking the sensation of "pain", visceral or somatic noxious stimuli elicit a characteristic pattern of "pseudaffective" reflex responses (Sherrington, 1906). These accompanying responses, including defensive behavior, cardiovascular, respiratory and visceromotor changes, are brainstem or spinal reflexes that suggest emotive or affective responses to noxious stimuli. When the noxious stimulus is terminated, these responses cease (Ness et al, 1990; Ness and Gebhart, 1990). Generally, it is believed that the accompanying responses with a visceral pain during the distension of the gut are conveyed by sympathetic nerves, because they can be blocked by splanchnectomy and produced by stimulation of the splanchnic nerves. However, there is also evidence of the

involvement of parasympathetic nerves (Ness and Gebhart, 1990). A recent study suggests that pseudoaffective cardioautonomic responses to gastric distension in rats are mediated by vagal afferents (Tougas and Wang, 1999)

Jänig posited four dimensions of pain, and they can be roughly assigned to certain brain areas as follows: the sensory-discriminative dimension is related to the somatosensory cortex and sensory thalamus, the motivational-affective dimension is assigned to the limbic forebrain and the limbic midbrain area, the cognitive dimension can be identified with the frontal cortex and the association cortex, and the motor and autonomic components are located in but not limited to the spinal cord. (Jänig, 1987). It is suggested that nociceptive input through the vagus nerve is important for the emotional-affective component of pain, but not the sensory-discriminative aspect (Traub et al., 1996). For example, cardiovascular reflexes produced by intraatrial administration of bradykinin begin prior to the onset of spinal unit responses; moreover, bilateral vagotomy changes the direction of blood pressure change caused by intracoronary injection of capsaicin (Ness et al., 1990). These studies support the idea that some components of esophageal nociceptive input depend on vagal afferent input. Thus the ECVR may represent an autonomic component of esophageal pain in the rat.

1.5.3 Vagal afferent modulation of nociception

Chemical, electrical or physiological activation of vagal afferents results in either facilitation or inhibition of spinal nociceptive processing in some species (Randich and Gebhart, 1992). The facilitation of the tail flick reflex produced by electrical stimulation of the cervical vagus occurs at lesser stimulation intensities, while the inhibition is usually

obtained with high intensities and is intensity dependent (Randich and Gebhart, 1992). The issue of whether activation of vagal afferents is either aversive or noxious and, in addition, whether the resultant states are responsible for either the facilitation or inhibition effects of activation of vagal afferents, remains to be resolved (Randich and Gebhart, 1992). Recent studies have shown that the vagus nerve has receptors for interleukin 1β and can detect stimulation caused by inflammation and infection (Ek et al., 1998; Goehler et al., 1999).

1.6 Research plan

1.6.1 Rationale and hypotheses

The foregoing overview summarizes currently available information about esophageal vagal afferents, especially those from mechanosensory receptors. Anatomically, the vagal mechanosensory afferent fibers originate from myenteric ganglia located between the two muscle layers of the esophagus, and terminate in the NTSc and its immediate vicinity. Functionally, the activation of these vagal afferents is known to evoke special and general visceral reflexes. Although important details have recently come to light, considerable gaps still remain in our knowledge of the range of functions served by esophageal mechanosensory vagal afferents and of the mechanisms by which they activate brainstem neurons responsible for programming special and general visceral reflex outputs.

1. Although morphologically homogeneous, rat esophageal vagal afferents display neurochemical differences as evidenced by immunostaining intensities for calcium binding proteins and SP (Kressel and Radespiel-Tröger, 1999). This suggests that these

afferents fall into different functional populations with distinct physiological features. Presumably these afferent fibers can be discriminated functionally by means of biophysical methods. However, to date this idea has not been tested.

2. Esophageal distension in the rat evokes a vagus nerve dependent rise in arterial blood pressure and heart rate that increases logarithmically with inflation pressure even in the high-pressure range (Loomis et al., 1997), suggesting that esophageal vagal afferents have the ability to detect tension signals in the high-pressure range. This inference contradicts available information from other species where tension-sensitive esophageal vagal afferents were shown to have a low saturation pressure (e.g. Sengupta et al., 1989). Thus, there is a need to determine the dynamic range of esophageal vagal afferents in the rat.

3. Given that vagal afferents can encode high-pressure signals, it appears warranted to examine if vagal mechanosensory input in the noxious range alters reflex esophagomotor output. As yet, the effects on esophageal motility of distension at supraphysiological pressure levels have not been investigated.

4. Anatomical evidence has shown that esophageal vagal afferents in the rat project to the NTSc and its immediate vicinity (Altschuler et al., 1989). In the esophageal motility control, the NTSc interneurons receiving vagal afferent input also serve as the premotoneurons to program esophageal motor output (Lu and Bieger, 1998a; 1998b). These neurons must have the ability to encode information over the entire dynamic range of vagal afferent inputs, and then program an appropriate motor output. Thus, presumably these interneurons respond to different intensities of vagal afferent input with varying

firing patterns and spike frequency. To date single neuron response patterns evoked at different intensities of esophageal vagal afferent input remain to be studied.

5. Present knowledge of inhibitory reflexes evoked by esophageal vagal mechanosensory afferent inputs is limited. In the rat esophagus whose TMP consists of only striated muscle, distal inhibition is presumably centrally organized, and likely to involve inhibitory neurotransmission between NTSc interneuron rather than inhibitory afferents. Accordingly, distal inhibition in the rat should be sensitive to antagonists of inhibitory neurotransmitters and this action should be localized to the NTS. This inference needs to be confirmed. Moreover, this issue is relevant to the deglutitive inhibition evoked by a swallow. As another inhibitory esophageal motor response, deglutitive inhibition could also involve inhibitory neurotransmission at the NTSc esophageal premotoneuron level. Thus, the mechanism of deglutitive inhibition needs to be studied and compared to that of distal inhibition.

6. The investigation of neurotransmitters used by esophageal vagal afferents has not been completed. Although an EAA has been implicated as the neurotransmitter conveying vagal afferent input to the NTSc esophageal premotoneurons (Lu and Bieger, 1998b), the question of whether an EAA also contributes to general visceral reflex afferent transmission remains to be answered. Moreover, for a better understanding of the mechanism by which esophageal vagal afferents evoke general visceral reflex responses, the central pathway of the ECVR should be explored. As an area playing a crucial role in the regulation of the blood pressure, the rostral ventrolateral medulla (RVLM) would be expected to form a link in the pathway controlling the ECVR.

The present investigations aim to provide further insight into the functional organization of afferent vagal mechanisms of the rat esophagus controlling special and general visceral reflexes. In light of the evidence summarized above, the following hypotheses are proposed:

Hypothesis I: Esophageal reflex motility control and esophageal autonomic reflexes involve different populations of vagal afferents. These vagal populations have distinct physiological properties that can be separated by means of biophysical methods.

Hypothesis II: Receptors that have a wide dynamic range (WDR) contribute to esophageal vagal mechanosensory afferent inputs from the rat esophagus.

Hypothesis III: The strength of vagal afferent input determines the pattern and force of esophageal contractions. In the NTSc region where esophageal vagal afferents terminate, activities of interneurons that respond to esophageal distension are correlated with the intensity of vagal afferent inputs.

Hypothesis IV: Distal inhibition is present in the rat esophagus, is programmed at the level of the NTS, and involves local inhibitory interneurons activated by vagal mechanosensory afferent input.

Hypothesis V: Esophageal primary vagal afferents mediating autonomic reflexes utilize an EAA as a neurotransmitter to activate interneurons in or near the NTSc. The RVLM bulbospinal neurons are part of the ECVR pathway.

1.6.2 Objectives

To test the above hypotheses, the research described in this thesis will aim:

i. to differentiate physiological subpopulations of mechanosensory vagal afferents in the rat esophagus by means of the cold block technique;

ii. to specify the dynamic range of these vagal afferents in the rat by means of single unit recording experiments, with particular emphasis on demonstrating the existence of afferents that have a wide dynamic range;

iii. to characterize esophageal motility responses triggered by incremental intensities of esophageal distension with a view to determining differences in motor output as reflected by the activities of esophageal motor neurons;

iv. to investigate the firing and the response patterns of interneurons in the NTSc and its immediate vicinity during esophageal distension at graded intensities;

v. to examine the contribution of spinal afferents to distension-evoked esophageal motility responses;

vi. to study the role of inhibitory aminoacidergic neurotransmission at the level of the NTSc in the mechanism of distal and deglutitive inhibition;

vii. to demonstrate the involvement of glutamatergic synapses in or near the NTSc in the mediation of the ECVR;

viii. to determine the contribution of the RVLM bulbospinal neurons to the ECVR.

Chapter two

Effects of vagal cooling on general and special visceral reflexes evoked by distal esophageal distension

2.1 Introduction

Mechanosensory vagal afferent input from the esophagus is held to be primarily involved in controlling esophageal reflex motility, but is also known to evoke general autonomic reflexes. Although rat esophageal vagal afferents are believed to represent a morphologically homogeneous population, they are neurochemically heterogeneous, as evidenced by immunostaining for calcium binding proteins and SP (Kressel and Radespiel-Tröger, 1999). The majority of rat esophageal vagal afferents appear to be capsaicin-resistant (Berthoud et al., 1997), suggesting that they are mostly myelinated. However, until now this idea has been tested physiologically only in the cat. Esophageal peristalsis in the latter probably depends on vagal myelinated fibers, as it is blocked by vagal cooling to a temperature at which conduction in C-fibers is not expected to be impaired, however, a contribution of the blockade of vagal efferent fibers can not be excluded (Reynolds and Effer, 1988).

The present investigation to be described below aimed to test the hypothesis that the cardiovascular reflex (ECVR) and esophageal reflex contractions evoked by distension of the rat esophagus are mediated by different populations of vagal afferents. The distal esophagus was chosen because distension of this portion can evoke maximum ECVR responses (Loomis et al, 1997) and a robust motility pattern of peristaltic esophageal

contractions (Lu and Bieger, 1998a). Since myelinated and unmyelinated fibers are differentially affected by cooling (Franz and Iggo, 1968), the technique of reversible local cold blockade of the cervical vagal trunk was employed to determine the blocking temperature for the ECVR, reflex rhythmic contractions and other vagally-regulated activities, such as basal heart rate and respiratory frequency.

2.2 Material and Methods

All procedures described in this and the following chapters were approved by the Institutional Animal Care Committee of Memorial University of Newfoundland in accordance with the Guidelines of the Canadian Council on Animal Care.

2.2.1 General Procedures

The experiments were performed in 30 male Sprague-Dawley rats (300-400 g) anaesthetized with urethane (1.2g/kg) given intraperitoneally. After tracheal intubation, the right external jugular vein and left carotid artery were cannulated for infusion of drugs and recording of arterial blood pressure. Rectal temperature was kept at 37-38 °C by means of a heating blanket (Homeothermic Blanket Control Unit, Harvard Apparatus Limited, U.S.A) in vagal cooling experiments, or radiant heat in vagal fiber recording experiments. Rate and depth of respiration were monitored via a tracheal cannula. Pressure signals were measured with pressure transducers. Heart rate was derived from the carotid pulse waves by means of a tachograph.

2.2.2 Esophageal distension

A small collapsible water-filled high compliance balloon made from PE-60 polyethylene tubing was placed in the distal part of the esophagus (11-12cm from upper incisors) for distending its muscle wall and simultaneous recording of intraluminal pressure. When fully distended, the oval-shaped balloon had a long and a short diameter of 15 and 9 mm, respectively, and a volume of 550 μl. As confirmed by autopsy, the center of

the balloon was positioned at the level of the diaphragm, that is, the diaphragmatic portion of the esophagus. Inflation of the balloon at 300 μl distended the esophagus to a cylindrical shape with an outer diameter of 6 mm and a length of 13 mm. Balloon volume was controlled with a syringe and intraluminal balloon pressure recorded by a transducer. Esophageal distensions were performed in 50 μl increments up to maximal balloon volumes of 300 μl.

2.2.3 Vagal cooling

For vagal cooling experiments, each cervical vagal trunk was dissected free and put onto a hook-shaped probe made of a glass capillary (Cat. 6020, A-M Systems, Inc., WA) loop, through which water was perfused by means of a refrigerated circulator pump (Lauda K-2/R, Brinkmann Instruments, Germany). To reduce thermal gradients, the nerves, glass loops and the surrounding area were covered with a 40 °C solution of 4% agar in saline, which on cooling gelled into a semisolid. The temperature at the tip of the glass loop was measured with a fine thermocouple wire (Cat. 52-1716, Harvard Apparatus). After each cooling period, warm water (38-40 °C) was manually injected into the probes. Vagal cooling was applied for 10 to 15 min at 20, 15, 12.5, 11, 9, 7.5, 6, and 4.5 °C. Between each successive temperature step, the nerve trunks were returned to 37 °C for at least 20 min. Bilateral cervical vagotomy was acutely performed at the end of the experiment. Esophageal distensions were performed before and during vagal cooling, and after rewarming, respectively. Blood pressure, heart rate, respiration and esophageal intraluminal pressure were recorded by means of a chart recorder (Model 79D Polygraph,

Grass Instrument Co, USA).

2.2.4 Data analysis:

To prevent desensitization of esophageal reflex responses, the interval between successive esophageal distensions was kept between 2 and 3 min. The cardiovascular data are presented as the maximum change in mean arterial pressure (MAP) and heart rate (HR) during esophageal distension relative to the 1-5 min prestimulus control period. MAP was calculated as diastolic pressure + 1/3 pulse pressure (mmHg). Variability of repeated measurements is expressed as the standard error of the mean (SEM). One-way ANOVA test was done with statistical software (Microcal Origin, Microcal Software, Inc.) to examine the significance of differences, and $P<0.05$ was considered to be significant.

2.3 Results

2.3.1 Effects of vagal cooling on ECVR

At the baseline temperature (37 °C), distal esophageal distension evoked pressor and cardioaccelerator responses that varied directly with balloon volume (Fig. 2.1). Stepwise cooling of both cervical vagal trunks resulted in a graded decline in both ECVR components (Fig. 2.1, 2.2). At balloon volumes of 100 or 200 μl, maximal inhibition of the ECVR was attained in the temperature range between 11 and 9 °C. At the largest balloon volume tested (300μl), a reversal of the pressor response occurred at 9 °C with an unmasking of a depressor component that again disappeared between 7.5 and 4.5 °C. This depressor component was not accompanied by a cardioinhibitor response (Fig. 2.1, 2.3). Bilateral vagotomy abolished all distension-evoked cardiovascular responses (Fig. 2.2).

2.3.2 Effects of vagal cooling on reflex esophageal rhythmic contractions

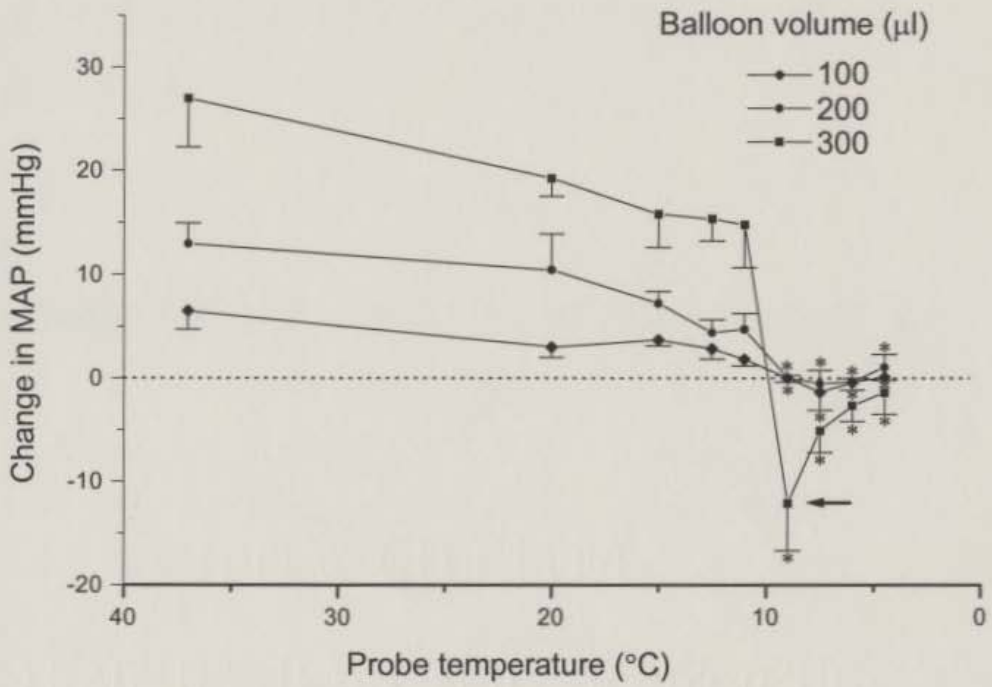
Distal esophageal distension at the volume between 50-100 μl evoked rhythmic esophageal contractions at the frequency of 0.7±0.1 Hz (n=6). At the 37°C, the rhythmic contractions lasted until the esophagus was deflated (15-20 s). Vagal cooling to 20 °C abolished the majority of rhythmic contractions and only left one to three early waves just after the start of balloon inflation. Vagal cooling at 12.5 °C totally blocked distension-induced esophageal reflex peristalsis (Fig. 2.4).

2.3.3 Other effects of vagal cooling

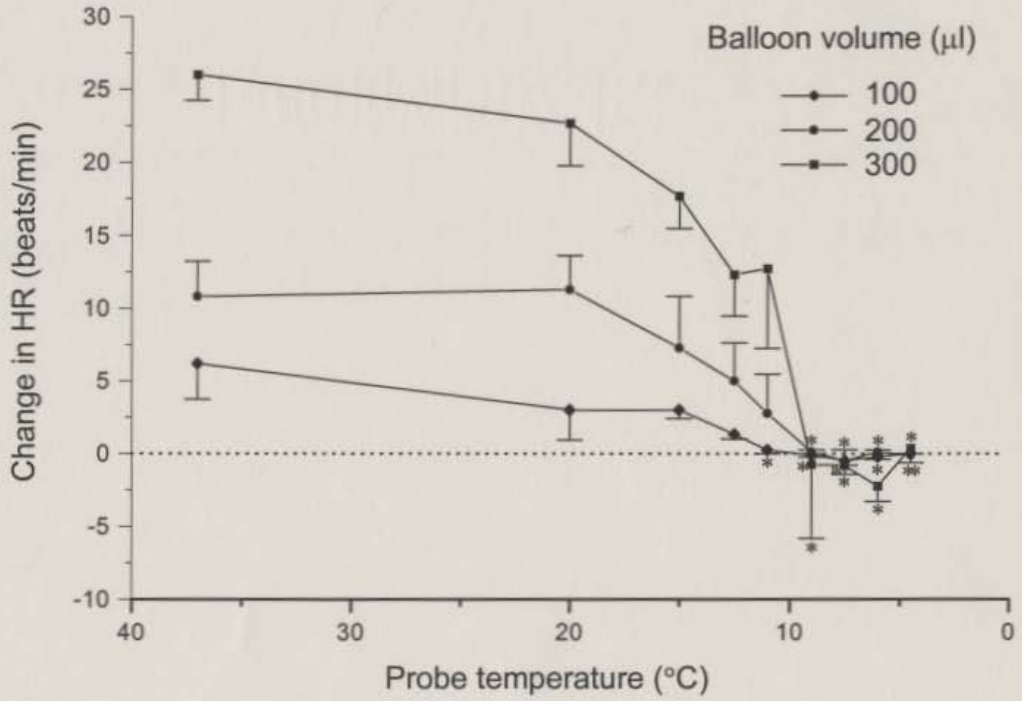
Basal HR and MAP: Cold-induced blockade of the ECVR was not accompanied by

Fig. 2.1 Effects of bilateral cooling of the cervical vagal trunk on esophageal distension-evoked cardiovascular responses (ECVR). Vagal cooling-induced inhibition of vasomotor component of ECVR differed at high and low balloon volumes. The depressor component (←) was observed only at maximal balloon volume in six of seven animals tested (A). Heart rate (HR) component of ECVR showed graded inhibition without evidence of a depressor component (n=7) (B). * $P<0.05$ vs. control (37°C).

A


Balloon volume (µl)
100
200
300
Change in MAP (mmHg)
30
20
10
0
-10
-20
40
30
20
10
0
Probe temperature (°C)

B


Balloon volume (µl)
100
200
300
Change in HR (beats/min)
30
25
20
15
10
5
0
-5
-10
40
30
20
10
0
Probe temperature (°C)

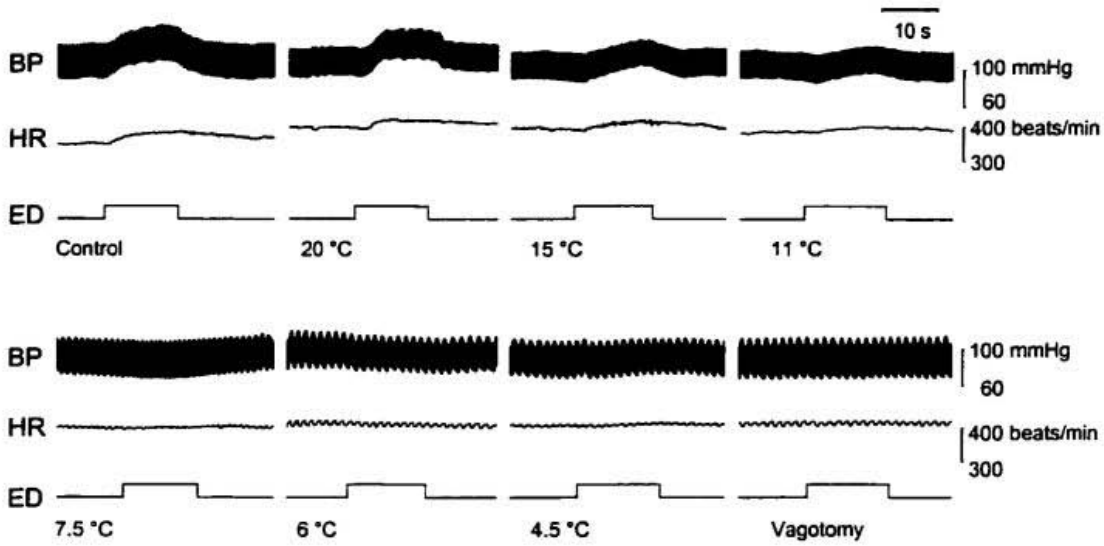


Fig.2.2 A representative example of the effects of bilateral vagal cooling on the ECVR. Data shown are obtained at balloon volume of 300 μl. Control responses sampled between cooling periods are not illustrated. A small depressor response is evident at 7.5 °C. Control temperature is 37°C.

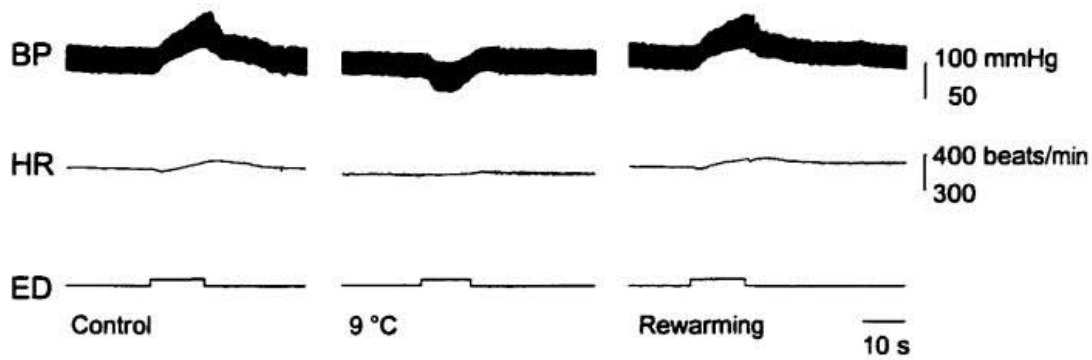


Fig. 2.3 A representative example of the depressor component is shown. This depressor component was obtained at a balloon volume of 300 µl. Control and rewarming temperatures are 37°C.

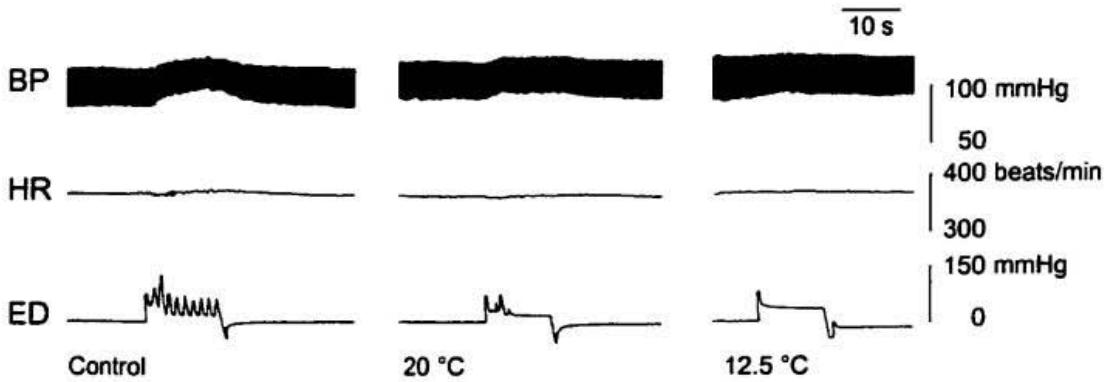


Fig. 2.4 A representative example of the bilateral cooling of cervical vagal trunks on esophageal reflex rhythmic contractions. The example was obtained at a balloon volume of 50 μl. Control temperature is 37°C.

significant changes in basal HR and MAP. A significant rise in basal HR occurred at 4.5 °C; however, this rise was about 50% less than that produced by bilateral vagotomy. Changes in basal MAP evoked by vagal cooling and vagotomy did not reach levels of statistical significance (Fig. 2.5).

Respiration: Spontaneous respiratory frequency decreased sharply between 12.5 and 6 °C (89% of the difference between control and vagotomy levels), and then decreased another 5% between 6 and 4.5 °C (Fig. 2.6).

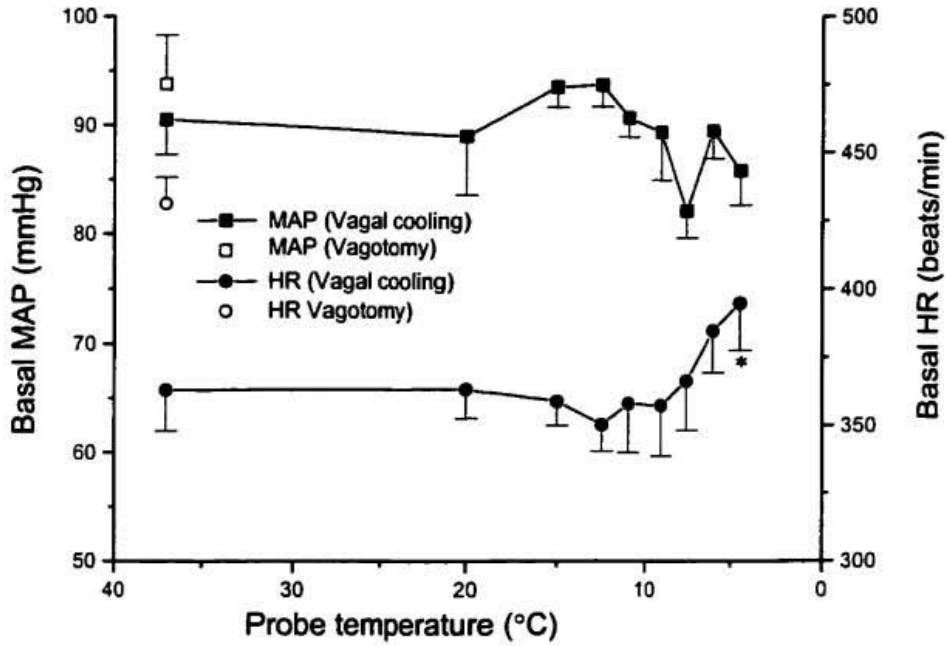


Fig. 2.5 Effects of bilateral cooling of the cervical vagal trunk on basal MAP and HR. Cooling caused insignificant changes in basal MAP and increased basal HR to about 50% of postvagotomy levels (n=7) at 4.5 °C (n=6). * $P<0.05$ vs. control (37 °C).

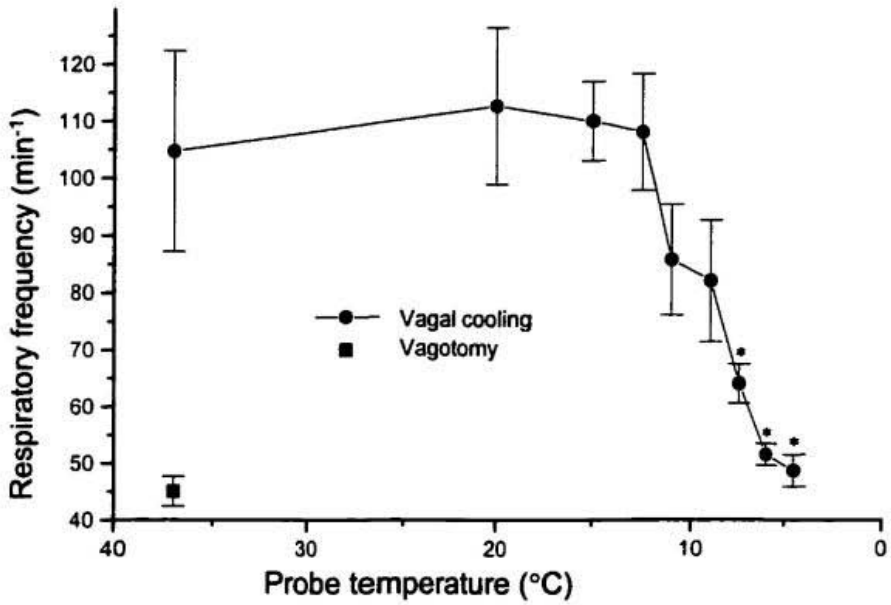


Fig. 2.6 Effects of bilateral cooling of the cervical vagal trunk on respiratory frequency. Decreases occurred in the same temperature range in which blockade of ECVR occurred (n=6). Respiratory frequencies measured at 6 °C and 4.5 °C did not show a significant difference from that measured after vagotomy. * $P<0.05$ vs. control (37 °C).

2.4 Discussion

In the present work, reflex cardiovascular and esophageal motility responses were blocked by graded bilateral cooling of cervical vagal trunks at different temperatures, and thus providing evidence for the existence of different functional groups in the esophageal vagal afferents.

The results of graded vagal cooling demonstrate that the ECVR consists not only an excitatory vasopressor and a cardioaccelerator, but also a masked inhibitory vasodepressor component. Both excitatory and inhibitory components were blocked well above the temperature known to block C-fibers (0 - 1 °C) (Franz and Iggo, 1968; Schultz et al., 1988), implying that the relevant afferent inputs are carried by myelinated fibers. Since the depressor component showed both a high mechanical threshold and relative resistance to cooling, it may involve a separate subpopulation of vagal afferent neurons. Previous work has shown that the ECVR is significantly attenuated, though not blocked, in adult rats treated neonatally with capsaicin (Loomis et al., 1997). This regimen would be expected to result in substantial degeneration not only of unmyelinated primary afferent fibers, but also of some A_δ fibers (Holzer, 1991). Taken together, the available evidence implicates at least two subpopulations of vagal A_δ fibers as the afferent limb of the ECVR responses.

Esophageal reflex peristalsis was blocked in a temperature range well above that where blockade of the ECVR occurred. This result is probably caused by the blockade of vagal efferent fibers to esophageal striated muscle. However, as the esophageal motor pattern is centrally generated at the NTS level (Lu and Bieger, 1998a; 1998b; Lu et al., 1997) a partial blockade of efferent fibers would not change the number of esophageal

contraction waves, but only reduce the force of each esophageal contraction wave because the maximum firing frequency of the fibers is reduced during cooling. In the present work, at a temperature of 20 °C, the contractions occurred just after esophageal inflation and only included two to three waves. This phenomenon is similar to a motor response to esophageal distension at a volume smaller than that used in the control, and thus suggests that the partial blockade of the reflex esophageal peristalsis at this temperature is due to a blockade of the afferent fibers. It thus appears that separate afferent pathways made up of myelinated fibers mediate esophageal reflex peristalsis and the ECVR.

Although it has been suggested that conduction is blocked in all myelinated nerve fibers at about the same temperature (Paintal, 1965), the decrease in conduction velocity and frequency response may vary among different myelinated fibers, so that the functions mediated by them are lost at different temperatures (Franz and Iggo, 1968). Thus, blocking temperatures alone do not permit precise discrimination between subgroups of afferent fibers.

As for the non-esophageal activities tested, basal HR did not show significant changes until vagal temperatures were below the blocking temperature of A-fibers (Franz and Iggo, 1968; Schultz et al., 1988). Although the rise in basal HR seen at 4 °C did not reach post-vagotomy levels, it probably resulted from partial blockade of vagal C-fibers. The degree of cooling-induced bradypnea was comparable to that reported in the dog (Pisarri et al., 1986), suggesting a major involvement of myelinated fibers (Hering-Breuer reflex afferents), and a residual minor component at 6-4.5 °C attributable to a partial blockade of C-fibers.

In summary, the ECVR in the rat is a vagally-mediated reflex that has excitatory and inhibitory components. As each component is blocked by bilateral vagal cooling at a temperature that is different from the blocking temperature of reflex peristalsis, the present work corroborates the hypothesis that different populations of vagal afferents mediate the ECVR and esophageal reflex contractions.

Chapter three

Vagal afferent input determines the volume-dependence of rat esophageal motility patterns

3.1 Introduction

As described in Chapter one and two, stimulation of mechanosensory vagal endings of the rat esophagus elicits both special visceral motor and autonomic cardiovascular responses. The latter increase in magnitude with inflation pressure (Loomis et al., 1997) and increment beyond the range in which secondary peristalsis is elicited (Lu and Bieger, 1998b). Thus, vagal mechanosensory input from the rat esophagus appears to encode information over a wide dynamic range. As yet, functional information on these primary vagal afferents is rather limited (Andrew, 1956c; Clarke and Davison, 1974), in particular, as regards the properties of mechanosensory fibers supplying the distal esophagus. However, recent studies in the opossum have reported that esophageal vagal tension receptors possess a low threshold and low saturation pressure (Sengupta et al., 1989), while esophageal mechanosensory receptors innervated by dorsal root ganglionic (spinal or "sympathetic") afferents have either a wide dynamic range or high threshold (Sengupta et al., 1990).

In exploratory experiments we noted that the reflex contraction pattern of the distal esophageal body changes from rhythmic to tonic as balloon distension increased. This observation led us to ask if the altered motor pattern was caused by other sensory inputs from the esophagus, specifically the spinal afferent pathway, which is believed to mediate nociceptive signals (Cervero and Laird, 1999; Euchner-Wamser et al., 1993). Furthermore,

it appeared necessary to examine the involvement of the esophageal smooth muscle tunica muscularis mucosae (TMM), as the latter receives its major sensory innervation via spinal afferents (Dütsch et al., 1998).

The present study was undertaken to examine the hypothesis that the vagal afferent input alone determines the pattern and force of distal esophageal contractions. Our specific aims were: 1) to characterize the motor response patterns evoked by distal esophageal distension at volumes exceeding physiological levels (Euchner-Wamser et al., 1993; Traub et al., 1994); 2) to examine the contribution of the TMM; 3) to determine the range in which vagal afferents from the esophagus encode intraluminal pressure signals; 4) to examine the role of spinal afferent input; and 5) to demonstrate alterations in neuronal activity patterns recorded at the level of the medulla oblongata.

3.2 Material and methods

3.2.1 General procedures and esophageal balloon distension

The experiments were performed in 72 male Sprague-Dawley rats (300-400 g) anesthetized with urethane (1.2 g/kg) given intraperitoneally. The general procedure was the same as that described in Chapter two. Rectal temperature was maintained at 37-38 °C by means of radiant heat.

The method of esophageal balloon distension is described in Chapter two. The balloon was placed in the esophagus 11-12 cm from upper incisors. In 4 experiments, the balloon was inserted into the gastro-esophageal junction, and autopsy showed the tip of the balloon to protrude into the stomach. The balloon was connected to a manually operated syringe and an infusion pump (Sage Instruments, model 355), permitting graded or constant rate incremental distension to be applied. Deflation was done manually, and the volume withdrawn was controlled closely to maintain a constant baseline. As shown in Fig. 3.1, the high compliance of this system enabled the detection of differences in intraesophageal pressure as small as 1 mmHg.

In medullary single unit recording experiments, the animals were mounted in a stereotaxic frame. The caudal roof of the fourth ventricle and surrounding structures of the dorsal medulla were surgically exposed under a dissection microscope. Cerebrospinal fluid was drained continuously with a wick. Extracellular single unit recordings were made by means of single barrel glass micropipettes filled with NaCl 3M. To ensure that electrode tracks aligned with the anatomical transverse plane of the NTS, the electrode carrier was tilted caudally by 27° out of its vertical axis. Under microscopic control, the

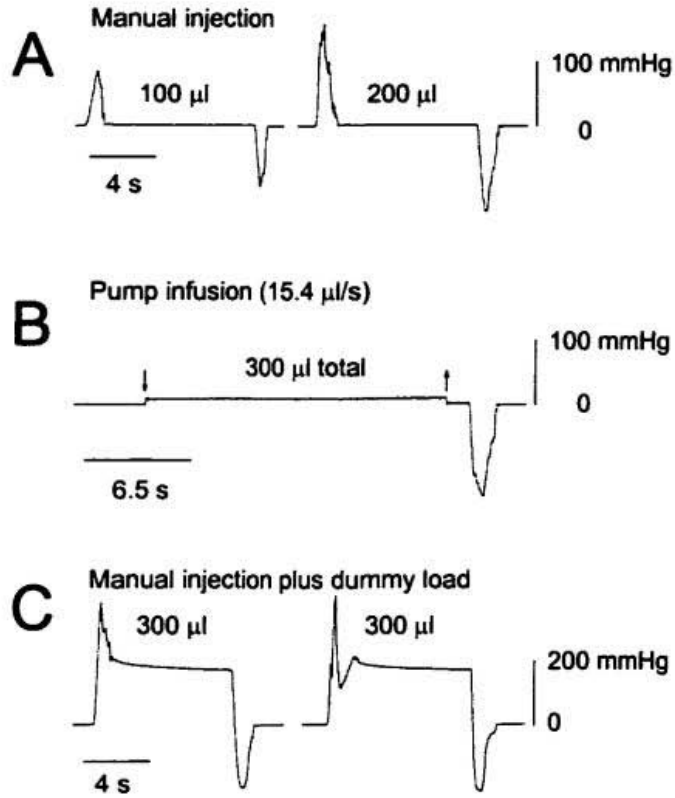


Fig. 3.1 Compliance profile of the polyethylene balloon catheter employed for esophageal distension. A. Rapid filling and emptying of balloon (100 and 200 µl) reveals negligible change in steady-state pressure between transient pressure gradients resulting from injection and withdrawal of fluid. B. When the balloon was filled by infusion pump up to a volume of 300 µl, a small constant increment in pressure was recorded. This reflects the hydraulic resistance of the catheter. Arrows mark the start (↓) and end (↑) of infusion. Transient downward deflection results from withdrawal of fluid from the balloon. C. Balloon was inserted into a soft silicone tubing (outer and inner diameter 0.6 and 0.4 mm, respectively), to simulate the *in vivo* recording condition. In both sample traces shown, a 300 µl fluid bolus was manually injected into balloon-catheter. Note variability of injection artifact.

micropipette was inserted into the ventral or dorsal medullary recording sites by means of a three-axis oil hydraulic micromanipulator (Model MMO-203, Narishige Co., Ltd). For the ventral medullary recording sites, stereotaxic coordinates were restricted to the nucleus ambiguus compact formation (800-1000 μm rostral to the cranial edge of the area postrema, 1800-2000 μm lateral to the midline, and 2200-2500 μm ventral to the medullary surface) as defined in previous work (Bieger and Hopkins, 1987; Lu and Bieger, 1998a). In the dorsomedial medulla at the level of the intermediate/caudal nucleus tractus solitarii (NTS), the area explored was confined to a circumscribed region (0-200 μm rostral to the cranial margin of the area postrema, 600-700 μm lateral). As shown previously (Bieger, 1984; Lu and Bieger, 1998a), at this level the cell dense caudal half of the NTS centralis (NTSc) lies 400-550 μm below the dorsal medullary surface. The pipette was advanced in 5-10 μm steps until units were located that responded in a consistent fashion during distal esophageal distension.

Recording from vagal afferent fibers was done following exposure of the cervical vagal trunk. The left vagal trunk was explored first, followed by the right trunk in some cases. A black synthetic rubber platform was placed under the nerve for improve visualization. Both the nerve and the platform were initially immersed in saline. After the perineural sheath of the nerve was removed by microdissection, the saline was replaced with warm mineral oil. Single afferent fibers were teased from the nerve trunk, carefully decentralized, and then placed on one pole of a bipolar platinum wire electrode, the other pole being connected to ground. Nerve action potentials were initially passed through a differential preamplifier (Duo 773 Electrometer, World Precision Instruments).

In some experiments recordings were made in the left nodose ganglion. After exposing the cervical vagus nerve, the nodose ganglion was surgically exposed and the supranodosal vagal trunk was cut. After the nodose ganglion and infranodosal 1 cm cervical vagal trunk were dissected free, the nodose ganglion was desheathed carefully and put onto a platform to dampen movements caused by carotid arterial pulsations and respiration. Extracellular single unit recordings were made by means of single barrel glass micropipettes filled with NaCl 3M.

Extracellular single unit signals were conditioned by conventional methods (NeuroLog modules NL102, NL126, NL106, NL120, Digitimer Limited, England), along with arterial blood pressure and esophageal pressure signals (Model 7D Polygraph, Grass Instrument Co., USA). All outputs were digitized at a sampling rate of 5 or 5.56 kHz, and then displayed and stored in a computer by means of a data acquisition system (DigiPack 1200, Axon Instruments, Inc., CA, USA).

For spinal cord transection, the animals were mounted in a stereotaxic frame. The cervical spinal cord (C1-C3) was surgically exposed through a dorsal midline incision and the dura was opened under a dissection microscope. The animal was artificially ventilated at 62-70 cycles per minute by means of a small animal respiration pump (Model 663, Harvard Apparatus Co., MA, USA). Spinal transection was done by cutting the spinal cord with a scalpel (blade size: 11) at the level of C2. Completeness of the cut was verified later at autopsy.

3.2.2 Drugs

Scopolamine methyl bromide (methscopolamine, MSCP), nifedipine and urethane were obtained from Sigma Chemical; tubocurarine chloride from Burroughs-Wellcome &Co (Canada). All the drugs were administered intravenously in aqueous solution, except for nifedipine, which was dissolved in ethanol.

3.2.3 Data analysis

To ensure reproducibility of responses, distensions at low volume and volumes over 200 µl were applied at 3-5 min and at 0.5-1 h intervals, respectively. In the case of rhythmic contractions, intraluminal pressure was taken at the peak of each wave and averaged. When rhythmic activity was evoked by constant rate incremental distension, the instantaneous frequency of contractions was calculated from the reciprocal of the interval between two successive waves, and expressed as the number of waves per second (Hz). Neuronal discharge rate was counted in spikes per second (Hz). Data are presented as means ± SEM, except where noted otherwise. Student's paired t-tests were done with statistical software (Microcal Origin, Microcal Software, Inc.). Differences were considered statistically significant at $P<0.05$. Numbers (n) given in parenthesis refer to individual separate experiments, and, in the case of extracellular unit recordings, to the total number of individual units. In preparing Fig. 2B, data files were imported into Microcal Origin, where digital subtraction of pressure signals could be performed.

3.3 Results

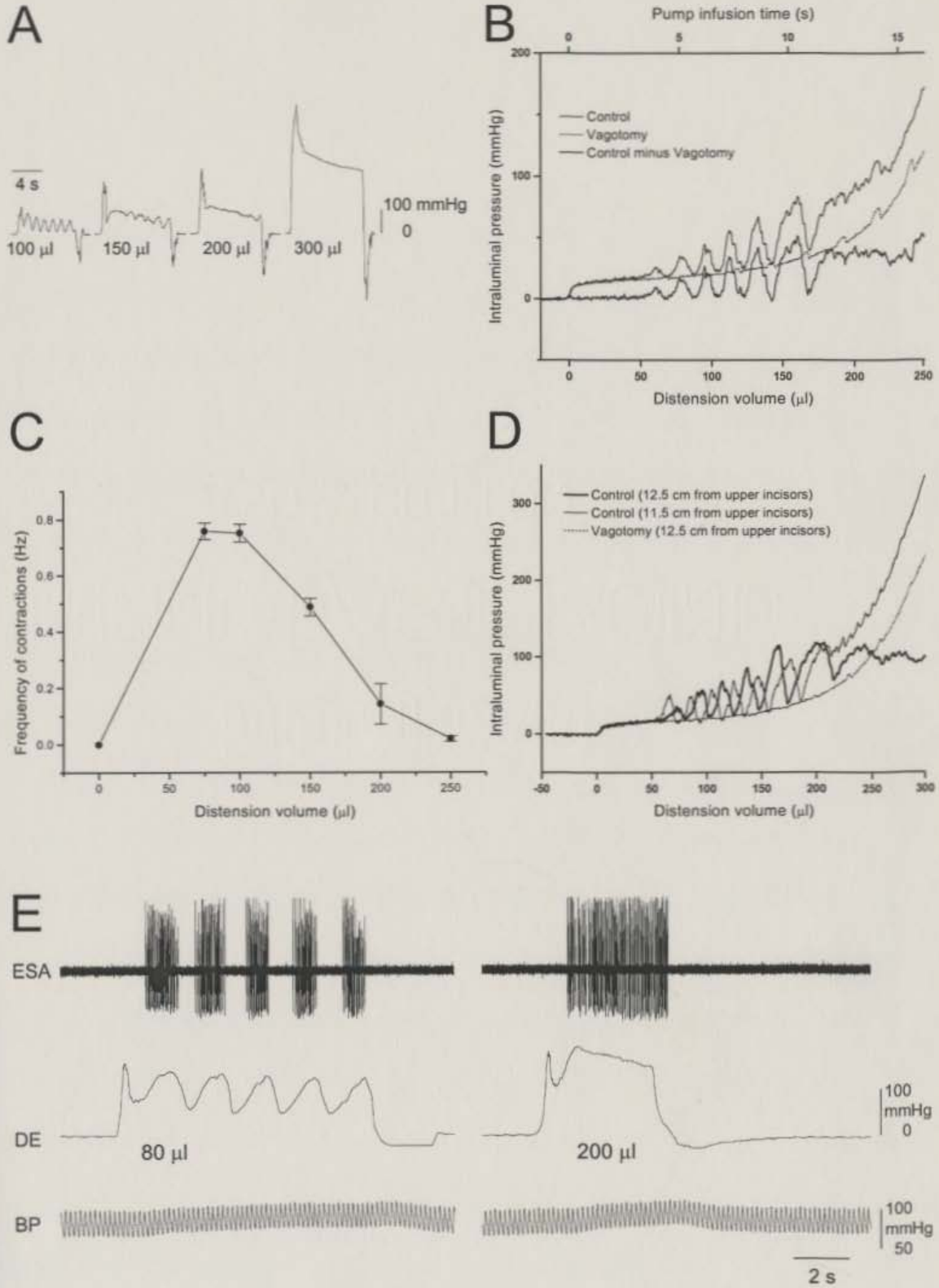
3.3.1 Volume dependence of distal esophageal motility pattern

Distension produced both rhythmic and tonic pressure responses that varied with balloon volume and required an intact vagal innervation (Fig. 3.2). During constant rate incremental distension, rhythmic contraction waves appeared at a threshold volume of 46.4±2.7 µl (n=9); they remained steady below 100 µl, slowed progressively between 100 to 200 µl, and were replaced by a tonic contraction between 150-250 µl (Fig. 3.2B, 3.2C). A single-step distension in the range of 150-200 µl evoked an initial slow or tonic contraction that changed to rhythmic contractions as the intraluminal mean pressure declined gradually (Figs. 3.2A, 3.7). Distension at volumes above 250 µl caused a persistent increase in reflex threshold, as evidenced by an increase in the minimal balloon volume required to elicit rhythmic activity (61.0±2.7 µl, n=9). Repeated testing at a volume of 300 µl revealed that the peak pressure evoked by subsequent distensions declined by 22% and remained depressed for up to 2 hours.

Placement of the balloon in the gastroesophageal junction revealed a modified response profile during constant rate incremental distension (Fig. 3.2D). When the balloon volume reached 250 to 300 µl, intraluminal pressure appeared to level off, indicative of an increase in esophageal compliance. Bilateral vagotomy abolished this apparent relaxation response, along with rhythmic pressure wave activity preceding it (n=4) (Fig. 3.2D).

Ventral medullary units (n=31) responding to distal esophageal distension were recorded in 17 rats. All units were silent at rest and did not fire unless a distension-evoked

Fig. 3.2 Volume dependence of reflex-evoked motility pattern in the rat distal esophagus. A. Representative sample traces obtained at four balloon volumes shown. Rhythmic pressure wave activity at low volume changes to nonrhythmic response at high volume. At intermediate volumes, tonic response precedes rhythmic activity, which appears during slow decline of intraluminal pressure. B. Representative example of reflex motility pattern changes revealed by constant rate incremental distension. With increasing volume, the rhythm of contractions is slowed, and changes into a tonic contraction pattern. Bilateral vagotomy eliminates rhythmic and tonic reflex contractions; remaining small pressure waves are of respiratory origin. The centrally mediated reflex component is obtained by digital subtraction of the post-vagotomy trace from the control response. C. Relationship between distension volume and frequency of rhythmic contraction as determined in 9 animals subjected to constant rate incremental distension. Between threshold volume and 100 μl, the frequency of pressure waves remains virtually stable, and then declines towards zero between 100 and 250 μl. D. Representative records obtained from one animal showing regional change in distal esophagus of motor response to incremental distension. Response recorded 12.5 cm from upper incisors in gastro-esophageal junction reveals a pressure plateau above balloon volume of 200 μl that is abolished by bilateral vagotomy. E. Correspondence between distension evoked distal esophageal motility pattern and activity of presumptive esophageal motoneuron. Single unit extracellular spike activity (ESA) recorded in rostroventral medulla at the level of rostral nucleus ambiguus is absent at rest and evoked by distal esophageal distension (DE) with a stationary balloon catheter. At low balloon volume, unit fires rhythmically in phase with each distal intraluminal pressure wave. Increase in balloon volume induces tonic firing that is associated with a non-rhythmic sustained pressure rise in inflated segment. Note accompanying rise in arterial blood pressure (BP).


A
4 s
100 mmHg
0
100 µl
150 µl
200 µl
300 µl
B
Pump infusion time (s)
0
5
10
15
200
100
0
Control
Vagotomy
Control minus Vagotomy
Intraluminal pressure (mmHg)
0
50
100
150
200
250
Distension volume (µl)
C
Frequency of contractions (Hz)
0.8
0.6
0.4
0.2
0.0
0
50
100
150
200
250
Distension volume (µl)
D
Control (12.5 cm from upper incisors)
Control (11.5 cm from upper incisors)
Vagotomy (12.5 cm from upper incisors)
Intraluminal pressure (mmHg)
300
200
100
0
-50
0
50
100
150
200
250
300
Distension volume (µl)
E
ESA
DE
80 µl
200 µl
100
mmHg
0
BP
100
mmHg
50
2 s

pressure wave occurred. The mean latency between the beginning of esophageal distension and the first evoked spike was 1.06±0.07 s. The pattern of evoked spike activity depended on the magnitude of balloon inflation. At balloon volumes of 50-100 µl, rhythmic activity prevailed and consisted of spike bursts associated with the rising phase of each pressure wave (Fig. 3.2E). The mean spike frequency of the initial burst was 29.3±2.2 Hz. Three units (9.6%) had a short burst discharge 1±0.5 s following rapid deflation of the balloon. Responses to 200 µl were obtained in 19 units. Evoked firing was continuous during distension (3.0±0.1 s) at a mean frequency of 34.4±3.4 Hz and was temporally correlated with a nonrhythmic pressure rise in the distal esophagus (Fig. 3.2E). About 2/3 of the units (68.4%) showed a burst discharge 0.7±0.2 s upon balloon deflation.

3.3.2 Differentiation of striated and smooth muscle components

Acute blockade with methscopolamine (0.2 µmol/kg, i.v.) of parasympathetic cholinergic efferents to the tunica muscularis mucosae (TMM) smooth muscle tunic (Bieger, 1984) did not result in a measurable change in the amplitude and pattern of distension evoked esophageal responses (Fig. 3.3A). During neuromuscular paralysis with tubocurarine (0.15 µmol/kg, i.v.), both rhythmic and tonic contractions were completely inhibited for 10 min, and the pressure-volume relationship was indistinguishable from that obtained after vagotomy (not illustrated). In vagotomized rats, esophageal compliance increased significantly at distension volumes ≤250 µl. This apparent increase in compliance was augmented by nifedipine (0.8 µmol/kg, i.v.), as evidenced by a further reduction in intraluminal pressure high-volume distension (Fig. 3.3B).

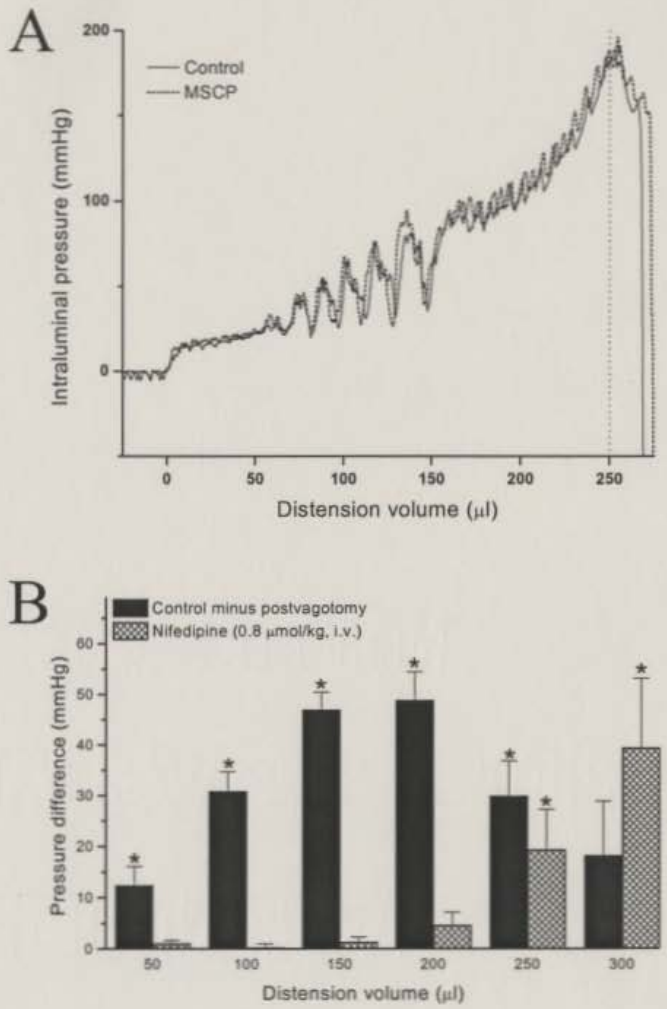


Fig. 3.3 Differentiation of striated and smooth muscle components. A. A representative example illustrating the absence of change in esophageal compliance and motility pattern after intravenous methscopolamine (MSCP). B. Subtraction of distension-evoked pressure increase after vagotomy from control response reveals the reflex neurogenic component of intraluminal pressure increase. Vagus-dependent response reaches its peak at a balloon volume between 150 and 200 µl. A second, nifedipine-sensitive, component is evident at large balloon volumes (250-300 µl; n=6) after vagotomy. * $P<0.05$.

3.3.3 Spinal afferent component

The intraluminal pressure responses to constant rate incremental esophageal distension were tested immediately (1-14 min) after acute spinal cord transection at the level of C2 (n=4). Distension evoked distal esophageal contractions with a pattern identical to that obtained 1-10 min before the spinal cord transection (Fig. 3.4). The responses remained stable for at least 14 minutes after spinal cord transection. Before and after spinal transection, the respective threshold volumes of rhythmic contractions were 55.0±9.7 and 54.2±9.4 μl ($P>0.05$). Tonic contractions appeared at approximately 200 μl. Thoracic or diaphragmatic respiratory movements were absent when the respiratory pump was temporarily turned off.

3.3.4 Recordings in vagal esophageal afferents

Vagal fibers (n=140) were recorded in 18 rats. The majority (n=92) had a respiratory rhythmic discharge, while the rest fired tonically (n=41) or had a cardiac rhythm (n=7). Ten fibers (7 from left, and 3 from right vagal trunk) that had been identified as single units and responded to distal esophageal balloon distension were chosen for this study. All of these afferents fired tonically at rest, without any evidence of cardiac or respiratory modulation. The resting discharge rate varied from 0.4 to 34 Hz (mean 9.7±2.8 Hz). During distal esophageal distension, the firing frequency increased and remained elevated until the balloon was deflated. Upon deflation a silent period ensued before spontaneous activity resumed (Fig. 3.5). Firing frequency increased linearly with the logarithm of esophageal inflation pressure in the test range and did not show saturation even at balloon

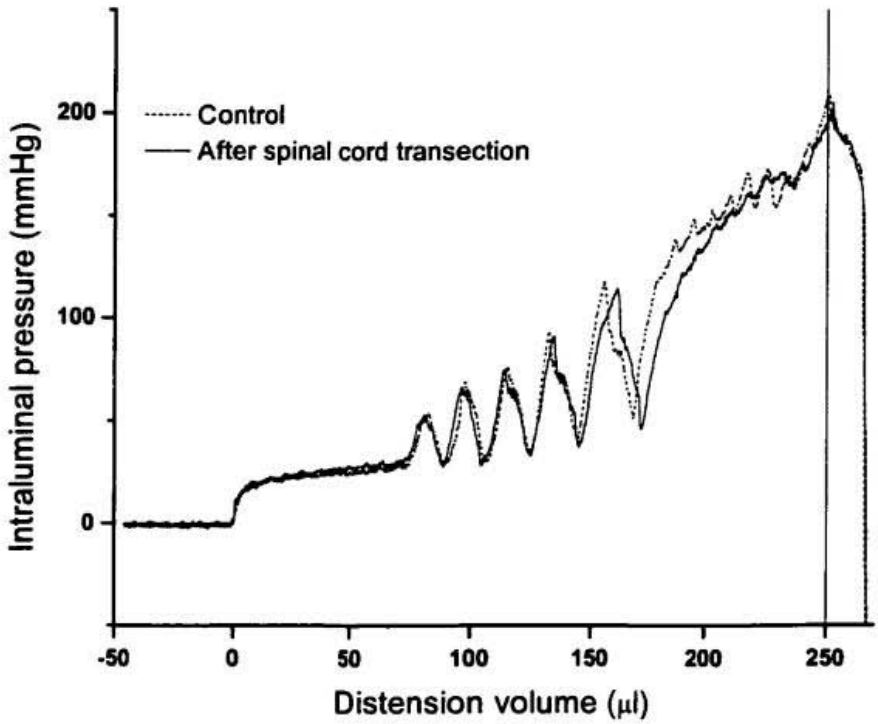


Fig. 3.4 Failure of C2 level spinal cord transection to alter volume-dependence of reflex motility in distal esophagus. Response to constant rate (15μl/s) incremental inflation of intraluminal balloon placed at 11.5 cm from upper incisors is shown before and 1.5 min after the transection.

Fig. 3.5 Distension-evoked activity in esophageal vagal afferent fibers. A. A representative example (fiber indicated by open inverted triangle in the following figure) illustrates volume-dependent increase in afferent impulse activity. The histogram (binwidth: 0.25 s) shows instantaneous firing frequency. B. Trace depicted in A is shown at slow speed to illustrate the inhibition following balloon deflation.

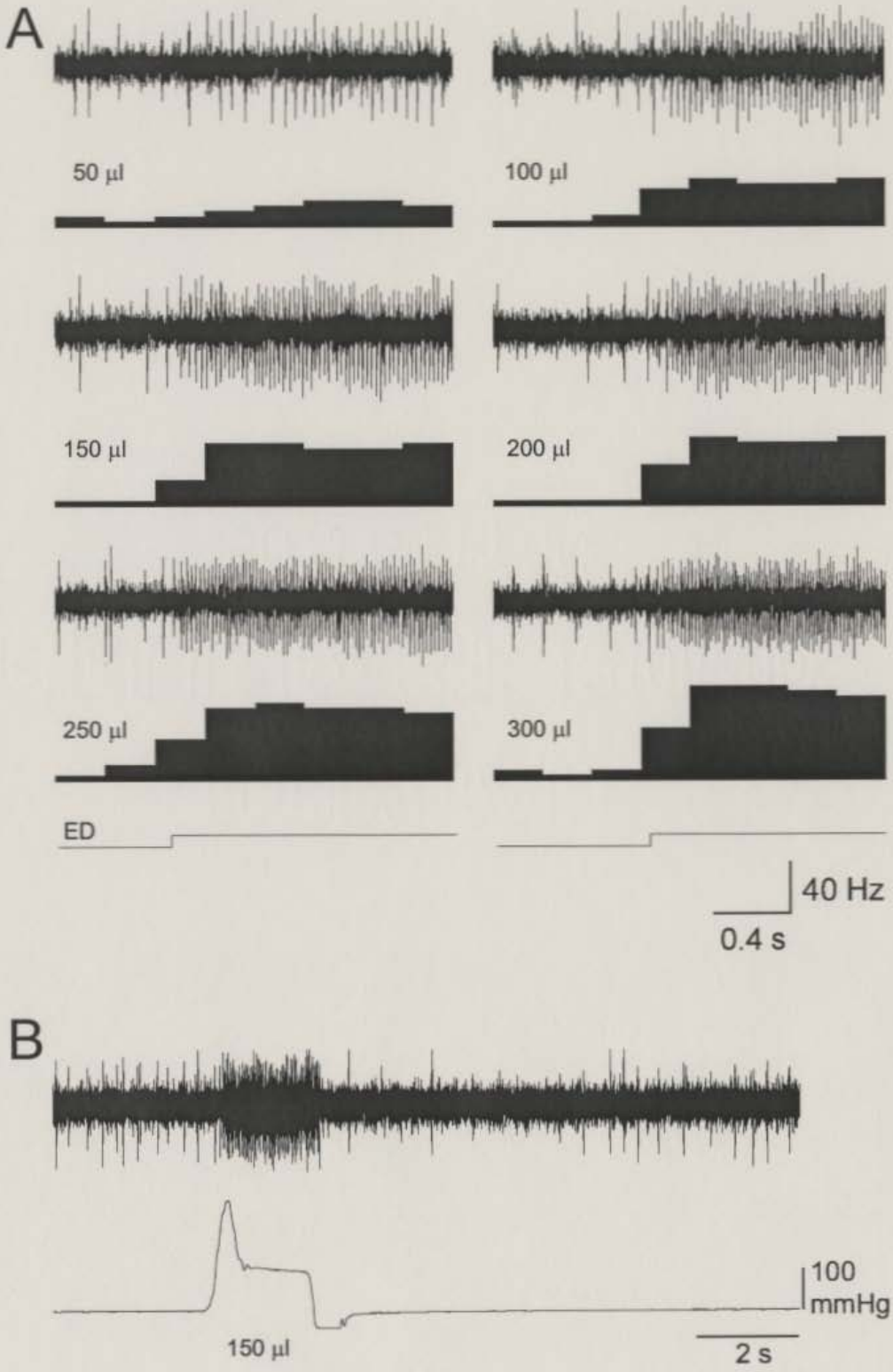
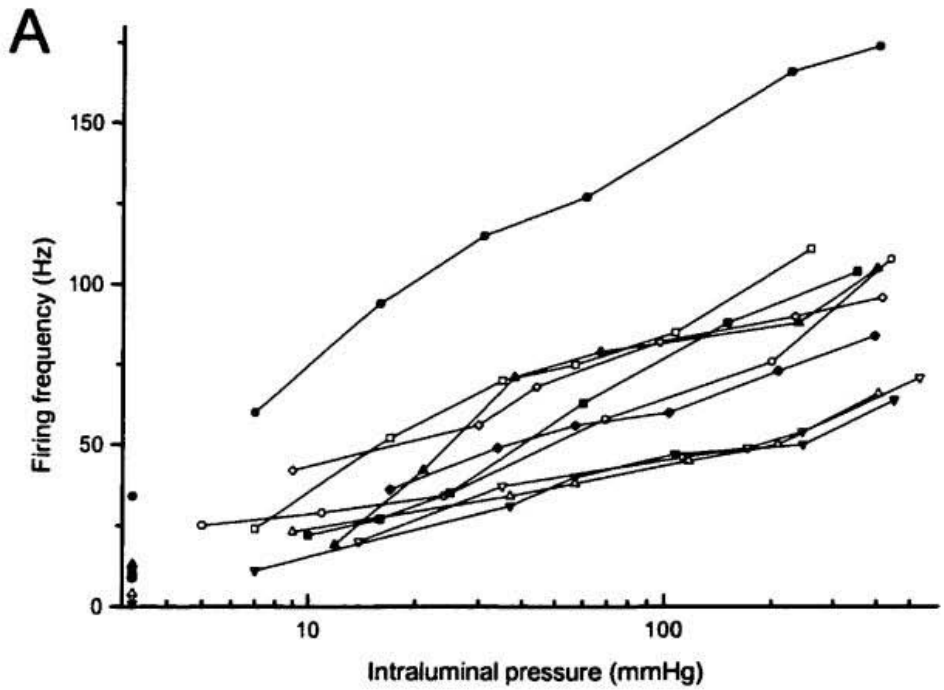
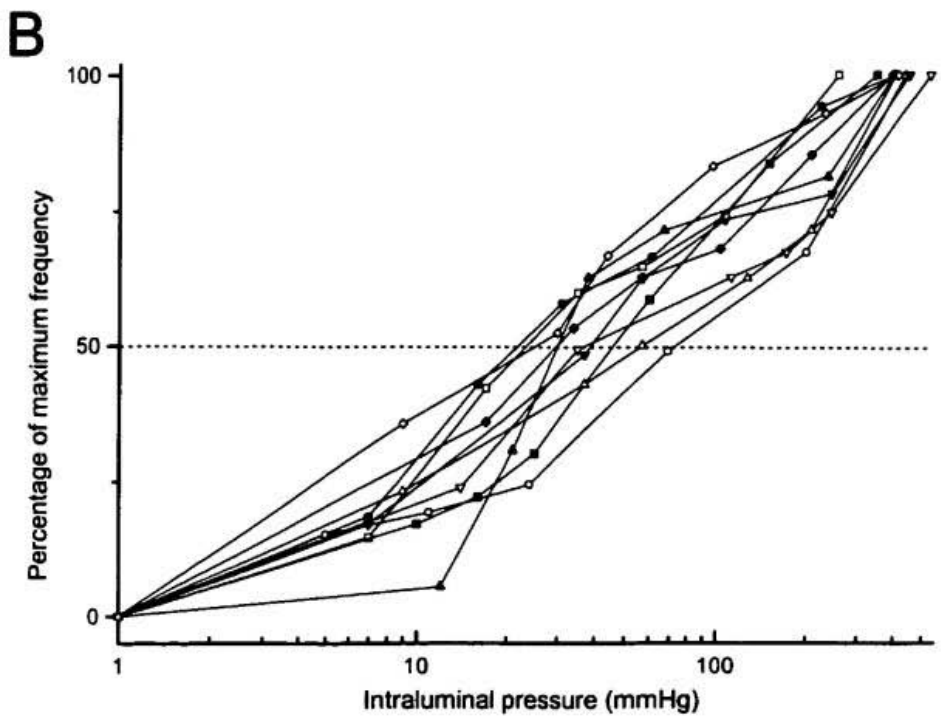

A
50 µl
100 µl
150 µl
200 µl
250 µl
300 µl
ED
40 Hz
0.4 s
B
100
mmHg
150 µl
2 s

Fig. 3.6 Relationship between inflation pressure and vagal afferent discharge. A. Firing frequency of individual fibers increases with the log of intraluminal pressure. According to the discharge frequency, these fibers appeared to fall into three subgroups. B. Normalized stimulus-response relationship for same group of fibers reveals similar rates of rise (slope) and dynamic range for majority of sample.


A
150
100
50
0
Firing frequency (Hz)
10
100
Intraluminal pressure (mmHg)


B
100
50
0
Percentage of maximum frequency
1
10
100
Intraluminal pressure (mmHg)

volumes as large as 300 µl. As suggested by the rate of resting and distension-evoked discharge, these fibers appeared to fall into three subgroups (Fig. 3.6A). When impulse frequencies obtained at 300 µl were arbitrarily set as the maximum, the fractional increase in firing frequency as a function of applied pressure showed a similar slope in all the fibers tested (Fig. 3.6B).

Extracellular single unit recordings from the nodose ganglion were done in 20 animals. Most units in the 23 units obtained had respiratory rhythm (n=14), others were firing with a cardiovascular rhythm (n=2), tonically (n=6), or had an unknown rhythm (n=1). Only one neuron responded to distal esophageal distension. This unit was not activated until the distension pressure exceeded 90 mmHg, and the largest increase in firing frequency occurred in a pressure range above 200 mmHg (not illustrated).

3.3.5 Volume dependence of dorsal medullary neuronal activities

Sixty-seven units that responded to distal esophageal distension were recorded in the intermediate/caudal NTS region. These unit discharges fell into three types as evidenced by their spontaneous background activity, firing patterns and responses to balloon inflation or deflation (Tab. 3.1). Average depths below the extraventricular surface of the NTS for type I, II, and III units were 471.6±38.9, 460.0±44.9 and 482.3±43.0 µm (mean ± SD, $P>0.05$), respectively. Within a given track type I units were separated by as little as 10 to 30 µm from type II and III units. All three types of evoked neural activity observed were superimposed on a continuous spontaneous background discharge. When bursting or

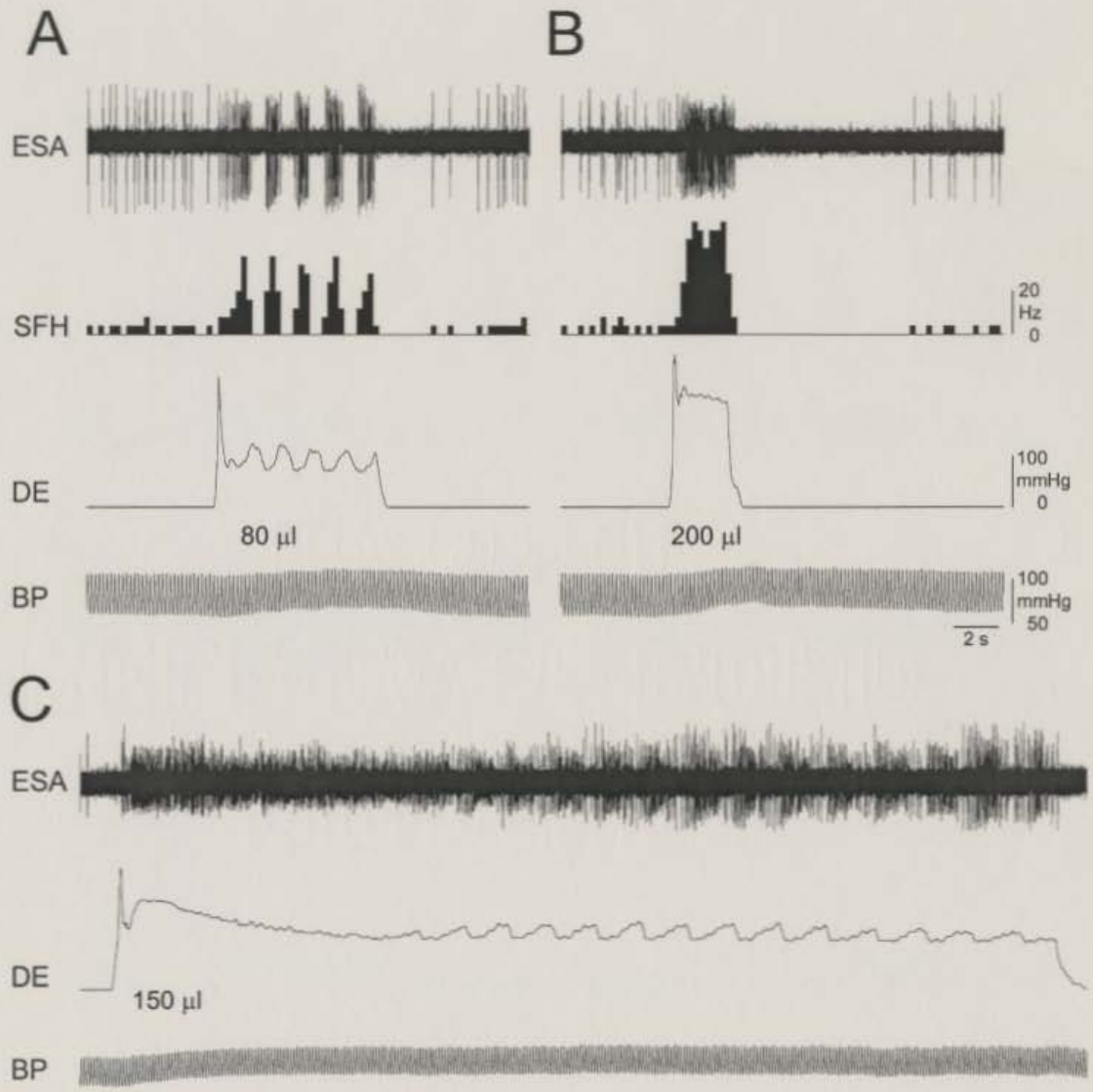
firing at a high frequency, the units typically showed a marked decrement in spike amplitude.

Type I units responded to 50-100 µl distensions with rhythmic spike bursts that coincided with the rising phase of the distal esophageal pressure waves. When balloon volume was increased to 200 µl, type I units produced high-frequency tonic spiking with an increased incidence of burst discharges after deflation (Tab. 3.1, Fig. 3.7). However, on deflation most units showed a silent period that varied directly with balloon volume (7.5±0.9 s at 200 µl vs. 5.1±0.8 s at 50-100 µl, $P<0.05$).

Type II units showed a sustained increase in activity during esophageal distension and fired continuously at rest, with the exception of two units that had a rhythmic basal discharge in phase with expiration. In the latter, distension evoked tonic activity reverted to an expiratory rhythm 2-3 seconds after deflation of the balloon. Type II units showed a volume-dependent poststimulation silent period before resuming their spontaneous discharge (4.2±0.7 s for 200 µl vs. 2.9±0.4 s for 50-100 µl, $P<0.05$). Both the increase and decrease in firing rate were volume dependent (Tab. 3.1, Fig. 3.8).

Type III units had a resting discharge significantly higher than that of type I units, and were inhibited by esophageal distension. Upon deflation of the balloon, they showed a rebound burst discharge (Tab. 3.1, Fig. 3.9). Intra-burst spike frequency and burst latency depended on the magnitude of the stimulus.

Fig. 3.7 Representative type I distension-sensitive units recorded in the caudal NTSc region. A. Moderate distension of esophagus evokes rhythmic bursting discharge in phase with intraluminal pressure wave of the distal esophagus. B. Strong distension induces high frequency tonic discharge of the neuron, with nonrhythmic pressure rise. The binwidth of spike frequency histogram (SFH) was set at 0.25 s. C. In another unit at intermediate volume, distension evokes initial nonrhythmic discharge that becomes rhythmic 15 s later as intraluminal pressure falls gradually. Note correlation between unit activity and esophageal contractions as response changes from tonic to rhythmic pattern.


A
B
ESA
SFH
20
Hz
0
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mmHg
0
80 µl
200 µl
BP
100
mmHg
50
2 s
C
ESA
DE
150 µl
BP

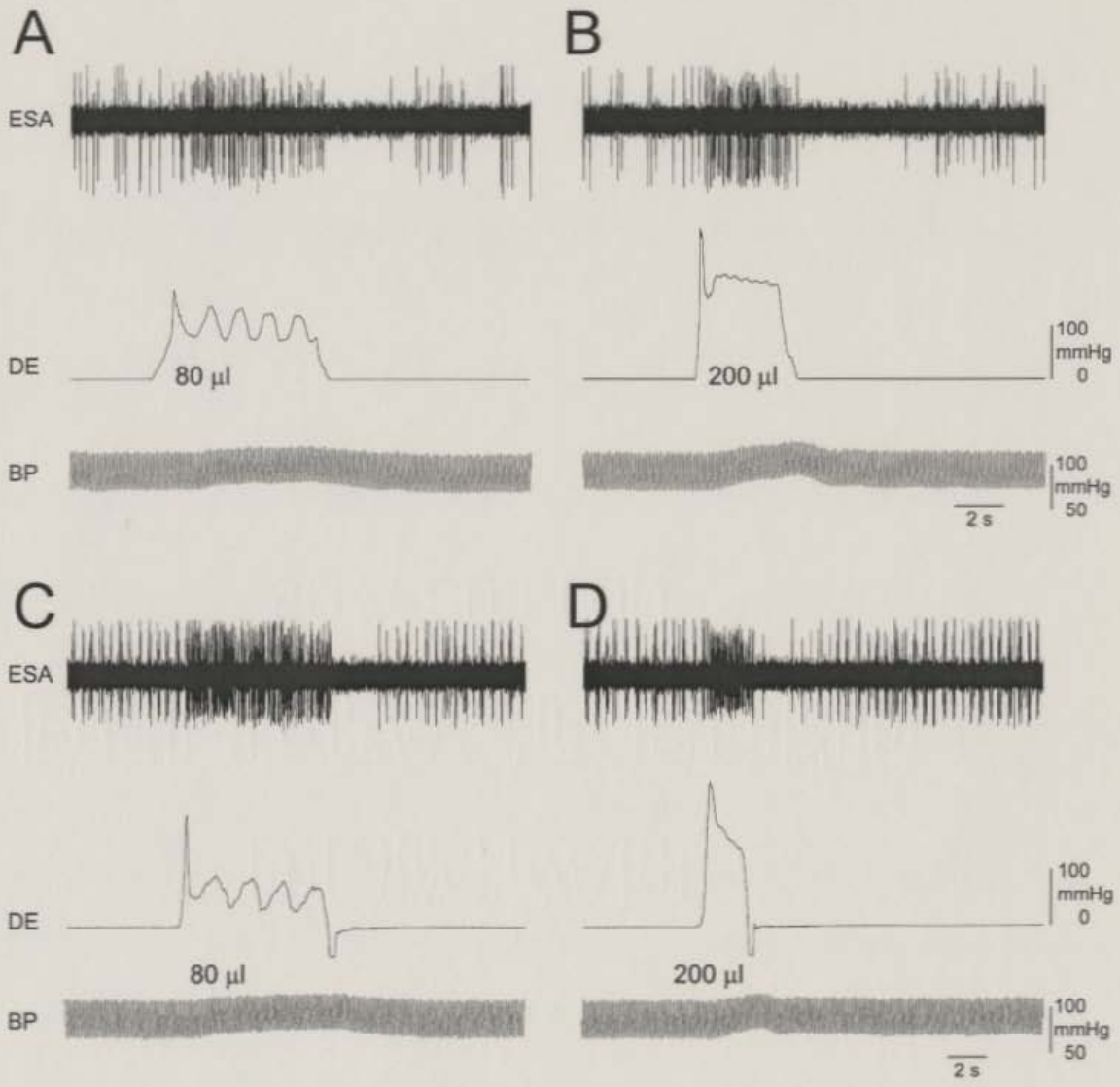


Fig. 3.8 Examples of type II distension-sensitive units recorded in the NTSc region. A, B: Tonic discharge at rest accelerates briskly during esophageal distension and undergoes a period of transient inhibition after balloon deflation. C, D: A subgroup of type II units exhibits expiratory background discharge and is tonically excited during distal esophageal distension.

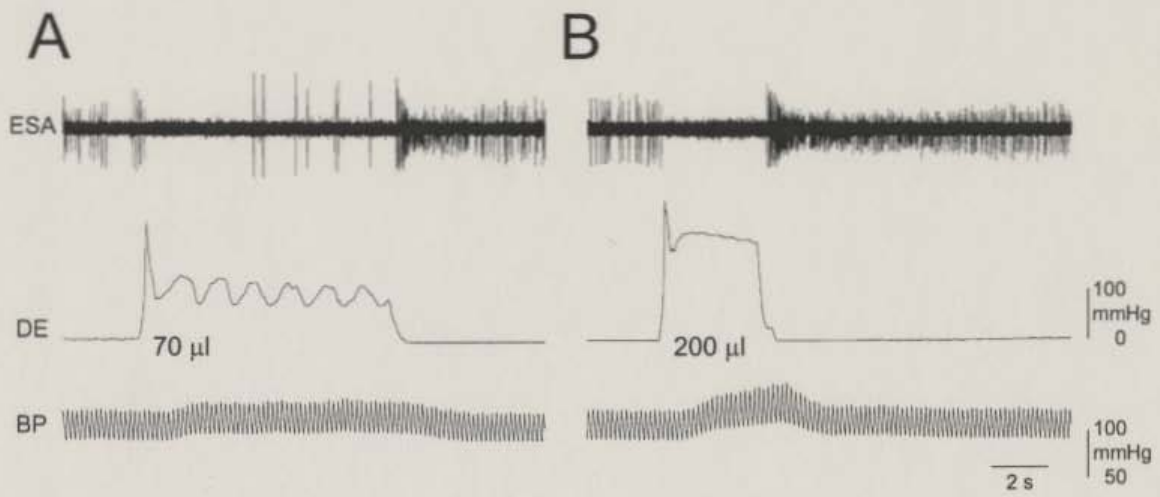


Fig. 3.9 Type III distension-sensitive unit recorded in the NTSc region. The resting discharge of this neuron type is inhibited by distal esophageal distension. A. At low balloon volume, the unit partially escapes from inhibition during the falling phase of the pressure waves. Deflation of the balloon is followed by a burst discharge. B. Strong distension causes total inhibition of the unit. Spike rate, duration, and latency of burst discharge triggered by deflation increase with balloon volume.

Tab. 3.1 Characteristics of esophageal distension responsive units recorded in rat NTS caudal central subnucleus area

Type	Resting Discharge (Hz)	Activity evoked at balloon volume of:		"Off" response[a] at balloon volume of:	
		50-100 µl	200 µl	50-100 µl	200 µl
I n=26	0.9±0.3	Rhythmic bursts 24.3±2.3 Hz[b]	Tonic 34.3±3.3 Hz	Inhibition (n=25) Burst discharge (n=1)	Inhibition (n=23) Burst discharge (n=3)
II n=28	1.6±0.4	Tonic 6.0±1.0 Hz	Tonic 14.2±2.5 Hz **	Inhibition	Inhibition
III n=13	2.6±1.0*	Complete cessation (n=7) Partial decrease (n=6)	Complete cessation	Burst discharge 19.2±2.6 Hz	Burst discharge 33.1±2.9 Hz **

* P<0.05 vs. type I units (8-12 rats per unit type). ** P<0.05 vs. 50-100 µl distension.

[a] "Off" response denotes change in activity caused by balloon deflation.

[b] spike frequency in leading burst.

3.3.6 Esophageal distension evoked rise in blood pressure

Distal esophageal distension was accompanied by a measurable arterial pressor response at every balloon volume tested. At 50-100 µl, systolic and diastolic pressure increased by 7.2±0.9 and 8.9±0.9 mmHg, respectively. At 200 µl, the respective increases were 12.6±1.4 and 14.6 ± 1.4 mmHg. The volume dependence of the pressor response was statistically significant ($P<0.05$).

3.4 Discussion

In the rat esophagus, motor control of the striated muscle tunica involves segmental vago-vagal reflex circuits composed of primary vagal afferents, esophageal premotoneurons in the NTSc, and esophageal motoneurons in the AMB compact formation (Altschuler et al., 1989; Barrett et al., 1994; Bieger, 1993; Bieger and Hopkins, 1987; Cunningham and Sawchenko, 1989; Lu and Bieger, 1998a; Wang et al., 1991). As demonstrated in the present study, brainstem esophagomotor control entails the generation of different motility patterns. Thus, the distal esophagus executes rhythmic or sustained (tonic) contractions depending upon the level of afferent impulse activity. The results not only confirm vagal input as the principal determinant in esophageal motor pattern control but also militate against a direct involvement of spinal afferent input. This idea agrees with previous work by Renehan et al. (1995) showing that after vagotomy rat NTS neurons do not respond to intestinal distension, although neurons in the dorsal motor nucleus of the vagus (DMV) continue to do so through a spinal afferent pathway. As discussed below, the volume-dependence of esophageal motor patterns and their central neuronal correlates need to be considered in relation to the dynamic range of vagal afferent input from the esophagus.

3.4.1 Volume-dependence of distal esophagomotor pattern

Both amplitude and pattern of distal esophageal contractions were found to depend on the volume of the intraluminal balloon, hence, the magnitude of sensory input. The pressure-volume relationship obtained would be expected to result from the interplay of four factors: reflex contraction of the striated muscle tunica muscularis propria,

neurogenic or myogenic tension of the smooth muscle tunica muscularis mucosae (TMM), neurogenic inhibition in the TMM, and the passive visco-elastic properties of both muscle tunics. Since muscarinic acetylcholine receptor blockade failed to change distension-evoked esophageal motility responses at methscopolamine doses likely to suppress vagal efferent transmission in the TMM, any centrally mediated reflex contraction of the TMM was evidently too small to be detected. Accordingly, the active neurogenic component of the intraluminal pressure change could be attributed to the muscularis propria, a conclusion corroborated by the complete loss of this response seen after vagotomy or curarization. At large balloon volumes (≥250 μl), a local myogenic contraction of the TMM occurred, as inferred from the decrease in intraluminal pressure induced by nifedipine, a blocker of smooth muscle L-type calcium channels (Triggle, 1992). The active neurogenic component attained peak amplitude at balloon volumes between 150 and 200 μl.

Although the possibility that high-intensity vagal input inhibits motor output cannot be ruled out, the observed decline in the active neurogenic component at balloon volumes ≥250 μl (Fig. 3.3B) is possibly due to excessive stretching of the striated muscle tunic. Indeed, distension at this volume resulted in a long-lasting decrease in esophageal contractility, suggestive of structural damage. This interpretation is consistent with other work in rats, in which esophageal distension with 1.25 ml air boluses was considered to be noxious (Traub et al., 1994). As described by Euchner-Wamser et al., (1993), inflation of the Swan-Ganz catheter used by the Traub et al. with air at a volume of 1.25 ml to 1.5 ml would distend the esophagus to an outer diameter of 6 mm, which is equivalent to a

distension produced by a balloon filled with 300 µl water (present study, see methods in Chapter two).

As reflected by the evoked activity of neurons in the region of the rostral AMB, that is, presumptive esophageal motoneurons innervating the TMP, a balloon volume of 200 µl resulted in a submaximal activation of the striated muscle component. This volume evoked a tonic discharge at an average frequency of 34 Hz. Unpublished experiments on *in vitro* vagus nerve-esophagus preparations in our laboratory show that stimulus frequencies between 40-50 Hz cause a maximal tetanic contraction of the esophageal TMP.

At the level of the gastro-esophageal junction, an apparent relaxation was evoked by high but not low to moderate volume distension. Since this relaxation was abolished by vagotomy, it appeared to be neurogenic. A recent report suggests that the gastric relaxation evoked by esophageal distension is dependent on a vago-vagal reflex (Rogers et al., 1999). Conceivably, the same neural control system extends to the gastro-esophageal junction.

3.4.2 Dynamic range of vagal afferents

To date very few studies have dealt with esophageal vagal afferents in the rat (Andrew, 1956c; Clarke and Davison, 1974), and information concerning the activity elicited at high intraluminal pressures is lacking. In this study, single fiber recording of vagal afferents revealed that all units sampled had a wide dynamic range. Thus, at balloon volumes of 250-300 µl, the firing frequency continued to increment while the active

vago-vagal component of the evoked intraluminal pressure rise appeared to decrement. Because unilateral damage to the vagal innervation of the esophagus impairs rhythmic reflex peristalsis (Lu and Bieger, 1998a), the vagal afferent activity during reflex-evoked esophageal rhythmic contraction was unable to be record. The present data differ from those reported in the opossum, in which esophageal vagal afferents were shown to have both a low threshold and low saturation pressure and, hence, to encode information only in the low-pressure range (Sengupta et al., 1989).

Anatomically, vagal afferent terminal endings are located in esophageal myenteric ganglia, whereas relatively sparse innervation is found in the muscularis mucosae or striated musculature (Dütsch et al., 1998; Kressel and Radespiel-Tröger, 1999). Vagal afferent neurons innervating the distal esophagus have smaller cell bodies and less or no staining intensity for calretinin and calbindin than do neurons projecting to other parts of the esophagus (Dütsch et al., 1998; Kressel and Radespiel-Tröger, 1999). As the presence of the calcium-binding proteins in the terminal structures is a characteristic of low threshold and rapid adapting sensors (Duc et al., 1994), vagal afferents from the distal esophagus must have other properties. According to the present study, the majority of this fiber population innervates slowly adapting receptors. Furthermore, the observed distension-evoked firing frequencies were much lower than those reported for afferents supplying the cervical esophagus (Andrew, 1956c). Thus, regional response patterns may differ significantly in esophageal vagal afferents.

In the present and previous (Loomis et al., 1997) studies, the magnitude of arterial blood pressure responses correlated with esophageal intraluminal pressure, functionally implying the similarity in dynamic range of vagal afferents mediating esophageal

cardiovascular and motility responses. This dynamic range agrees with that observed in vagal fiber recordings. The data presented in Chapter two suggest that different populations of rat esophageal afferents in the vagus mediate distension-evoked reflex motility and two types of cardiovascular responses. Furthermore, anatomical studies suggest that substance P exists in some esophageal vagal afferents in the rat (Kressel and Radespiel-Tröger, 1999). Because of the limited number of esophageal vagal afferent fibers from which successful recordings were obtained, the present study cannot as yet discern different populations clearly. However, the data suggest that although all fibers showed an overlapping dynamic range, individual activity levels differed. Based on the discharge rate, the recorded fibers appeared to fall into three subgroups. Clearly, more work is needed to classify mechanosensory vagal afferents of the rat esophagus in functional terms.

By virtue of their wide dynamic range, rat esophageal vagal afferents may represent sensory neurons that have the ability to generate both normal motility-regulating and nociceptive signals depending on the intensity of stimulation (Cervero and Laird, 1999). Esophageal distension in humans is known to evoke pain (De Caestecker et al., 1992), and esophageal spasm and intense peristaltic contractions are considered to be painful (Cervero, 1994). In humans, distension-evoked pain has been reported to wax during relaxation and to wane during contraction; invariably, however, isometric contraction on an incompressible balloon was noted to be painful (Payne and Poulton, 1927). Accordingly, the slow wave tonic contractions in the rat esophageal body that result from high-volume distension by means of a water-filled incompressible balloon may be equivalent to a "pain spasm" (Payne and Poulton, 1927). Although it is generally believed

that esophageal pain is mediated by spinal afferents (Cervero, 1994; Lynn, 1992), there is evidence implicating an involvement of vagal afferent pathways (Andrews and Lawes, 1992; Loomis, 1997; Traub et al., 1994). The present work supports the idea that esophageal vagal afferents contribute to nociceptive processing.

3.4.3 Responsivity of NTS interneurons

Single unit recordings in the NTSc area revealed three different neuron groups that responded to distal esophageal distension. Although these units were obtained in a narrowly restricted region, the ventralmost recording sites in some tracks may have encroached on the DMV. However, the firing pattern of the neurons concerned is clearly different from that of units recorded in the DMV that respond to esophageal distension (Rogers et al., 1999). These DMV units were shown to fall into two types and to be activated or inhibited by esophageal distension (Rogers et al., 1999). In the present work, although type III units showed a burst discharge and superficially resembled the excitatory DMV units, the burst discharge of our type III units occurred well *after* deflating the esophagus, while excitatory DMV units displayed a burst discharge *before* deflation of the esophagus. During high-volume distension, our type I units changed their firing pattern from rhythmic bursting to a tonic discharge, type II units were further activated, and type III units fell silent but rebounded with enhanced deflation bursts. These results demonstrate that the pattern and strength of NTSc interneuron activity is dependent on the level of vagal afferent input. Since the type I units and esophageal motoneurons had corresponding firing patterns over the entire pressure range tested, and since both reflected the motility pattern recorded in the esophagus, the present results

suggests that the type I unit represents a premotoneuron. The functions of type II and type III units are currently not clear; however, it is reasonable to believe some of type II units represent interneurons mediating esophageal distal inhibition (see Chapter five) or esophageal cardiovascular reflex responses (Loomis et al., 1997). Recordings in the rat NTSc described by others (Rogers et al., 1999) have shown that most units are silent at rest and do not fire rhythmically during esophageal distension. This discrepancy could be attributable to differences in the depth of anesthesia; moreover, in the rat thoracic esophagus, distension may evoke a single phasic motor response (Lu and Bieger, 1998a).

3.4.4 Summary

The evidence obtained in the present study suggests that the reflex motor response pattern and contractile force in the rat esophagus vary with the strength of vagal afferent input. The role, if any, of spinal afferent input in esophageal motor control is yet to be defined. The responsiveness of vagal afferent neurons and NTS interneurons to high volume distension indicates that this system has the ability to encode information in a high-pressure, noxious range.

Chapter four

Distal and deglutitive inhibition in the rat esophagus: role of inhibitory neurotransmission in the nucleus tractus solitarii

4.1 Introduction

Neurophysiological evidence from studies in the sheep supports the hypothesis that the central motor pattern generating network controlling the upper alimentary tract is functionally polarized such that interneurons governing proximal segments exert a powerful inhibition of interneurons coordinating the activity of aboral segments (Roman, 1986; Jean 1984). This mechanism is inferred to operate in both the so-called distal inhibition, i.e., a cessation of peristalsis in aboral segments during distension of the proximal esophagus, and deglutitive inhibition, i.e., the arrest of ongoing primary or secondary esophageal peristalsis during a swallow. Distal and deglutitive inhibition have been investigated extensively in the human (Creamer and Schlegel, 1957; Sifrim et al., 1992; Sifrim and Janssens, 1996; Vanek and Diamant, 1987; Diamant, 1989; Williams et al., 1993), and several studies have shown that in some patients with esophageal symptoms, these inhibitions are impaired (Deschner et al., 1989; Kendall et al., 1987; Richter et al., 1989; Sifrim et al., 1994). However, the underlying neural mechanisms are still poorly understood and no previous reports have documented their presence in the rat esophagus. Because the rat esophagus effects peristaltic bolus transport principally by the striated musculature of its muscularis propria, a central network presumably constitutes the predominant source of neuroregulatory reflex control (Bieger, 1993).

The aims of the present investigation were threefold: 1) to establish the existence of esophageal relaxant responses induced by proximal distension or swallowing, 2) to correlate these responses with firing patterns of esophageal reflex interneurons or motoneurons, and 3) to obtain pharmacological evidence for the involvement of inhibitory amino acidergic neurotransmission. For the latter purpose, antagonists of γ-aminobutyric acid (GABA) and glycine receptors were targeted at the region of the medulla oblongata known to contain esophageal premotor neurons and their primary visceral afferents.

4.2 Material and methods

4.2.1 General Procedures

The experiments were performed in 48 male Sprague-Dawley rats (300-400 g). General procedures are the same as described in Chapters two and three.

4.2.2 Esophageal distension

To test distal inhibition, two small inflatable high compliance balloons made from PE-60 polyethylene tubing were filled with water and placed in the mid-thoracic and distal part of esophagus (7.5-9 and 11-12cm from upper incisors, respectively) for distending the esophagus and simultaneous recording of intraluminal pressure. The balloons had a diameter of 9 mm and a volume of 400 μl when fully inflated. The volume of the balloons was controlled by means of a manually operated syringe. Distal esophageal distension at a volume of approximately 80 μl was applied for 7-15 s; thoracic esophageal distension at a volume of 50 μl for 2-7 s. To test deglutitive inhibition, a third water filled polyethylene balloon (50 μl) was inserted into the pharynx for recording pharyngeal pressure. Swallows were evoked by mechanical or chemical (distilled water or 50% ethanol, 1-4 μl) stimulation of the airway or by gentle displacement of the pharyngeal balloon. In addition, brief bouts of fictive swallowing were evoked by application of bicuculline to the surface of the NTS (Wang and Bieger, 1991).

4.2.3 Medullary single unit recording and vagotomy

The technique of single unit recording in medulla oblongata is the same as described in Chapter three.

Bilateral vagotomy was acutely performed in 5 rats. The upper cervical vagal trunks were dissected free, snared with loose silk threads and cut at the level of the cricoid cartilage. Animals were allowed to stabilize for 5 min before the start of data collection.

4.2.4 Pharmacological Procedures

The $GABA_A$ receptor antagonist bicuculline methiodide, the glycine receptor antagonist strychnine hemisulfate salt, the chloride channel blocker picrotoxin, and urethane were obtained from Sigma Chemical (St. Louis, MO); the $GABA_B$ receptor antagonist 3-amino-2-(4-chlorophenyl)-2-hydroxy propyl sulphonic acid (2-(OH)-Saclofen) and the $GABA_C$ receptor antagonist 3-aminopropylphosphonic acid (3-APPA) from Tocris Neuramin (England). 2-(OH)-Saclofen and picrotoxin were dissolved in dilute aqueous base (1.5% $NaHCO_3$).

Aqueous solutions of drugs were applied to the NTS surface from a Hamilton microliter syringe (1 μl) fitted with a thin flexible polyethylene catheter. The volume applied was kept within 0.02 μl, to minimize spread beyond the targeted region (Lu and Bieger, 1998b; Wang and Bieger, 1991). As visualized by local application of the fluorophore bisbenzimide (0.05%, 0.02 μl), this volume covered the entire extraventricular surface of the NTS, spreading ventrally by 400 μm, and the transverse axis by about 500 μm, with only traces of fluorescence being detectable on the contralateral NTS surface

adjoining the area postrema. For control, dissolved drugs were applied at adjacent sites on the dorsal surface of the medulla oblongata as follows: 1) the midline at the caudal edge of the area postrema; 2) 1,700-1,800 μm lateral to the midline at the rostral edge of area postrema.

Picrotoxin was given intravenously at an initial dose of 3 mg/kg, and repeated over 15-20 min up to a total dose of 10 mg/kg, until the animal showed prodromal signs of seizure activity.

5.2.5 Data analysis

To ensure reproducibility of esophageal reflex responses, the interval between successive esophageal distensions was kept between 2 and 3 min. Drug applications were performed after at least three consistent control responses were obtained. When the effect of a drug was no longer detectable, a 30 min rest interval was observed before sampling of subsequent control responses for the next drug challenge. Drugs (including application to the control sites) were tested in random order. The amplitude of esophageal contraction was taken as the pressure difference between the peak and the trough of the contraction wave; the interval between two contractions was measured at the peak of the pressure wave; and the duration of the falling phase of a pressure wave was taken as the time difference between the peak and the following trough of the wave. The wave frequency was computed by averaging 4-8 cycles. Data are presented as means ± SEM. Student's paired t-tests were done with statistical software (Microcal Origin, Microcal Software, Inc.). Statistical significance is defined as $P<0.05$. Unless stated otherwise, the number (n)

given in parenthesis refers to individual separate experiments, except in the case of extracellular unit recordings where it specifies the total number of individual units.

4.3 Results

4.3.1 Distal inhibition

Motor and ventral medullary single unit responses to esophageal distension. In the distal esophagus, rhythmic pressure waves (mean frequency 0.66±0.03 Hz; n=14) occurred during local distension at low to moderate volume (50-150 μl). Concurrent mid-thoracic esophageal distension invariably resulted in an instantaneous cessation of this rhythmic response (i.e. distal inhibition). Upon deflation of the proximal esophageal balloon, the distension-evoked rhythmic contractions resumed promptly. Bilateral cervical vagotomy (n=5) abolished distension-evoked reflex contractions and demonstrated that distal intraluminal pressure did not change during proximal inflation (Fig. 4.1C).

Units recorded in the ventral medulla at the level of the rostral nucleus ambiguus (n=15, from 6 rats) were silent at rest and fired only during esophageal contractions. When contractions were rhythmic, unit activity displayed a crisp burst discharge that was phase-locked with each pressure wave. Proximal inflation caused an immediate cessation of both rhythmic firing and phase-locked pressure wave activity evoked by distal distension (Fig. 4.1A, 4.1B).

Dorsal medullary single unit responses. As described in Chapter three, three types of evoked neural activity were observed in the intermediate/caudal NTS region (Fig. 4.2). Type I units (n=8, from 5 rats) discharged in distinct bursts in phase with distal esophageal activity; type II units (n=16, from 7 rats) fired tonically without a discernible rhythm. Type III units (n=8, from 4 rats) were either partially or totally inhibited during balloon inflation at both levels, and showed a prominent rebound response upon deflation. Evoked rhythmic

Fig. 4.1 Distal inhibition in rat esophagus.

Single extracellular spike activity (ESA) recorded in rostroventral medulla oblongata at level of rostral nucleus ambiguus. Note absence of baseline discharge at rest. Rhythmic burst discharge and phase-locked pressure wave activity elicited by distal esophageal (DE) distension (80μl) (**A**) are suppressed during concurrent distension (50μl) of the mid-thoracic esophagus (TE) (**B**). After bilateral cervical vagotomy, esophagus is not responsive to distal or combined distal and mid-thoracic esophageal distension (**C**). Initial pressure wave results from rapid injection of fluid into balloon catheter. Return of pressure below zero level marks rapid withdrawal of fluid from balloon.

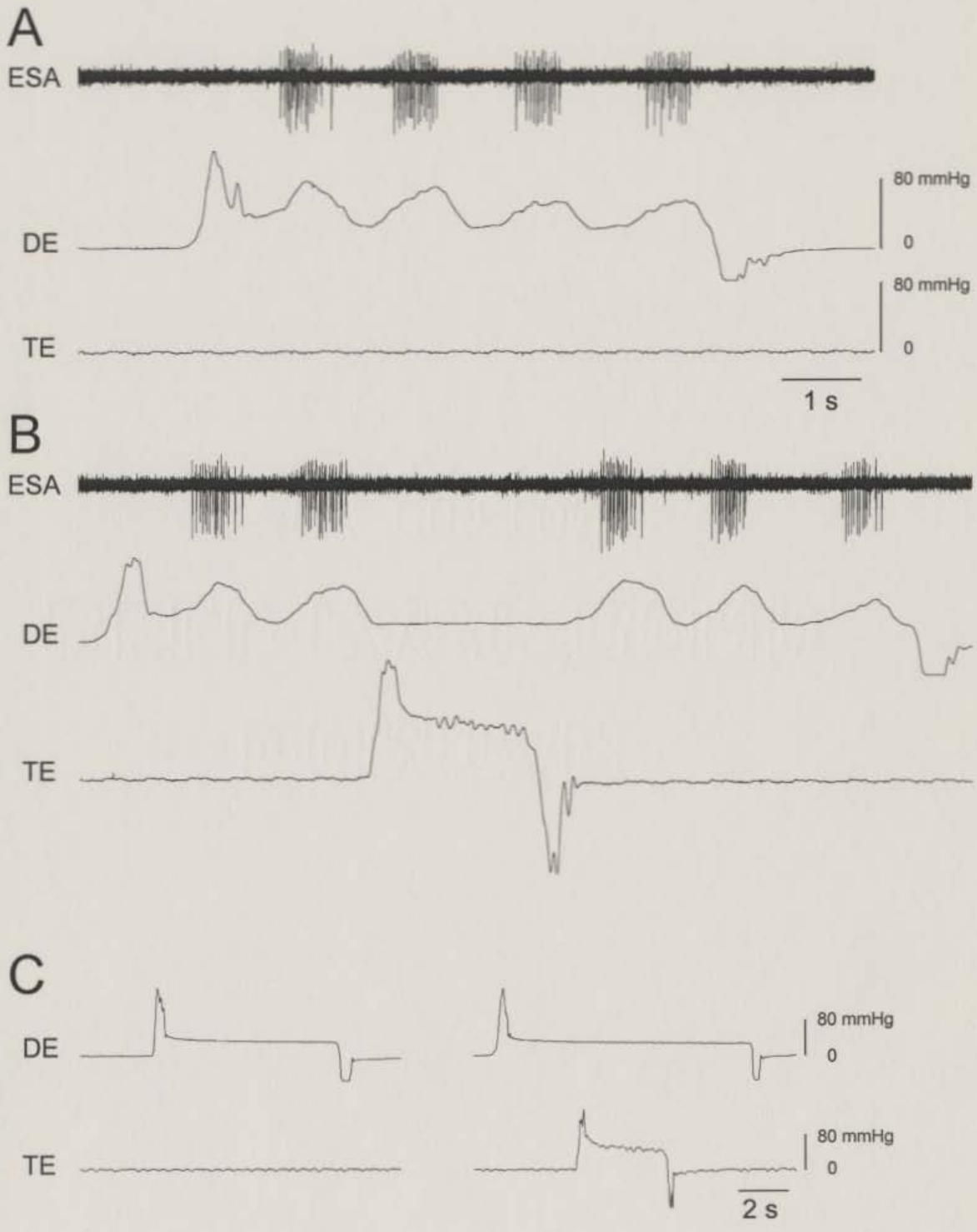
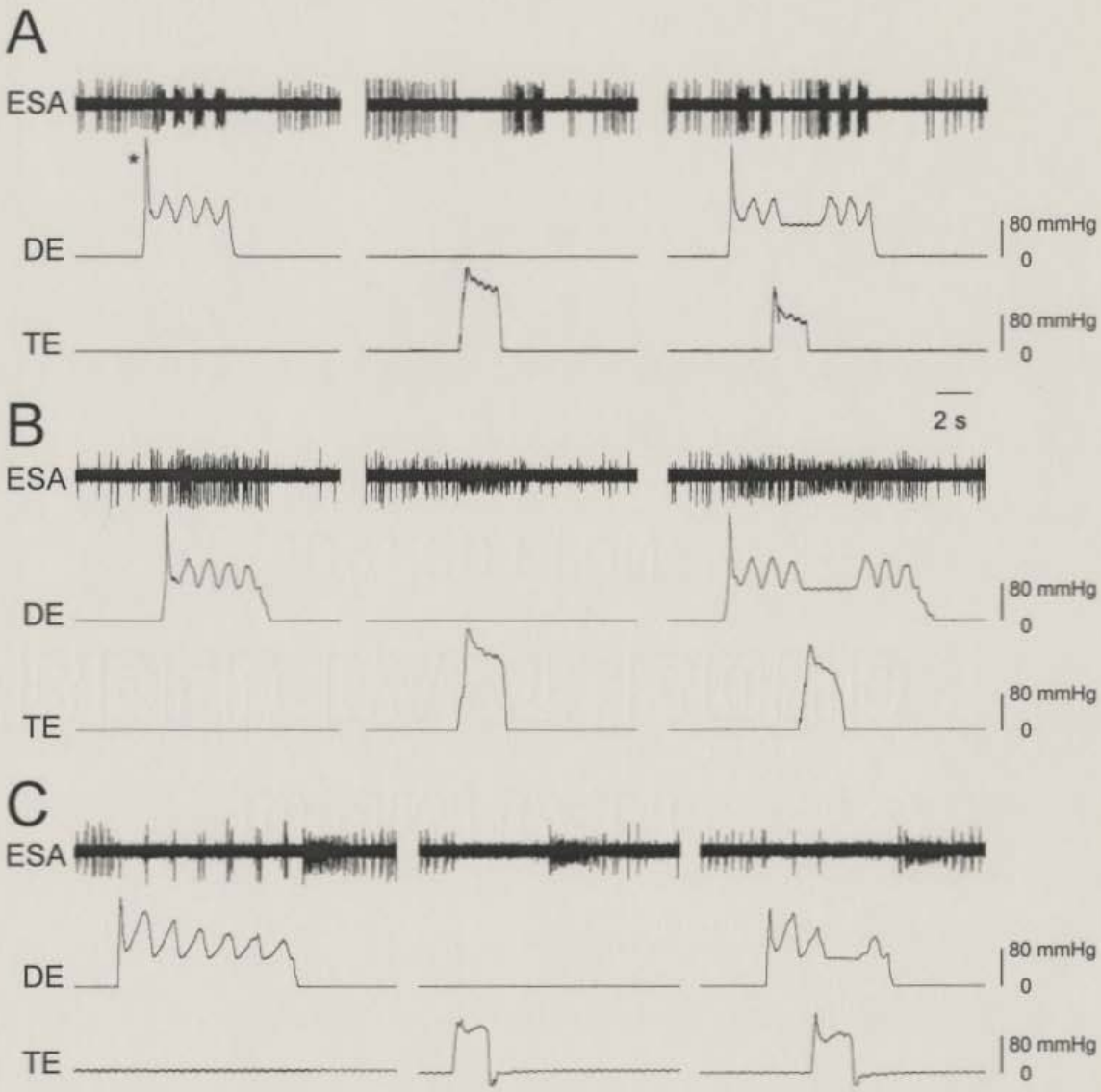

A
ESA
DE
80 mmHg
0
80 mmHg
TE
0
1 s
B
ESA
DE
TE
C
DE
80 mmHg
0
TE
80 mmHg
0
2 s

Fig. 4.2 The firing patterns of distension-responsive units in the NTSc region during distal inhibition. Type I unit (**A**) shows slow rhythmic bursting in phase with intraluminal pressure waves during distension of the distal esophagus and prominent inhibition during mid-thoracic inflation followed by rebound discharge not associated with detectable esophageal pressure change (see text). During superimposed mid-thoracic inflation, distal inhibition is evident as indicated by cessation of both unit burst activity and pressure waves; note immediate return of both responses upon deflation of thoracic balloon. Type II unit (**B**) fires non-rhythmically during either thoracic or distal esophageal distension. Concurrent distension (right panel) augments unit discharge but simultaneously arrests rhythmic pressure wave activity in distal esophagus. Type III ("deflation") unit (**C**) fires 1 to 2 spikes in phase with the falling phase of the esophageal pressure wave during distal inflation. During mid-thoracic inflation alone or combined with distal inflation unit falls silent. Balloon deflation at either level triggers single "off" discharge of similar strength; however, note longer latency of discharge with thoracic esophageal distension. Esophageal distension was performed at fixed balloon volumes (80 μl DE; 50 μl TE). Initial sharp peak in pressure trace (*) is an experimental artifact due to rapid injection of fluid into balloon catheter. Small pressure fluctuations are due to respiration.

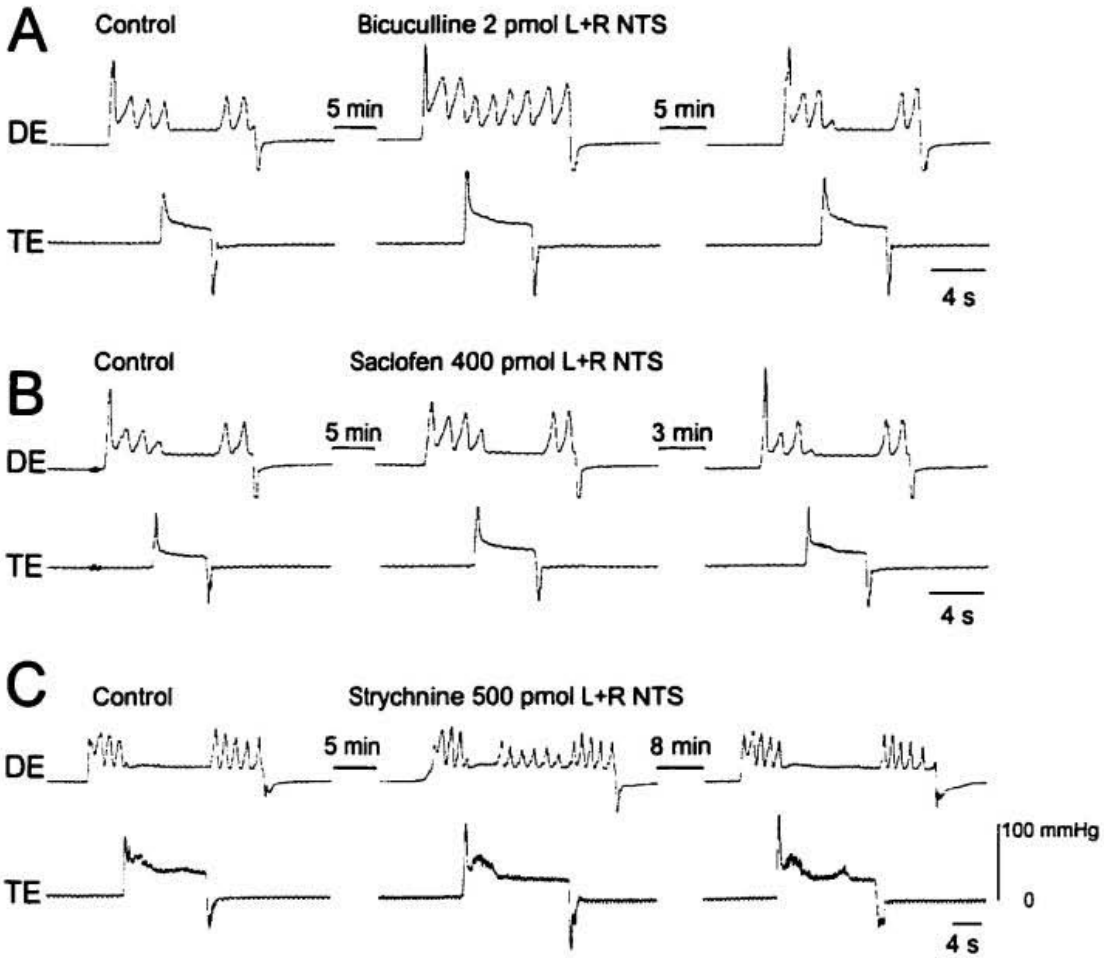

A
ESA
DE
TE
80 mmHg
0
80 mmHg
0
2 s
B
ESA
DE
TE
80 mmHg
0
80 mmHg
0
C
ESA
DE
TE
80 mmHg
0
80 mmHg
0

burst responses of type I units were temporarily inhibited during proximal distension. During proximal inflation alone, type I units fell silent, but showed one or two rebound burst discharges upon balloon deflation. These were not associated with a detectable pressure change in the deflated balloon (Fig. 4.2A). However, at balloon filling volumes of 20 μl, an associated esophageal pressure signal was consistently observed (not shown).

Type II units showed an increase in firing during proximal inflation that was augmented during combined proximal and distal inflation (Fig. 4.2B). In type III units, proximal distension alone or in combination with distal distension inhibited firing with a rebound discharge following deflation; however, the latency of rebound firing was longer than that seen with distal inflation (2.70±0.45 s vs. 0.53±0.09 s, $P<0.05$) (Fig. 4.2C).

Effects of bicuculline and 2-(OH)-saclofen. After local NTS surface application of bicuculline (2 pmol, each side), distal inhibition was clearly diminished as evidenced by a persistence of distal esophageal rhythmic contractions during mid-thoracic distension (n=13). In 9 cases, the distal rhythmic pressure wave activity recovered before deflation of the mid-thoracic balloon; in 4 cases, mid-thoracic distension ceased to slow down distal rhythmic activity. The amplitude of evoked esophageal pressure waves was decreased during mid-thoracic esophageal distension in all cases (27.5%±2.6% lower than amplitude before mid-thoracic distension, $P<0.05$). The effects were evident in 3 min and wore off between 10 and 15 min (Fig. 4.3A). After a larger dose of bicuculline (20 pmol, each side), autorhythmic buccopharyngeal swallowing ensued, which obviated testing of distal inhibition (see below). By contrast, the $GABA_B$ receptor antagonist 2-(OH)-saclofen (400 pmol, each side) was without effect in 9 animals at the doses tested (Fig. 4.3B).

Fig. 4.3 Effects of bicuculline, 2-(OH)-saclofen and strychnine on distal inhibition. Bilateral application of bicuculline to the extraventricular surface of NTS reversibly attenuates distal inhibition (**A**). 2-(OH)-Saclofen fails to alter distal inhibition (**B**). Following bilateral NTS surface application of strychnine, suppression of distal esophageal distension evoked rhythmic pressure wave activity during mid-thoracic balloon inflation is reversibly attenuated (**C**). Balloon inflation volumes: 80μl in DE, 50μl in TE. Traces in **A** and **B** are from same animal, **C** from another animal. Pharyngeal pressures traces (not illustrated) remained inactive in all cases.


A
Control
Bicuculline 2 pmol L+R NTS
DE
TE
5 min
5 min
4 s
B
Control
Saclofen 400 pmol L+R NTS
DE
TE
5 min
3 min
4 s
C
Control
Strychnine 500 pmol L+R NTS
DE
TE
5 min
8 min
100 mmHg
0
4 s

Effect of strychnine. Distal inhibition was partially inhibited after applying strychnine (500 pmol, each side, n=11) to the NTS surface. Thus, distal esophageal rhythmic activity resumed before deflation of the mid-thoracic balloon, however, the amplitude remained depressed (34.4%±7.2% lower than amplitude before mid-thoracic distension, $P<0.05$) during residual distal inhibition. Responses returned to baseline levels between 10 and 15 min after drug application (Fig. 4.3C).

4.3.2 Deglutitive inhibition

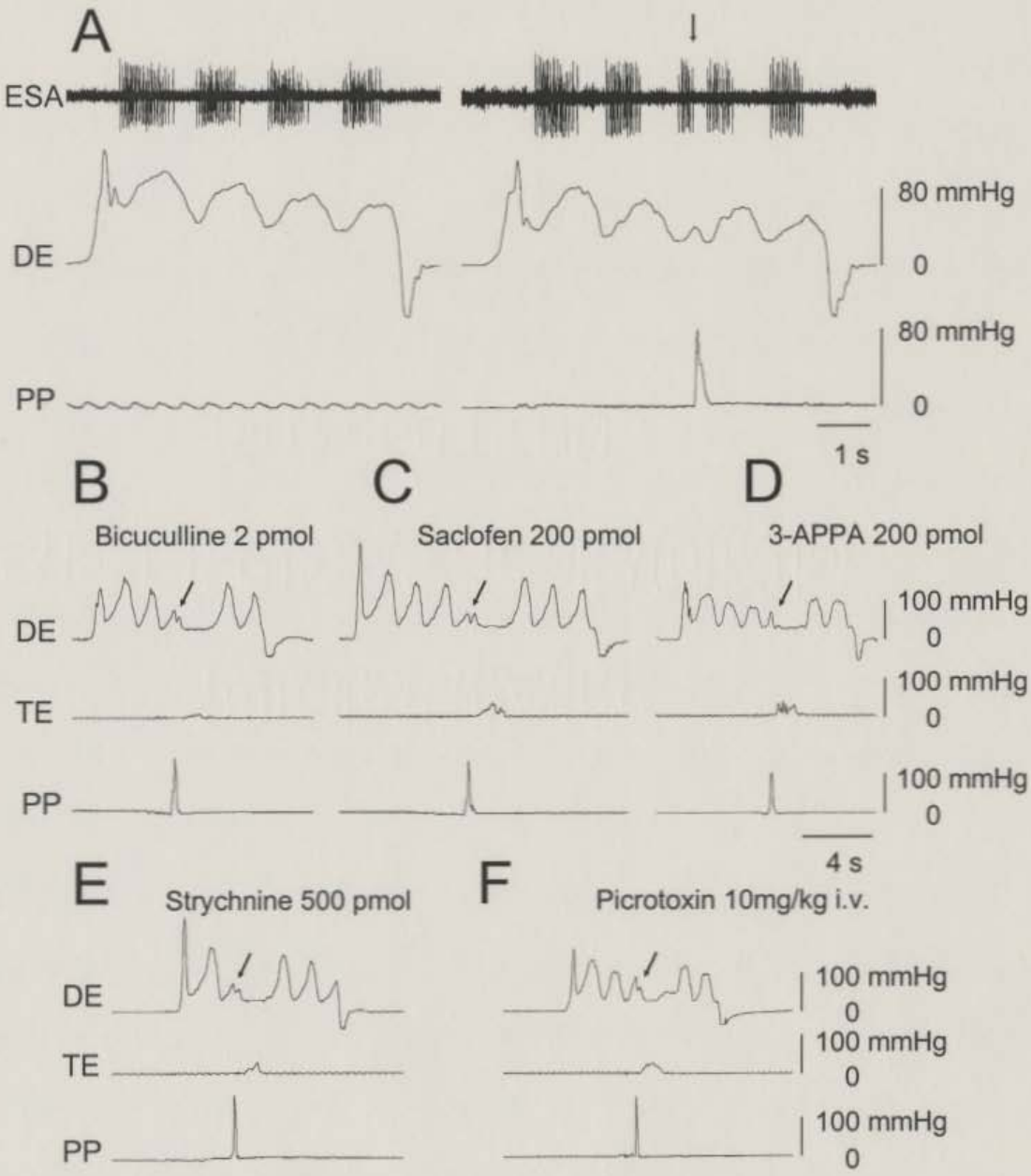
Motor and ventral medullary single unit responses to a swallow. When a swallow coincided with the rising phase of a distension-evoked distal esophageal pressure wave, an immediate drop in esophageal pressure occurred after the start of the pharyngeal contraction. The lag time between both events lasted up to 100 ms (mean 54.4±9.5 ms, n=12). Typically, this was evident either as a sharp notch in the rising phase of the esophageal pressure wave, or a premature termination of the rising phase (Fig. 4.4). When the swallow coincided with the falling phase of a pressure wave, the duration of the latter was shortened from 0.36±0.02 s to 0.28±0.02 s (n=7, $P<0.05$). In most cases (n=14), the subsequent rhythmic pressure wave was reduced in amplitude, or even absent, if it occurred during a propagated pharyngoesophageal response recorded in the upper balloon as a phasic pressure rise. In the latter case, distal esophageal activity remained inhibited until completion of the proximal pressure wave. The interval between pharyngeal pressure change and onset of the following distal esophageal rhythmic pressure wave was 2.0±0.1 s. An identical delay was noted between pharyngeal pressure change and the onset of the

esophageal pressure wave as recorded in the distal balloon during a normal swallow in the same animals (2.0±0.1 s, $P>0.05$). When the swallow was not propagated (n=5), the subsequent rhythmic waves had unaltered amplitudes and timing.

Extracellular single unit recording in the ventral medulla showed that, during a swallow, rhythmic burst firing was punctuated by an brief period of inhibition (Fig. 4.4A). Similar recordings were attempted in the NTS but were unsuccessful because deglutitive movement artifacts caused displacement of the recording micropipette.

Effects of antagonists. When applied to the NTS surface, neither bicuculline (n=12), nor 2-(OH)-saclofen (n=9), nor 3-APPA (n=5), nor strychnine (n=7) blocked swallow-induced fast relaxations of reflex evoked pressure waves in the distal esophagus. (Fig. 4.4B-E). In two other animals treated with bicuculline (20 pmol each side), autorhythmic buccopharyngeal swallowing occurred at a rate of 0.55 Hz. When swallows coincided with the rising phase of distension-evoked pressure waves, they were consistently associated with a fast relaxation of esophageal pressure. Intravenous application of picrotoxin (3 to 10 mg/kg) did not block swallowing-evoked esophageal relaxation (n=3) (Fig. 4.4F).

Fig. 4.4 Deglutitive inhibition of distension-evoked esophageal activity. Presumptive esophageal motoneuron responds with rhythmic bursts during distal esophageal inflation (**A**). Trace shown in right panel reveals brief pause (↓) coincident with pharyngeal pressure (PP) wave, indicative of buccopharyngeal swallow. The pressure traces recorded in upper balloon (not illustrated) remain inactive. Note concomitant notch in rising phase of esophageal pressure wave. Local blockade of GABA (**B-D**) or glycine (**E**) receptors in the NTS fails to change deglutitive inhibition. The antagonists were applied bilaterally to the NTS surface. Doses refer to amount of drug applied to each side. Intravenous application of picrotoxin also fails to block deglutitive inhibition (**F**). Traces in A; B, C, E; and D, F are from three different animals, respectively. All responses were recorded 5 min after each drug application. Oblique arrows mark swallow-induced fast relaxation. Distal balloon distension was performed at a volume of 80 μl. Swallowing was evoked by intratracheal injection of 40% ethanol (2 μl) in trace A; and gentle displacement of the pharyngeal balloon in trace B-F.


A
ESA
DE
PP
80 mmHg
0
80 mmHg
0
1 s
B
Bicuculline 2 pmol
C
Saclofen 200 pmol
D
3-APPA 200 pmol
DE
TE
PP
100 mmHg
0
100 mmHg
0
100 mmHg
0
4 s
E
Strychnine 500 pmol
F
Picrotoxin 10mg/kg i.v.
DE
TE
PP
100 mmHg
0
100 mmHg
0
100 mmHg
0

4.4 Discussion

Although both distal and deglutitive inhibition of the esophagus have been extensively studied in humans (Creamer and Schlegel, 1957; Sifrim et al., 1992; Sifrim and Janssens, 1996; Vanek and Diamant, 1987; Diamant, 1989; Williams et al., 1993), and laboratory animals (Hellemans et al., 1974; Diamant and El-Sharkawy, 1977), information about the underlying neural mechanism(s) is still limited. Two recent studies in the opossum, which has a mixed striated and smooth muscle esophagus, suggest that deglutitive and distal inhibition are not the same (Paterson et al., 1988), and that the latter depends in part on a local intramural reflex utilizing nitric oxide as the final mediator (Paterson and Indrakrishnan, 1995). In the rat, the entire tunica muscularis propria is made up of striated muscle whose motor endplates are innervated by motoneurons located in the compact formation of the nucleus ambiguus (Bieger and Hopkins, 1987; Bieger, 1993). Although a nitrergic co-innervation of those motor endplates has been reported (Wörl et al., 1994), there is at present no evidence to implicate such a pathway in the inhibitory events studied in this investigation. On the contrary, the present study reinforces the concept that both distal and deglutitive inhibition in the rat striated muscle esophagus reflect neural events organized at the level of the medulla oblongata. The absence of distension evoked reflex responses in bilateral vagotomized preparations precluded testing of distal inhibition per se; nonetheless, the results fail to provide evidence in favor of a locally generated smooth muscle component under neuronal control. Moreover, in the case of deglutitive inhibition, the relaxation occurring in the esophageal body was evident only during active esophageal

contraction, and hence not likely to arise from activation of intramural inhibitory ganglia controlling smooth muscle of the tunica muscularis mucosae.

As evidenced by extracellular single unit recordings from presumptive esophageal motoneurons in the ventral medulla, neuronal activity and esophageal pressure changes were closely correlated, suggesting that the esophageal inhibition seen during two-balloon inflation or swallowing mirrored central esophagomotor output instead of a local mechanical artifact. As discussed below, these inhibitions appear to involve different neural mechanisms.

4.4.1 Distal inhibition

The observed suppressant effect of bicuculline strongly implicates a GABA-ergic inhibition of NTS esophageal premotoneurons as the principal responsible mechanism. Since this effect was not mimicked by the $GABA_B$ receptor antagonist 2-(OH)-saclofen, $GABA_A$ receptors probably play a primary part. Previous work has shown that localized blockade of $GABA_A$ receptors within the NTSc region releases esophageal premotoneurons from a tonic inhibition, as evidenced by slow rhythmic contractions of the esophagus (Wang and Bieger, 1991). Conversely, the present observations demonstrate that, during distal inhibition, the activity of distal esophageal premotoneurons is subject to a phasic inhibition that depends on the activation of $GABA_A$ receptors.

The partial sensitivity of distal inhibition to the glycine receptor antagonist strychnine raises the possibility that glycinergic inhibitory interneurons in the NTS region also contribute to distal inhibition. Although glycine is generally believed to act as the major

inhibitory neurotransmitter at the spinal level, it is distributed throughout the central nervous system. In the NTS area, glycinergic inhibitory interneurons are known to play a part in the control of blood pressure (Kubo and Kihara, 1987; William and Robertson, 1989). The phenomena observed in this study did not result from the blockade of the glycine binding site at the NMDA receptor, since this site is known to be strychnine resistant (Thomson, 1989). Indeed, strychnine did not inhibit esophageal secondary peristalsis which relies on esophageal vagal input and activation of NMDA receptors at the level of the NTSc (Loomis et al., 2001).

Neither bicuculline nor strychnine totally abolished distal inhibition as the amplitude of esophageal contraction waves during mid-thoracic distension remained below the control level. This decrement is unlikely to be a mechanical artifact due to upper balloon inflation, because the two balloons were separated by at least 2 cm, and furthermore, upper balloon inflation did not change the pressure level recorded between waves in the distal balloon. Interestingly, however, bicuculline appeared to cause a complete block of the decrease in contraction frequency in about 1/3 of the animals. Although we did not test the combined effects of bicuculline and strychnine, our findings suggest that GABA- and glycinergic inhibition operate in a synergistic manner. Recently, glycine and GABA were shown to effect synergistic inhibition of low threshold mechanical input in the spinal cord of the rat (Khandwala et al., 2000). Possibly, GABA and glycine mediated distal inhibition comes from the same neuron population, as co-release of GABA and glycine from the same spinal interneuron has recently been reported (Jonas et al., 1998).

Extracellular single unit recording in or near the NTSc region revealed two groups of neurons that increased their activity with different firing patterns during esophageal distension. As inferred in the previous Chapter, Type I neurons had the firing pattern expected of esophageal premotoneurons, i.e., elements programming the motoneuronal output to the tunica muscularis propria. Type II neurons did not show a clear rhythmic burst activity and had wide receptive fields. Tentatively, some of these neurons may be considered inhibitory interneurons; however, more work is needed to corroborate this idea. Type III neurons also had wide receptive fields, but were inhibited by esophageal distension at both levels, and displayed strong rebound burst discharges upon balloon deflation. Most probably these neurons are esophageal premotoneurons controlling segments caudal to the level at which the distal balloon was stationed; conceivably, they receive summated postsynaptic inhibition via type II neurons from proximal levels. Previous work in this lab has shown that the majority of neurons in the NTSc display a prominent postinhibitory rebound upon termination of a hyperpolarizing current injection (Lu, 1996).

4.4.2 Deglutitive inhibition

The current data implicate at least two underlying components. An early fast relaxation of ongoing distal esophageal pressure waves occurred immediately after the onset of buccopharyngeal activity, even if the swallow was not propagated. As this is a very fast and short-lasting inhibition, it is unlikely to be mediated by a complex neuromodulatory system. The second component, evidenced by a phase-shift in esophagomotor

rhythm, coincided with swallow-induced activity in the proximal esophagus. Since the interval between pharyngeal pressure rise and the onset of the following distal esophageal rhythmic pressure wave corresponded to that of a primary peristaltic wave propagating to the distal esophagus, a common inhibitory process may be inferred. Since both components appeared to be resistant to bicuculline, strychnine or 3-APPA applied directly to the NTS, or systemic picrotoxin, they are unlikely to result from a postsynaptic GABA- or glycine-mediated inhibition of NTSc neurons. A previous study in this laboratory has shown that bicuculline is ineffective in blocking the uncoupling of primary esophageal peristalsis when the rate of buccopharyngeal swallowing exceeds 10-12/min (Wang and Bieger, 1991). Since deglutitive inhibition was also resistant to 2-(OH)-saclofen, it does not appear to be mediated by postsynaptic $GABA_B$ receptors either. Although some studies report certain subtypes of $GABA_B$ presynaptic receptors to be insensitive to 2-(OH)-saclofen, these receptors are implicated in inhibition of GABA release, i.e. inhibition of inhibition (Davies and Collingridge, 1993; Deisz et al., 1997). If deglutitive inhibition were to involve a presynaptic inhibition, it would arise from inhibition of synaptic excitation, not inhibition. Another alternative is that this inhibition is mediated by interneurons that directly project to esophageal motoneurons controlling the distal esophagus. Such an arrangement would require the generation of an inhibitory post-synaptic potential (IPSP) in esophageal motoneurons. A recent report has shown $GABA_A$ receptors to be present in esophageal motoneurons (Broussard et al., 1996). However, previous work has revealed only excitatory post-synaptic potentials (EPSPs) in these neurons during microstimulation of the solitarioambigual pathway in a brainstem sagittal

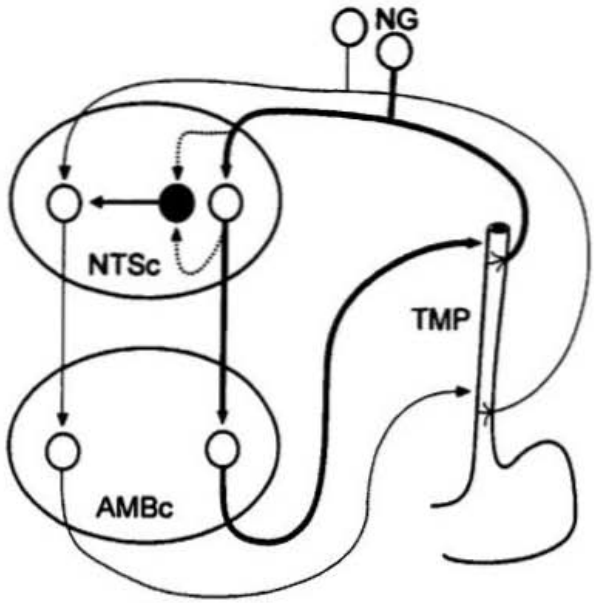


Fig. 4.5 Schematic diagram outlining proposed mechanism for distal inhibition. The premotor neurons in the NTS *subnucleus centralis* (NTSc) receive segmentally organized esophageal vagal afferent input and control the activity of motoneurons in the compact formation of the nucleus ambiguus (AMBc) that innervate the striated muscle tunica muscularis propria (TMP) of the esophagus. Distal inhibition results from excitation of GABA-ergic and/or glycinergic interneurons in the NTSc (filled symbol) that are activated by collaterals (dotted lines) of either vagal afferent or esophageal premotor neurons controlling proximal esophageal segments. The inhibitory output of these putative local interneurons impinges on NTSc neurons controlling distal esophageal segments and is mediated by postsynaptic ligand-gated chloride channels. The system mediating deglutitive inhibition may involve a different type of NTSc interneuron and/or different sites of synaptic inhibition, e.g., primary afferent terminals, or motoneurons. NG: nodose ganglion.

slice preparation (Wang et al., 1991). In the present work, systemic picrotoxin did not block this inhibition. Therefore, extrasolitary GABA systems are most probably not involved in deglutitive inhibition.

The present data support the working hypothesis that esophageal distal inhibition is mediated by inhibitory interneurons in the NTSc region (Fig. 4.5). These interneurons receive inputs from vagal afferents or premotoneurons controlling the mid-thoracic esophagus, and inhibit esophageal premotoneurons controlling the distal esophagus via post-synaptic $GABA_A$ and glycine receptors. The neural mechanism of deglutitive inhibition seems to be different, at least in terms of the pharmacological criteria applied in this investigation.

Both distal and deglutitive inhibition probably form an integral part of the central swallowing program. Functionally, both would serve to promote bolus transport. Swallow-induced suppression of ongoing distal esophageal contractions would aid passage of a succeeding bolus through the active segment. Distal inhibition would reduce the number of boluses in transit through the esophagus. Several studies have shown that patients with esophageal symptoms such as dysphagia, achalasia, diffuse esophageal spasm, and chest pain, may have abnormalities in deglutitive and/or distal inhibition (Deschner et al., 1989; Kendall et al., 1987; Richter et al., 1989; Sifrim et al., 1994).

4.4.3 Summary

Both distal and deglutitive inhibition are present in the rat esophagus. Distal, but not deglutitive, inhibition depends upon activation of $GABA_A$ and glycine receptors in the

solitary complex. $GABA_B$ and $GABA_C$ receptors in this area do not appear to contribute to either deglutitive or distal inhibition. The observed differences in responses of NTSc neurons point to the existence of functional sub-groups, with wide receptive field inhibitory amino acidergic local interneurons being a putative source of distal inhibition.

Chapter five

Mediation by nucleus tractus solitarii and rostral ventrolateral medulla glutamatergic neurotransmission of the cardiovascular reflex evoked by distal esophageal distension

5.1 Introduction

Previous studies (Loomis et al., 1997, Chapter two) have shown that the ECVR is a vagally mediated reflex. As yet information about the synaptic organization of the ECVR is limited. In particular, the neurotransmitter mechanisms operating at the level of the medulla oblongata need to be specified and compared to that of other cardiovascular reflexes such as baro- and chemoreceptor reflexes.

Anatomical tracing studies have shown that esophageal vagal afferent fibers terminate in the nucleus tractus solitarii (NTS) subnucleus centralis (NTSc) or its immediate vicinity (Altschuler et al., 1989). However, the involvement of this region in the ECVR has not been determined. Glutamate is believed to be the principal neurotransmitter released from vagal afferents (Lawrence, 1995; Schaffar et al., 1997). It is implicated as a potential mediator of esophageal motility reflexes (Lu and Bieger, 1998b) and of cardiovascular reflexes such as the baroreflex (Talman et al., 1980; Lawrence and Jarrott, 1994) and the chemoreceptor reflex (Vardhan et al., 1993). Therefore, the question arises if

glutamatergic neurotransmission in the NTSc and/or its immediate vicinity play a part in the ECVR.

The rostral ventrolateral medulla (RVLM) plays a crucial role in the tonic and phasic regulation of blood pressure. As a common output area for vasomotor components of cardiovascular reflexes such as the baro- and chemoreceptor reflex (Dampney, 1994), the RVLM has been hypothesized to act as a link between the central structure(s) mediating the ECVR and the spinal sympathetic motoneurons (Loomis et al., 1997). Available data suggest that GABA-ergic and glutamatergic neurotransmissions in this area mediate the baro- and the chemoreceptor reflex, respectively (Sun and Guyenet, 1985; Koshiya et al., 1993; for review see Dampney, 1994). In the ECVR and the chemoreceptor reflex, the increase in afferent input evokes a pressor response, and the opposite is true for the baroreflex, where an increased input evokes a depressor response. Thus, the ECVR and chemoreflex presumably share a common mechanism to activate the RVLM sympathetic bulbospinal neurons. However, the actual role of the RVLM in the ECVR remains unclear.

The purpose of the present study was to determine 1) whether interneurons mediating the ECVR are located in or near the NTSc; 2) whether synaptic excitation of these NTS interneurons depends on activation of glutamate receptors; and 3) whether the putative link between the NTS and the RVLM operates via a glutamatergic rather than a GABA-

ergic synapse.

5.2 Materials and Methods

5.2.1 General procedures and esophageal distension

The experiments were performed in 48 male Sprague-Dawley rats (300-400 g) anesthetized with urethane (1.2 g/kg) given intraperitoneally. After tracheal intubation, the right external jugular vein and left carotid artery were cannulated for infusion of drugs and recording of arterial blood pressure. Rate and depth of breathing were derived from tidal pressure fluctuations recorded via a catheter inserted into the tracheal cannula. Rectal temperature was maintained at 37-38 °C by means of radiant heat. Carotid arterial, esophageal and respiratory pressure signals were collected through transducers, amplified and recorded on a polygraph (model 7D, Grass Instruments, Quincy, MA) together with heart rate, which was computed from the carotid pulse by means of a tachograph.

The technique of esophageal balloon distension was the same as that described in Chapter one. The balloon was placed in the distal part of the esophagus (10-12cm from upper incisors) for distending the esophagus and simultaneous recording of intraluminal pressure. The esophageal distension-evoked cardiovascular responses at this level exceed those evoked at more proximal levels of the esophagus (Loomis et al., 1997). Distension was performed at a balloon volume of 200 μl for 15-20 s by means of a manually operated syringe.

5.2.2 Drug application

Drugs were directly applied to the medulla oblongata. The rat was placed in a stereotaxic apparatus with its head held in moderate ventroflexion, to facilitate surgical exposure of the caudal roof of the fourth ventricle and surrounding structures of the dorsal medulla. Ventricular cerebrospinal fluid was drained continuously with a wick. For NTS topical application, aqueous solutions of drugs were applied to the medullary surface from a Hamilton microliter syringe (1 μl) fitted with a thin flexible polyethylene catheter. The working volume was kept between 20-40 nl to minimize spread beyond the targeted region. The target area lay 0-100 μm rostral to the cranial edge of the area postrema (AP) immediately rostral to the obex, and between 500-700 μm lateral to the midline (Site 1 in Fig. 5.1E). The caudal (control) site was located 800-1200 μm caudal to the rostral margin of the AP and 100-200 μm lateral, corresponding to the surface of the caudal gracile nucleus where the latter overlies the NTS commissural subnucleus (Site 2 in Fig. 5.1E). In 19 animals, drugs were pressure-ejected at predetermined the NTS sites from a three-barrel glass micropipette by means of a nitrogen-pressured Picospritzer pump (General Valve Corporation, East Hanover, NJ). Glutamate antagonists were targeted at the NTSc region, extending 600-700 μm laterally from the midline at the cranial margin of the AP and centered at 450 μm below the NTS surface. The ejected volume was kept in the range of 20-200 pl. Pressure pulse-ejection of glutamate (0.2 M, 20-40 pl) was performed at a depth between 200 and 500 μm below the NTS surface and responses were tested in dorsoventral steps of 50 μm during the initial descent of the pipette. For RVLM pulse-ejection, the solutions were targeted at an area 800-1000 μm rostral to the cranial margin

of the AP, 2000-2200 μm lateral to the midline and 2800-3000 μm below the dorsal medullary surface. The ejected volume was in the range of 200 pl - 2 nl. The volume delivered with each pressure pulse was estimated by measuring the diameter of the ejected droplet under a microscope equipped with an ocular micrometer.

Bicuculline, glutamate, 2-amino-5-phosphonovaleric acid (AP-5), γ-D-glutamyl-glycine (γ-DGG), 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX), kynurenic acid, tetrodotoxin (TTX), urethane, and lucifer yellow were purchased from Sigma Chemical (St. Louis, MO). All the drugs were administered in aqueous solution, except for DNQX, which was dissolved to a maximal concentration (0.02 mol/l) in dilute aqueous base (1.5% $NaHCO_3$).

5.2.3 Histological examination of injection sites

Aqueous solutions of glutamate antagonists and fluorescent dye (2% lucifer yellow) were mixed and co-ejected from the same pipette barrel at sites in the NTSc or RVLM region. In NTS chemostimulation experiments, the fluorophor was ejected from another barrel of the pipette to mark the locus where glutamate application evoked an esophageal contraction. After the experiment, the animals were perfused transcardially with 150-250 ml of saline, followed by 250 ml of 4% paraformaldehyde. The medulla was removed, stored overnight in the same fixative at room temperature, transferred to phosphate buffered saline and cut in serial 50 μm thick transverse sections on a vibratome. The sections were examined under darkfield ultraviolet illumination. NTS subnuclear parcellations were determined by reference to previous tracing studies (Bieger 1984; Altschuler et al., 1989; Cunningham and Sawchenko, 1989) and NADPH-diaphorase

stained serial brainstem sections, in which the NTSc is readily discerned (Bieger and Sharkey, 1993).

5.2.4 Data analysis

The collection of the cardiovascular data was the same as that described in Chapter two. In cases in which biphasic HR changes were evoked by esophageal distension, only the cardioaccelerator component was measured for statistical analysis. Glutamate pulse-ejection was repeated at least 3 times at a given response locus to ensure reproducibility of observed response patterns. Response latency was measured as the time difference between the picospritzer pulse event mark and the beginning of the response. Variability of repeated measurements was expressed as the standard error of the mean (SEM). Student's t-test was done with statistical software (Microcal Origin, Microcal Software, Inc.) to examine the significance of differences ($P<0.05$) between two groups.

5.3 Results

5.3.1 Characterization of the ECVR

Distal esophageal distension evoked a monophasic pressor response in all animals tested with MAP increasing from 98±3 to 117±2 mmHg ($P<0.05$, n=28). Following balloon deflation, arterial blood pressure showed a small (<5 mmHg) residual increase that disappeared after 0.5-2 min. The HR response was variable. In the majority of animals a monophasic cardioaccelerator response was seen, during which HR rose from 338±11 to 350±12 beats/min, ($P<0.05$, n=21), while a biphasic HR response was noted in 5 other cases. The latter showed an initial transient (3.2±0.4 s) decrease in HR that preceded the increase (see below). In the two remaining animals, esophageal distension did not produce any HR change although the pressor responses were no less than in other animals and basal HR was comparable. The experiments described below focused on the pressor and cardioaccelerator components of the ECVR.

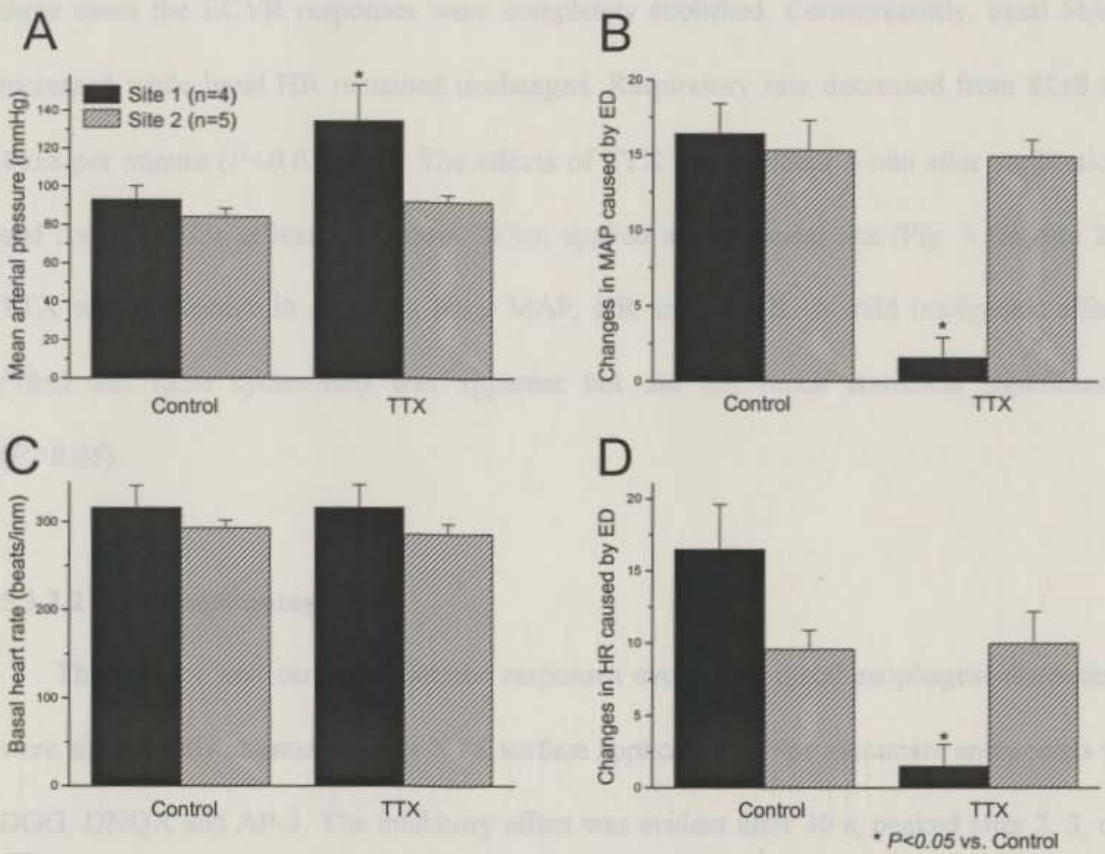
5.3.2 Effects of agents applied in the dorsal medulla

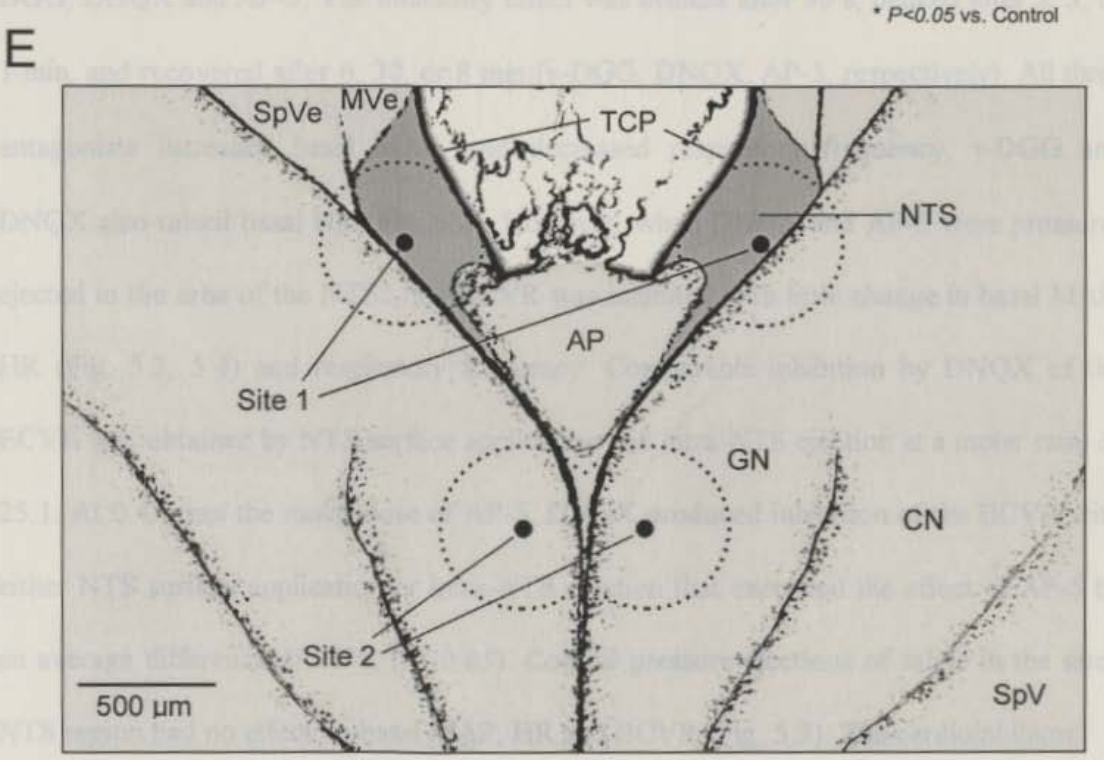
At the level of the NTS, the effects of blockade of neuronal impulse generation with TTX, blockade the glutamate receptors with antagonists, and chemical stimulation of the glutamate receptors with the glutamate on the ECVR were observed.

5.3.2.1 TTX

As summarized in Fig. 5.1, local bilateral application of TTX (0.04 nmol each side) to the NTS surface (panel E, site 1) resulted in an immediate persistent inhibition of the pressor and cardioaccelerator responses evoked by distal esophageal distension (n=4). In

Fig. 5.1 Effects of bilateral medullary surface application of tetrodotoxin (TTX) on esophageal cardiovascular reflex (ECVR), basal mean arterial pressure (MAP) and heart rate (HR). TTX was applied bilaterally at a dose of 0.04 nmol per side. At site 1, centered 100 μm rostral to the cranial edge of the area postrema (AP), and 600-700 μm lateral to the midline, TTX significantly increased basal MAP (A) and reduced the arterial pressor response evoked by distal esophageal distension (ED) (B). Although basal HR was not affected (C), TTX significantly inhibited the HR component of ECVR (D). TTX did not change basal MAP, HR, and ECVR when applied at site 2, centered 800-1200 μm caudal to the rostral margin of the AP and 100-200 μl lateral to the midline. In the camera lucida drawing of the dorsal medullary surface (E), the dotted circles show the estimated area of solute spread from the point of application. The extraventricular surface (darkly shaded area) of the nucleus tractus solitarii (NTS) is bounded medially by the tenia of the choroid plexus (TCP), cranially and laterally by the vestibular and gracile nuclei, and caudally by the AP. Other abbreviations: CN: cuneate nucleus, GN: gracile nucleus, MVe: medial vestibular nucleus, SpV: spinal tract trigeminal nerve, SpVe: spinal vestibular nucleus.


A
Site 1 (n=4)
Site 2 (n=5)
Mean arterial pressure (mmHg)
Control
TTX
B
Changes in MAP caused by ED
Control
TTX
C
Basal heart rate (beats/inm)
Control
TTX
D
Changes in HR caused by ED
Control
TTX
* P<0.05 vs. Control

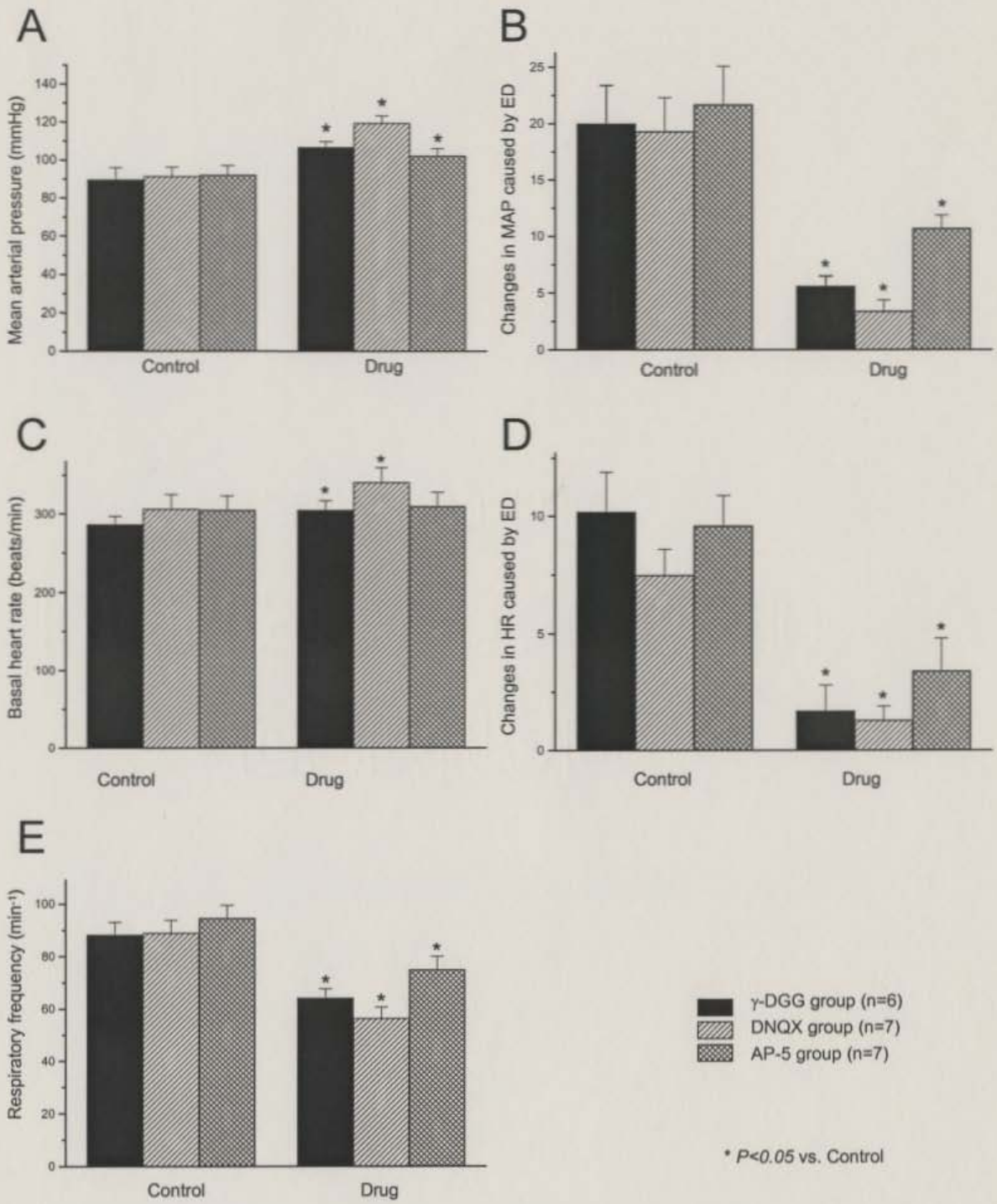

E
SpVe
MVe
TCP
NTS
AP
Site 1
GN
CN
Site 2
500 µm
SpV

three cases the ECVR responses were completely abolished. Concomitantly, basal MAP increased while basal HR remained unchanged. Respiratory rate decreased from 81±8 to 44±8 per minute ($P<0.05$, n=4). The effects of TTX were evident 5 min after application and continued for at least 1.5 hours. When applied at the caudal site (Fig. 5.1E, site 2), TTX was ineffective in changing basal MAP, HR and ECVR. A mild bradypneic effect (78±5 vs. 72±6 cycles/min) was apparent but did not reach statistical significance ($P>0.05$).

5.3.2.2 Glutamate antagonists

The pressor and cardioaccelerator responses evoked by distal esophageal distension were significantly attenuated after NTS surface application of the glutamate antagonists γ-DGG, DNQX and AP-5. The inhibitory effect was evident after 30 s, peaked after 2, 3, or 1 min, and recovered after 6, 30, or 8 min (γ-DGG, DNQX, AP-5, respectively). All three antagonists increased basal MAP, and decreased respiratory frequency; γ-DGG and DNQX also raised basal HR (Fig. 5.2). However, when DNQX and AP-5 were pressure-ejected in the area of the NTSc, the ECVR was inhibited with little change in basal MAP, HR (Fig. 5.3, 5.4) and respiratory frequency. Comparable inhibition by DNQX of the ECVR was obtained by NTS surface application and intra-NTS ejection at a molar ratio of 25:1. At 0.4 times the molar dose of AP-5, DNQX produced inhibition of the ECVR with either NTS surface application or intra-NTS ejection that exceeded the effect of AP-5 by an average difference of 21%, ($P<0.05$). Control pressure-ejections of saline in the same NTS region had no effect on basal MAP, HR and ECVR (Fig. 5.3). The cardioinhibitor

Fig. 5.2 Effects of NTS surface applied glutamate antagonists. All agents significantly increased basal MAP (A) and reduced the pressor response evoked by distal esophageal distension (B). Although the increase in basal HR was only evident after γ-DGG and DNQX application (C), all agents significantly inhibited the HR component of the ECVR (D) and also reduced respiratory frequency (E). Doses (nmol) were applied to each NTS at site 1 (Fig. 5.1E) in a volume of 40 nl as follows: γ-DGG 12, DNQX 0.8, AP-5 2.0.


A
Mean arterial pressure (mmHg)
Control
Drug
B
Changes in MAP caused by ED
Control
Drug
C
Basal heart rate (beats/min)
Control
Drug
D
Changes in HR caused by ED
Control
Drug
E
Respiratory frequency (min-1)
Control
Drug
γ-DGG group (n=6)
DNQX group (n=7)
AP-5 group (n=7)
* P<0.05 vs. Control

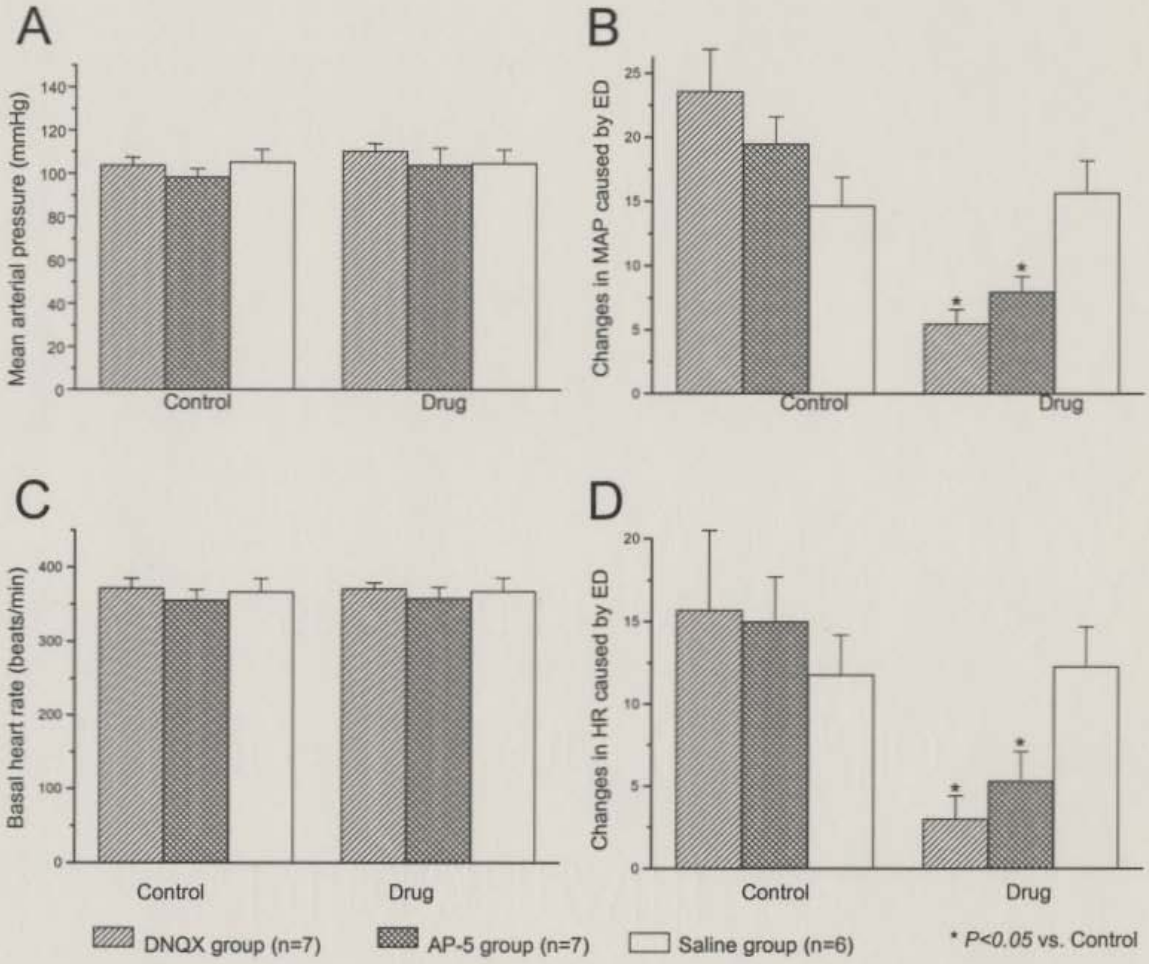


Fig. 5.3 Effects of pulse ejection of glutamate antagonists in the region of NTS central subnucleus (NTSc). The bilateral application of DNQX or AP-5 did not change basal MAP (A) and HR (C) but significantly inhibited both pressor (B) and cardioaccelerator (D) components of the ECVR. Pulse ejection of an equivalent volume of saline did not cause any noticeable change. DNQX and AP-5 were delivered by pulse-ejection in doses of 32 pmol and 80 pmol, respectively, per side, at a depth of 400-500 μm below the NTS surface point marked Site 1 in Fig. 5.1E.

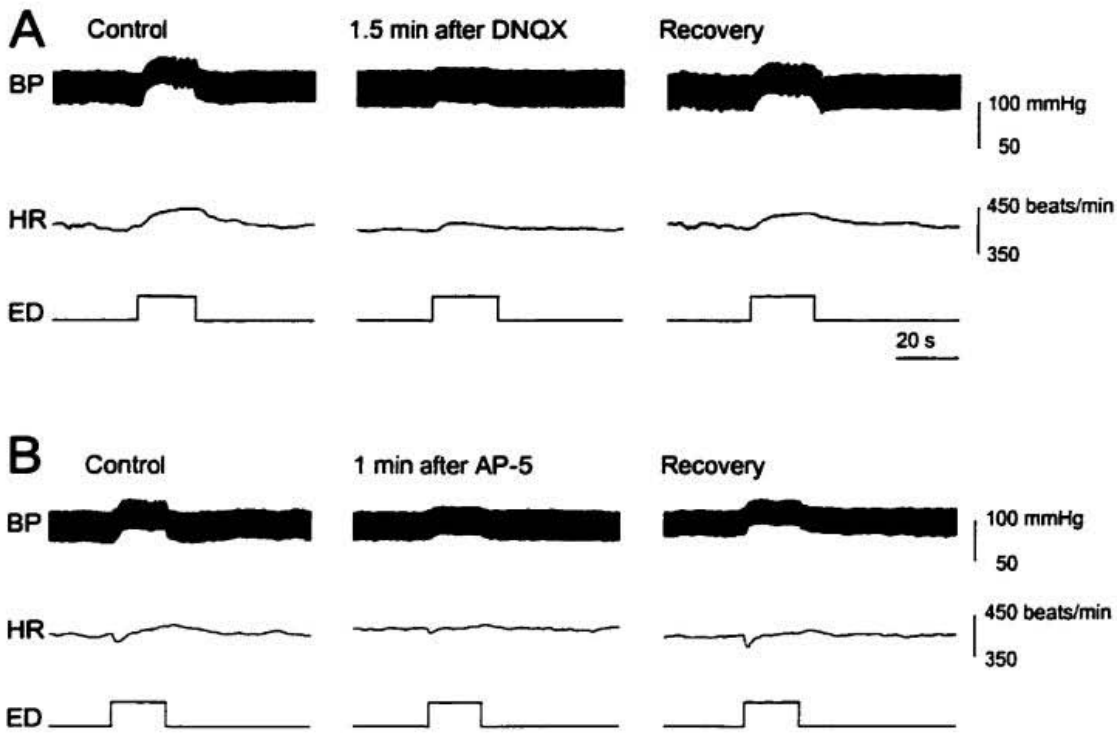


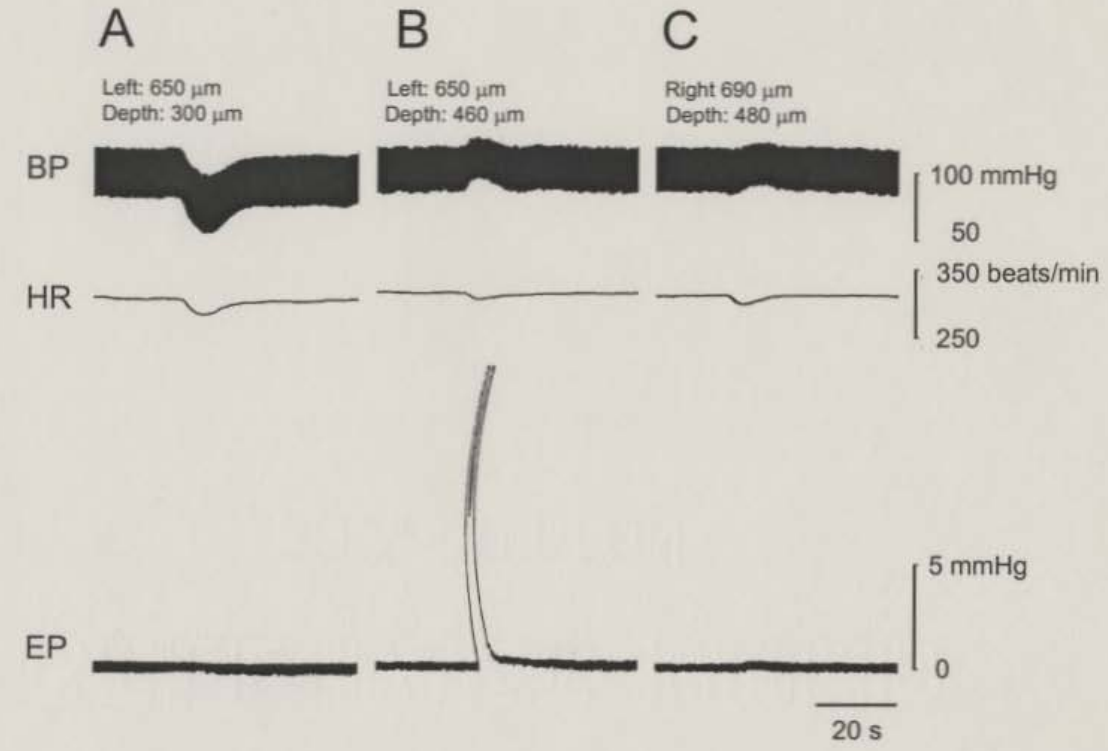
Fig. 5.4 Representative examples of pulse ejection of glutamate antagonists in the NTSc region resulting in inhibition of the ECVR with negligible change in basal blood pressure and heart rate. A and B were obtained from different animals. ED: esophageal distension. Note in B that HR response is biphasic, and both components are attenuated after AP-5 application.

component of the ECVR was also inhibited by intra-NTS injection of AP-5 (from 13±4 to 6±2 beats/min, P<0.05, n=4, Fig. 5.4). Histological examination confirmed that the fluorescent dye ejected with the glutamate antagonists labeled an NTS region that included the NTSc on both sides of the medulla.

5.3.2.3 Glutamate

At a depth between 200-400 μm and 400-900 μm lateral to the midline, pulse-ejection of glutamate (targeted at the same NTS level where the glutamate antagonists were pulse-ejected) consistently evoked depressor and cardioinhibitor responses in all animals tested (Fig. 5.5A, Tab. 5.1). At a depth of 400-500 μm and 600-700 μm lateral to the midline, unilateral pressure-ejections of glutamate (8 on left, 8 on right side) produced three distinct response patterns: 1) a pressor response (from 92.9±3.3 to 99±3.8 mmHg) coinciding with a rapid phasic contraction of the distal esophagus (Fig. 5.5B), 2) a pressor response accompanied by a minor slow pressure rise in the distal esophagus (Fig. 5.5C), and 3) an isolated rapid contraction of the distal esophagus (Tab. 5.1). The average latency of the pressor response was 0.8±0.2 s, which was not statistically different from that of esophageal contractions (0.8±0.1, $P>0.05$), with the notable exception of two experiments in which the esophageal contraction consistently either started 0.2 s before the pressor response or lagged behind it by 0.8 s. The pressor response lasted for 8.9±0.6 s, significantly surpassing the duration of the accompanying esophageal contraction (3.6±0.3 s, $P<0.05$). The pressor response occurred without change in HR in 8 animals, and was accompanied by cardiac slowing in five other animals that showed a cardioinhibitor

Fig. 5.5 Representative examples illustrating the blood pressure response to pulse-ejection of glutamate into NTS. Traces shown in panel A, B and C are taken from the same experiment. Pulse-ejection of glutamate at a depth of 300 μm below the dorsal surface of NTS caused transient hypotension and cardiac slowing with no obvious change in distal esophageal pressure (A). Upon lowering the micropipette by an additional 160 μm, the same pressure pulse of glutamate evoked a short latency esophageal pressure wave, a small pressor response and a diminished cardioinhibitor response (B). In the contralateral NTS, glutamate ejection at a depth of 480 μm produced a distinct pressor response with a minor increase in distal esophageal pressure (C). The camera lucida drawing (D) illustrates the approximate location of ejection sites in the NTS, as reconstructed from seven experiments and shown only in one half of the medulla. The transverse plane lies immediately caudal to Site 1, marked in Fig. 5.1E. The two shaded areas illustrate the locations of the dorsal depressor (A) and ventral pressor (B or C) response sites. The ventral shaded area overlaps the NTSc, as visualized by NADPH-diaphorase histochemistry (Bieger and Sharkey, 1993) and retrograde neural tracing (Bieger, 1984). Other abbreviations: DMV: dorsal motor nucleus of vagus; NTSc: NTS subnucleus centralis; NTSg: NTS subnucleus gelatinosus; TS: tractus solitarii; V4: fourth ventricle; XIIm: hypoglossal nucleus.


A
B
C
Left: 650 μm
Depth: 300 μm
Left: 650 μm
Depth: 460 μm
Right 690 μm
Depth: 480 μm
BP
100 mmHg
50
HR
350 beats/min
250
5 mmHg
EP
0
20 s

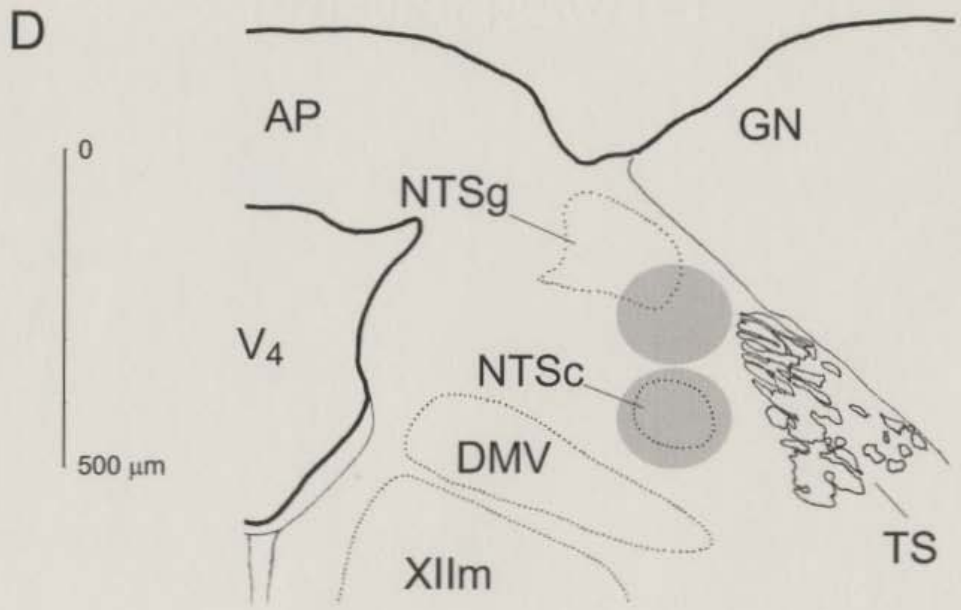

D
AP
GN
0
NTSg
V4
NTSc
DMV
500 μm
TS
XIIm

Table 5.1 Cardiovascular and esophageal responses evoked by pulse-ejection of glutamate in NTS immediately rostral to obex.

		Incidence of response pattern observed		
Site coordinates		D: 200-400 L: 400-900 (29)[a]	D: 400-500 L: 600-700 (16)	D: 400-500 L: 400-600 or L: 700-900 (13)
Response pattern	I: pressor cardiac slowing or no change esophageal contraction	0	11	0
	II: pressor cardiac slowing	0	2	0
	III: no cardiovascular change esophageal contraction	0	3	0
	IV: depressor cardiac slowing	29	0	13

Abbreviations: D: μm below the dorsal extraventricular medullary surface; L: μm lateral to the midline.

[a] the number in brackets refers to the total sites tested in each region.

response to esophageal distension (Fig. 5.4, 5.5). Glutamate ejection did not produce any change in breathing pattern. At loci 200 μm lateral or medial to the region yielding the pressor response, glutamate-evoked depressor and cardio-decelerator responses had a smaller amplitude compared to those obtained at corresponding depths between 200-400 μm.

In 9 animals in which pressor response sites were dye-labeled, histological examination confirmed that the area marked by the dye encroached on the NTSc on both sides (Fig. 5.5D).

5.3.3 Effects of agents applied in the RVLM

Pulse-ejection of the nonselective glutamate receptor antagonist kynurenic acid significantly inhibited the pressor response evoked by distal esophageal distension without changing the basal MAP. The inhibition was evident after 3 min, peaked after 6 min and vanished after 20 min. The $GABA_A$ receptor antagonist bicuculline significantly increased the basal MAP. The pressor component of ECVR was also increased, but this change did not reach statistical significance ($P>0.05$). The effects of bicuculline were evident within 1 min, reached a peak after 2 min, and disappeared after 10 min. None of the agents significantly changed the basal HR or HR component of ECVR (Fig. 5.6), and the respiratory frequency. A decrease in respiratory frequency was only seen in one case after bicuculline pulse-ejection. Histological examination (n=7, in 9 animals) confirmed that the ejection sites were in the RVLM and, judging by the spread of fluorescent dye, covered an area with a diameter of 400-600 μm. Their center was located 600-1000 μm ventral and 0-

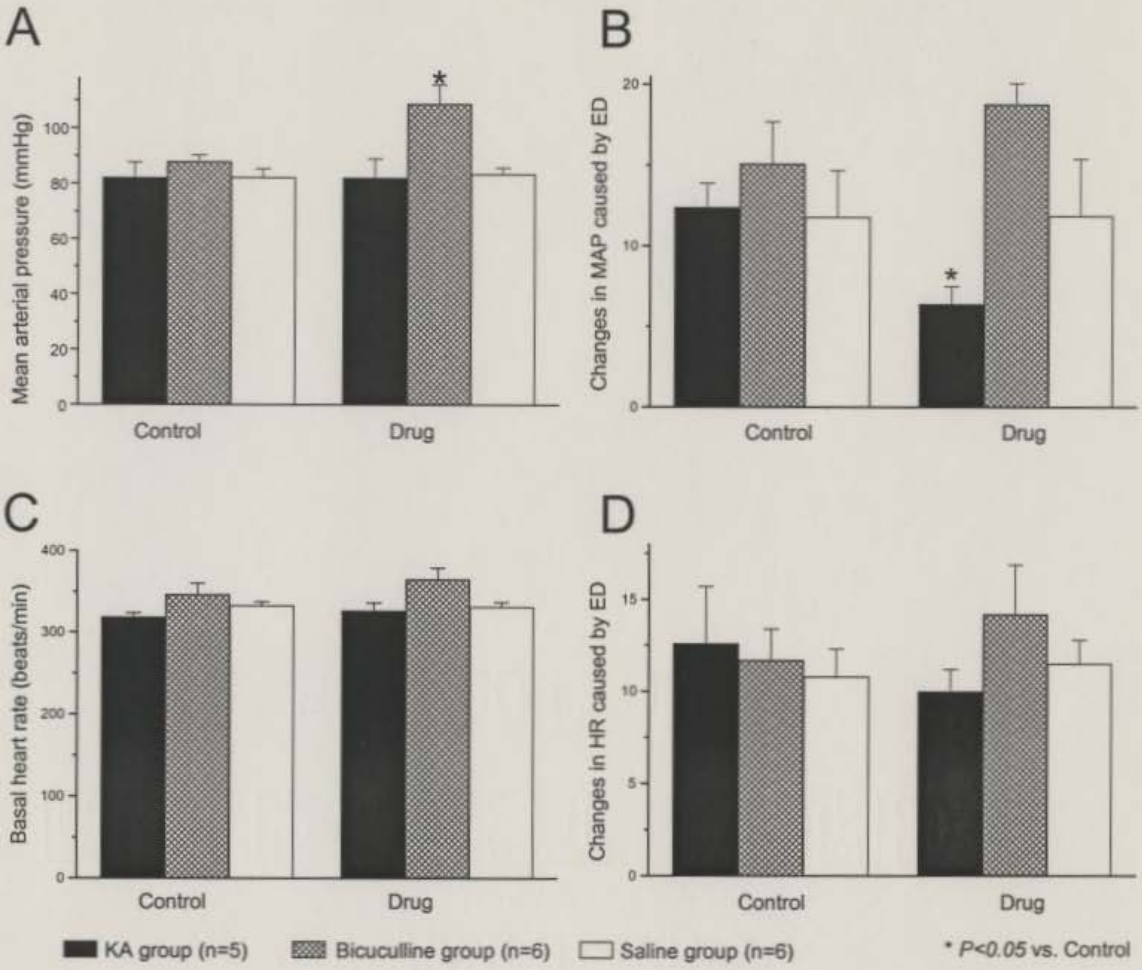


Fig. 5.6 Effects of pulse-ejection of agents in the RVLM region. The bilateral application of kynurenic acid (KA) did not change basal MAP (A) but significantly inhibited the pressor component of the ECVR (B). Bicuculline application increased the basal MAP (A) without a significant change of the pressor component of the ECVR (B). None agents evoked a significant change in the basal HR or the HR component of the ECVR (C, D). Pulse ejection of an equivalent volume of saline did not cause any noticeable change. KA and bicuculline were delivered in doses of 3.2 nmol and 20 pmol, respectively, per side.

300 μm lateral to the rostral nucleus ambiguus.

5.4 Discussion

The vagally mediated ECVR evoked by distal esophageal distension has both a vasomotor and a heart rate component (Loomis et al., 1997). As shown in Chapter two, the former consists of a low threshold pressor and a high threshold depressor response that is usually concealed. The data presented here concern the central afferent representation of the pressor and attendant cardioaccelerator component. In particular, the present study provides evidence regarding 1) the central location of neurons mediating the ECVR and 2) the dependence of this reflex on glutamatergic neurotransmission. The following discussion will consider both aspects with regard to the two major neural structures forming the intramedullary pathway of the ECVR.

5.4.1 Involvement of NTS interneurons

As demonstrated by the elimination of the ECVR following application of TTX to the extraventricular NTS surface, the subjacent NTS region plays a critical role in generating the ECVR. Based upon our estimate of surface-applied drug solute spread, the affected area extends up to 400 μm ventrally and would include the NTSc region (see methods in Chapter four). The present data would suggest that surface application of the antagonist doses used can affect neurons as deep as 400-500 μm below the dorsal surface. At the caudal site (Site 2 in Fig. 5.1), TTX would likewise be expected to penetrate first into the dorsal column nuclei that mediate abdominal visceral pain (Willis et al., 1999; Al-Chaer et al., 1997), and next into the subjacent commissural nucleus of the NTS, an area receiving cardiovascular chemoreceptor afferent inputs (Spyer, 1994; Dampney, 1994; Sapru, 1996).

The present results thus suggest that neither of these structures is involved in the ECVR.

Since the ECVR was inhibited by NMDA or non-NMDA glutamate antagonists applied to the NTS surface or the NTSc region itself, ECVR afferents presumably activate second order neurons via release of glutamate acting at receptors of both the NMDA and non-NMDA subtype. However, at the doses of the antagonists used in the present study, none of the glutamate receptor subtype blockade eliminated the ECVR. Although the data suggest that selective blockade of non-NMDA receptors by DNQX (Watkins et al., 1990a) was more effective in inhibiting the ECVR than blockade of NMDA receptors by AP-5, detailed analysis of dose-response relationships is needed to determine the relative contribution of each receptor subtype. Conversely, at the level of NTSc esophageal premotor neurons, NMDA receptor stimulation was more effective than non-NMDA receptor stimulation in eliciting an esophageal motility response (Hashim and Bieger, 1989). Moreover, NMDA receptor blockade with AP-5 at the same dose as used in the present study, or non-NMDA receptor blockade with CNQX at a dose equivalent (Watkins et al., 1990b) to that of DNQX, as used in the present study, were sufficient to eliminate esophageal reflex peristalsis (Lu and Bieger, 1998b). As regards NTS-mediated cardiovascular responses, both NMDA and non-NMDA receptors are known to contribute to the transmission of baro- and chemoreceptor reflex afferent impulses (Dampney, 1994; Ohta and Talman, 1994; Sapru, 1996; Machado and Castania, 2000). Blockade of one receptor subtype alone was insufficient to eliminate baroreflex (Machado and Castania, 2000). Thus, the ECVR and the cardiovascular reflexes may share a similar dependence on glutamate receptor subtypes.

As regards the neurocardiovascular actions of glutamate, microinjection of this neuroexcitant into the NTS is known to evoke dose-dependent depressor and cardioinhibitor responses in the anesthetized rat (Talman et al., 1980; Talman, 1989; Le Galloudec et al, 1989). The NTS areas delineated by these studies correspond to the central terminations of baroreflex afferents (Ciriello, 1983; Spyer, 1994). These sites lie dorsal, medial or lateral to the solitarial area that was shown to harbor esophageal premotor neurons by Bieger (1984) and was subsequently defined as the NTSc by Ross et al. (1985). In conscious rats, microinjection of glutamate into the commissural or medial subnuclei of the NTS is reported to evoke pressor responses that become depressor during anesthesia with urethane (Machado and Bonagamba, 1992, Colombari et al., 1994). The pressor responses depend on the integrity of the midline area in the commissural nucleus of NTS, which plays an important role in the chemoreceptor reflex pathway (Colombari et al., 1996; Chitravanshi et al., 1994; Vardhan et al., 1993). As confirmed here, pulse ejection of glutamate in urethane anesthetized rats at baroreflex sites dorsally adjoining the NTSc evoked depressor and cardioinhibitor responses. Moreover, our study has identified a highly circumscribed ventral subregion overlapping the NTSc, where glutamate elicited a *pressor* response that was present in anesthetized rats and appeared to mimic the pressor component of the ECVR. The evoked pressor response could have been a reflex response triggered by the esophageal contraction resulting from the excitation of distal esophageal premotoneurons of the NTSc. However, in the majority of experiments the pressor response did not lag behind the esophageal contraction, and in some instances the two responses could be reliably dissociated (see Tab. 5.1). Moreover, glutamate-evoked

pressor responses and esophageal contractions had a different time course, while the reflex pressor response resulting from an active esophageal contraction closely matches the time course of the latter (Loomis et al., 1997).

Taken together, the data support the idea that glutamate is released at terminals of ECVR afferents. Because blockade of neuronal activity in the midline area of the commissural nucleus with TTX failed to change the ECVR, its pressor component is not likely to utilize an afferent pathway similar to that of the chemoreceptor reflex (Chitravanshi et al., 1994; Vardhan et al., 1993). The reason why glutamate microinjection into the NTSc failed to mimic the cardioaccelerator component of the ECVR remains unclear. Possibly, interneurons involved in the parasympathetic cardioinhibitory component of the baroreflex extend into the vicinity of the NTSc. Activation of these elements by glutamate application could evoke a cardioinhibitory response that would override the response to activation of neurons mediating the ECVR. The variability of the HR component of the ECVR also suggests that its mechanism may be different from that of the vasomotor component.

The present results reveal an initial decrease in HR in less than one fifth of animals. This response is frequently seen during distension of the mid-thoracic segment of esophagus (unpublished observations). As the cardioinhibitor component was also sensitive to NTS NMDA receptor blockade (Fig. 5.4), its vagal mediation appears likely.

As regards the location of NTS ECVR interneurons, the present data accord with anatomical observations indicating that vagal afferents arising from the rat esophagus terminate in the NTSc and its immediate vicinity (Altschuler et al., 1989). Based upon the

available information, the NTSc is the principal source of axons projecting to the rostral nucleus ambiguus (Bieger, 1984; Ross et al., 1985; Cunningham and Sawchenko, 1989; 2000) where esophageal motoneurons are located (Bieger and Hopkins, 1987). The NTSc also projects to the dorsal motor nucleus of the vagus (Rogers et al., 1999). Its presumed physiological functions include esophageal motility control (Bieger, 1993; Lu and Bieger, 1998a), and receptive relaxation of the stomach (Rogers et al., 1999). Neurons in the immediate vicinity of the NTSc are known to project to the rostral ventrolateral medulla (Ross et al., 1985). In the present study, the glutamate ejection-evoked pressor response and the distal esophageal contraction could not be dissociated in the majority of cases, implying that cell groups mediating these two responses are closely adjacent or even intermingled.

5.4.2 Involvement of the RVLM

Since the glutamate antagonist kynurenic acid inhibited the pressor component of ECVR, a glutamatergic synapse in the RVLM is likely to activate the vasomotor component of the ECVR. Thus, the ECVR and the chemoreceptor reflex (Koshiya et al., 1993) may employ a common neurotransmitter system to activate the RVLM sympathetic premotoneurons. As the decrease of the cardioaccelerator response of the ECVR did not reach statistical significance, the role of the RVLM glutamatergic synapse in this component remains to be clearly determined. Conversely, local blockade of $GABA_A$ receptors with bicuculline did not inhibit the pressor component of the ECVR. The GABA-ergic synapses in the RVLM are essential components of the central baroreceptor

reflex pathway, and blockade with bicuculline abolishes the vasomotor component of the reflex (Sun and Guyenet, 1985; 1987; Dampney et al., 1988). Thus, the vasomotor components of the ECVR and the baroreflex use two different central pathways. In the present work, although the increase of the pressor and cardioaccelerator components of the ECVR after bicuculline application failed to reach statistical significance ($P=0.063>0.05$), it would be expected to result from inhibition of the baroreflex.

Microinjection of glutamate in the RVLM has been shown to increase arterial blood pressure (Ross et al., 1984; Willette et al., 1987; Bachelard et al., 1990; Maeda et al., 1991). As this well documented effect mimics many cardiovascular reflexes that depend on glutamatergic synapses in the RVLM, including the chemoreceptor reflex and the ECVR, the present work did not exmine responses to RVLM glutamate microejection. Glutamate application also was reported either to increase the heart rate in the anesthetized animal (Ross et al., 1984; Willette et al., 1987), to have no change in the heart rate in the anesthetized animal (Maeda et al., 1991), or to evoke a bradycardia in the anesthetized animal that changed to a tachycardia in the conscious rat (Bachelard et al., 1990). The variability in heart response could be due to differences in glutamate dosage employed in these studies. Moreover, a possible sympathoadrenal activation caused by glutamate application would increase the heart rate (Dampney, 1994).

Both the NTS and the RVLM are cardiovascular reflex relay structures, and are linked through direct or indirect connections. In the baroreflex, the NTS interneurons that receive afferent inputs send glutamatergic projection to interneurons in the caudal ventrolateral medulla, and the latter tonically inhibit the RVLM sympathetic premotor neurons through

GABA-ergic synapses (Aicher et al., 1995; Cravo et al., 1991; Sun and Guyenet 1985). In the chemoreceptor reflex, there is a direct projection to the RVLM neurons from the commissural subnucleus of the NTS that receives the afferent input; however, there may be other interneurons subserving the reflex (Koshiya and Guyenet, 1996; for review see Dampney, 1994). The same maybe true for the ECVR. Although a direct projection from neurons in the vicinity of the NTSc to the RVLM neurons has been shown (Ross et al., 1985), its inferred role as the central pathway of the ECVR requires further study.

5.4.3 Summary

The present work demonstrates that the afferent fibers of this reflex terminate in the NTSc or its immediate vicinity and activate second-order neurons via glutamate receptors of both the NMDA and non-NMDA subtype. The RVLM glutamatergic neurotransmission is involved in mediation of the vasomotor component of the ECVR.

Chapter six

Summary and synthesis

6.1 Summary

1. The ECVR in the rat is a vagally-mediated reflex that has a low threshold excitatory and a high threshold inhibitory component. The former includes an arterial pressor and a cardioaccelerator response; the latter component consists of an arterial depressor response that is observed only when the pressor response is blocked or impaired.

2. Vagally mediated reflex esophageal contractions and both components of the ECVR evoked by distal esophageal distension are blocked at different temperatures during graded bilateral cooling of the cervical vagal trunks. The blocking temperatures for reflex esophageal contractions, and the excitatory and inhibitory components of the ECVR are in the range of 12.5-15, 9-11 and 4.5-7.5 °C, respectively. These blocking temperatures are well above that known to block C-fibers.

3. As determined by single vagal afferent fiber recording, all the units responding to distal esophageal distension are spontaneously active; however, their resting and distension-evoked discharge frequencies diverge. Impulse frequencies in these fibers increase logarithmically with esophageal inflation pressure and do not show saturation when the esophagus is distended to an outer diameter of 6 mm (300 μl distension). Thus, these vagal mechanosensory afferent fibers have the ability to encode information over a wide dynamic range.

4. The reflex motor response pattern and the contractile force of the esophageal body vary with the strength of afferent input. At balloon volumes between 75 to 200 μl, distal esophageal contraction rate decreases with increasing stimulus size, and at volumes of 150-250 μl contractions become tonic. The contraction amplitude reaches peak at distension volumes between 150 and 200 μl. Putative esophageal motoneurons recorded in the rostral nucleus ambiguus respond to 50-100 μl distensions with rhythmic burst discharges and to 200 μl distension with a nonrhythmic discharge at a higher frequency than that evoked by 50-100 μl distensions.

5. At distension volumes less than 250 μl, the above change in motor pattern involves mainly the striated musculature of the esophageal body. A nifedipine-sensitive local contraction of the TMM occurs only at large distension volumes (≥250 μl) and persists after cervical vagotomy.

6. The reflex motor responses to esophageal distension are dependent on vagal rather than spinal afferent inputs. The acute blockade of esophageal spinal afferents by means of spinal transection at the level of C2 does not cause measurable changes in the esophageal motor patterns evoked by distal esophageal distension.

7. In the NTSc region, units that respond to distal esophageal distension at a volume between 50-100 μl fall into three different types. Type I units show rhythmic burst activities correlating with intraluminal pressure waves, type II units respond with a sustained nonrhythmic increase in spike frequency, and type III units are inhibited by the distension and show a rebound burst discharge upon deflation of the balloon. With

increasing strength of distension, type I responses change to nonrhythmic, while type II and III responses are intensified.

8. A swallow or proximal esophageal distension simultaneously inhibits both rhythmic local contractions and burst discharges of presumed esophageal motoneurons evoked by distal esophageal distension. In NTSc interneurons, type I discharges are suppressed by mid-thoracic esophageal distension; type II excitatory responses, like type III inhibitory responses, are evoked by distension of either the thoracic or the distal esophagus.

9. Proximal distension-evoked distal inhibition is sensitive to antagonists acting at $GABA_A$ and glycine receptors in the area of the NTSc. $GABA_B$ and $GABA_C$ receptors in this region are not involved in distal inhibition.

10. Neither GABA-ergic nor glycinergic synapses in the NTS region contribute to deglutitive inhibition.

11. Afferent fibers of the ECVR terminate in the vicinity of esophageal premotoneurons comprising the NTSc and activate second-order neurons via glutamate receptors of both the NMDA and non-NMDA subtype.

12. In the RVLM, glutamatergic, but not GABA-ergic neurotransmission is involved in the mediation of the vasomotor component of the ECVR.

6.2 Synthesis

The findings presented in this thesis broadly support the concept that the distension-evoked reflex motor responses and the ECVR in rat esophagus involve different vagal

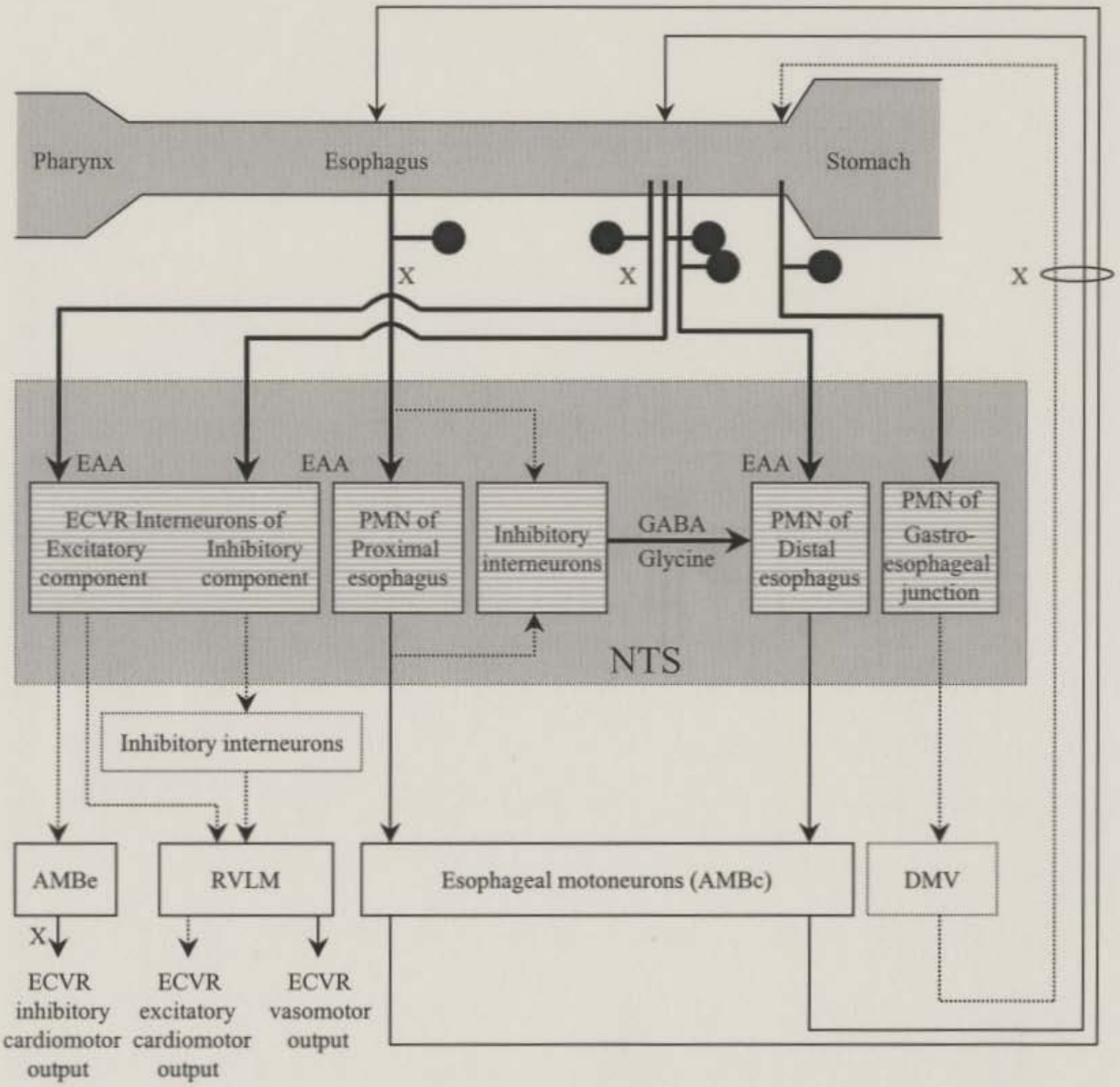


Fig. 6.1 Schematic diagram of postulated model of general and special visceral reflexes evoked by esophageal distension. The dotted lines illustrate the hypothesized pathways. Abbreviations: AMBc, compact formation of the nucleus ambiguus; AMBe, external formation of the nucleus ambiguus; DMV, dorsal motor nucleus of vagus; EAA, excitatory amino acid; ECVR: esophageal cardiovascular reflex; NTSc, central subnucleus of the nucleus tractus solitarii; PMN, premotoneurons; RVLM, rostral ventrolateral medulla; X, vagus nerve. Filled circles represent sensory neurons in the nodose ganglion.

mechanosensory afferent fibers and affect distinct central pathways. Fig. 6.1 illustrates a proposed circuit diagram.

6.2.1 Characterization of esophageal vagal mechanosensory afferents

Although the anatomy and neurochemistry of vagal afferents arising from the rat esophagus have been examined in some detail (Neuhuber, 1987; Kuramoto and Kuwano, 1994; Dütsch et al., 1998; Kressel and Radespiel-Tröger, 1999), the physiological implications of these studies are not well understood. The findings presented in this thesis provide insights into the functional diversity and dynamic range of vagal mechanosensory afferent fibers from the rat distal esophagus based upon two approaches: bilateral cervical vagal cooling and single vagal fiber recording.

As described in Chapter two, reflex esophageal contractions, and both excitatory and inhibitory components of the ECVR are blocked at different temperatures well above that known to block C-fibers. Thus, these reflexes are likely to involve vagal myelinated afferent fibers. Although all myelinated nerve fibers are believed to have the same blocking temperature (Paintal, 1965), the impairment of repetitive activity during cooling occurs well above the blocking temperature (Franz and Iggo, 1968). In the intact animal, functions requiring high frequency nerve conduction will be lost first during nerve cooling (Franz and Iggo, 1968). As esophageal reflex peristalsis is most sensitive to vagal cooling, it conceivably requires vagal afferent input at a frequency higher than that needed for the ECVR. Conversely, the high-threshold inhibitory component of the ECVR is most resistant to vagal cold blockade, because it is triggered presumably by vagal afferent input

in a frequency range lower than that needed for eliciting the excitatory component of the ECVR and esophageal reflex contractions. In contrast, as the significant rise in basal HR occurs at a cooling temperature that is below the temperature known to block myelinated fibers (Franz and Iggo, 1968), it is probably due to the blocking of vagal efferent C-fibers to the heart (Nosaka et al., 1979).

These findings raise the question whether the three visceral reflexes studied have afferent limbs made up of separate groups of vagal mechanosensory afferent fibers, or a single population of fibers acting on three different sets of NTS interneurons requiring different input frequencies. As argued below, the evidence obtained favors the first interpretation.

First, consider the possibility that the excitatory component of the ECVR and the esophageal reflex contractions share the same afferent fibers that project via collaterals to different interneurons. As the firing frequency required for triggering the former is lower than that for eliciting the latter, the interneurons mediating the ECVR would have to be activated at an intraluminal pressure level lower than that needed to activate the interneurons mediating the reflex esophageal contractions. This conclusion contradicts the observation that the excitatory component of the ECVR and the esophageal reflex contractions have the same activation threshold.

Second, let us assume that the inhibitory component of the ECVR and the excitatory component or the esophageal reflex contractions share the same afferent fibers that send collateral projections to separate groups of NTS interneurons. Since the discharge frequency required for triggering the inhibitory component of the ECVR is lower than that

needed for triggering the other two reflexes, the activation threshold for interneurons mediating the inhibitory component must be lower than that for interneurons mediating the excitatory component or the esophageal reflex contractions. Accordingly, an esophageal distension at an intraluminal pressure just lower than the threshold pressure for the excitatory component or the esophageal reflex contractions would be expected to evoke the pure inhibitory component of the ECVR. However, the experiments show that the inhibitory component of the ECVR has a high activation threshold.

Taken together, the present data thus support the concept that at least three groups of thinly myelinated fibers mediate reflex esophageal contractions, the excitatory and the inhibitory components of the ECVR, respectively.

Unlike afferents reported in the opossum (Sengupta et al., 1989), the rat esophageal vagal mechanosensory afferent fibers have a wide dynamic range. The spike frequency continues to increase even when the esophageal wall is being stretched to a degree presumed to be in a noxious range. Thus, these afferents in the rat have the ability to encode both physiological and noxious information, and may contribute to esophageal nociception. The intraluminal pressure ceiling at which the discharge of these fibers saturates remains unknown; however, based on the present data, at this ceiling structural damage would be expected to occur. Although all the units in the vagal single fiber recordings show a similar dynamic range and rate of rise during graded esophageal distension, the resting and distension evoked discharge frequencies of these fibers appeared to fall into three groups. In light of the above consideration, it is tempting to speculate that the group with lower firing frequencies represents the afferent fibers

mediating the excitatory component of the ECVR; whereas, the group with intermediate firing frequencies may represent the afferent fibers mediating the reflex esophageal contractions. The function of the group consisting of only one fiber that has a firing rate much higher than the other two groups is still more uncertain. Possibly, this unit originates from the mucosa, like the high frequency SLN mucosal afferents innervating the cervical esophagus (Andrew, 1956b; Sengupta and Gebhart, 1994). As the vasodepressor component of the ECVR has a high threshold, one would predict the existence of esophageal vagal afferents with high thresholds. In the present study, a single unit recorded in the nodose ganglion potentially falls into this category. More work is needed to confirm this type of vagal mechanosensory afferent neuron.

Calcium-binding protein immunoreactivity has been found in vagal afferent neurons innervating the rat esophagus (Dütsch et al., 1998; Kressel and Radespiel-Tröger, 1999). Calretinin and calbindin immunoreactivities are present in 5% and 40%, respectively, of nodose ganglion neurons innervating the distal esophagus; and in 80% and 87%, respectively, of nodose ganglion neurons innervating the cervical esophagus (Dütsch et al., 1998). The presence of these calcium-binding proteins in esophageal vagal terminal structures may indicate a low threshold, rapidly adapting mechanoreceptor (Duc et al., 1994). As all vagal afferent fibers recorded in the present work, and most vagal mechanosensory afferent fibers from the cervical esophagus (Andrew, 1956a), appear to belong to a slow adapting type, esophageal vagal mechanosensory afferents with fast adapting features remain to be identified.

6.2.2 Excitatory motility responses evoked by vagal mechanosensory afferent input

Previous studies have revealed a distinct circuitry for the reflex motility control of the rat esophagus, specifically its striated muscle TMP (for review, see Bieger, 1993a). Vagal mechanosensory afferents project to interneurons located in the NTSc (Altschuler et al., 1989; Lu and Bieger, 1998a), and the latter serve as esophageal premotoneurons to program esophageal motility (Lu and Bieger, 1998a; 1998b; Lu et al., 1997).

The present study demonstrates that the distal esophagus executes rhythmic or tonic contractions dependent on the intensity of vagal mechanosensory afferent input to the brainstem esophagomotor control system. In the NTSc region, type I distension-sensitive units respond with rhythmic burst and high frequency tonic discharges to low and high level esophageal distension, respectively. Because this pattern change reflects the motility responses recorded in the esophagus, and discharge patterns of presumed esophageal motoneurons in the AMBc, type I units appear to represent the activities of esophageal premotoneurons. Thus, the intensity of vagal mechanosensory afferent input determines the pattern and strength of the activities of the esophageal premotoneurons and, hence, the pattern and contractile force of the esophageal motor response.

Since the present work did not reveal any measurable changes of esophageal motility pattern after spinal cord transection, spinal afferents are unlikely to be involved in esophageal reflex control of the striated muscle TMP. However, it remains unknown if spinal afferents are involved in reflex control of the smooth muscle TMM, since this component, if it exists, is too small to be detected with the technique used in the present study. The TMM component of esophageal contractions evoked by large volume

distensions appears to be local myogenic, because it remains unchanged after bilateral vagotomy. Nonetheless, the TMM of the rat esophagus is richly innervated by spinal afferents (Dütsch et al., 1998). Spinal afferents from rat intestine project directly to DMV neurons (Renehan et al., 1995). Spinal afferents from the esophagus may have similar projections to DVM neurons and thus affect the activity of the smooth muscle TMM.

6.2.2 Inhibitory motility responses evoked by vagal mechanosensory afferent input

Distal inhibition is the muscle activity that distension of the proximal esophagus inhibits any contractions in progress in aboral segments. This phenomenon has been described in the human (Creamer and Schlegel, 1957; Sifrim and Janssens, 1996; Williams et al., 1993) and in some experimental animals that have a mixed striated and smooth muscle esophagus such as the cat, opossum (Roman and Tieffenbach, 1971; Paterson et al., 1988; Paterson and Indrakrishnan, 1995). In the smooth muscle segment of the esophagus, distal inhibition is believed to be mainly organized via the intramural peripheral control system (Roman and Tieffenbach, 1971; Paterson and Indrakrishnan, 1995; and for review, see Diamant and El-Sharkawy, 1977). Although the inhibition of neurons responsible for excitation of the esophagus below the balloon is also suggested, the mechanism has remained unknown (for review, see Diamant and El-Sharkawy, 1977). The present study demonstrates that, in the striated muscle TMP of the rat esophagus, distal inhibition is an inhibitory motor reflex that is: (i) evoked by esophageal mechanosensory afferent input, (ii) organized at the NTS esophageal premotoneuron level, and (iii) mediated by inhibitory reflex interneurons.

In principle, the inhibitory input to premotoneurons controlling the distal esophagus could come from local inhibitory interneurons or directly from vagal primary afferents. However, available evidence does not support the idea that vagal afferents directly release inhibitory neurotransmitters acting on the NTS interneurons. In a brainstem-cranial nerve preparation, a glutamatergic mediated EPSP is the only response to vagal nerve stimulation (Smith et al., 1998). Moreover, in a brainstem horizontal slice preparation, electric stimulation of the solitary tract evokes only EPSPs in the NTSc region (Lu and Bieger, 1998b). Thus, the present data indicate that distal inhibition involves local GABA-ergic and/or glycinergic interneurons in the NTSc region. These inhibitory interneurons are activated by collaterals of either vagal afferent or esophageal premotoneurons controlling proximal esophageal segments. The inhibitory output acts on NTSc premotoneurons controlling distal esophageal segments and is mediated by postsynaptic ligand-gated chloride channels. Both GABA-ergic and glycinergic systems are present in this region (Kubo and Kihara, 1987; William and Robertson, 1989; Broussard et al., 1996).

Conversely, another inhibitory esophageal motor response, deglutitive inhibition evoked by a swallow, does not appear to involve inhibitory amino acid neurotransmission in the NTSc region, at least not by the inhibitory amino acid receptor types known at present. Therefore, deglutitive inhibition may involve a different type of NTS interneuron and/or different sites of synaptic inhibition.

In the gastro-esophageal junction, high-volume distension evokes a relaxation in the distended segment. Since this relaxation is abolished by vagotomy, it appears to be an inhibitory motor reflex elicited by vagal mechanosensory afferent input. It would be of

interest to investigate the possible involvement of nitrergic inhibition of both esophageal striated and smooth muscle via intramural ganglia.

6.2.4 General visceral reflexes evoked by vagal mechanosensory input

As known earlier, the ECVR is a vagally mediated reflex that consists of a pressor and a cardioaccelerator component; increases logarithmically with inflation pressure; and increases in magnitude in distal relative to other segments of the esophagus (Loomis et al., 1997). In revealing a hidden depressor response as a third component of the ECVR the present results add another facet to this complex response. Furthermore, the data afford new insight into the reflex pathway and neurotransmitter utilized in the excitatory components.

The depressor response evoked by distal esophageal distension is only observed when the pressor response is blocked or impaired. As noted before, since this inhibitory component of the ECVR has a high threshold and is blocked by bilateral vagotomy, it is presumably mediated by high threshold vagal mechanosensory afferents from the esophagus. Moreover, the mechanism of this inhibitory component remains unclear. Conceivably, this component and the arterial baroreflex share a similar mechanism, because in either the increased afferent input lowers blood pressure.

A depressor response is frequently seen during distension of the mid-thoracic segment of the esophagus. As shown in the pentobarbital sodium anesthetized dog (Satpathy and Al-Sattar, 1983) and rat (Euchner-Wamser et al., 1993), the duration of this response is more or less restricted to the distension period. In the urethane-anesthetized rat, large

volume middle to upper thoracic esophageal distension evokes a similar depressor response that persists after bilateral vagotomy and/or ganglionic blockade (unpublished observation, also see Loomis et al., 1997). Most probably, this is an artifact arising from mechanical compression of major intrathoracic blood vessels by the inflation balloon.

Concerning the excitatory components of the ECVR, the present study shows that glutamatergic synapses are involved in mediating the ECVR at the level of both the NTS and the RVLM.

In the NTS, local application of the NMDA or non-NMDA glutamate receptor antagonists in the NTSc region inhibits both pressor and cardioaccelerator components of the ECVR. Moreover, the application of glutamate into the same region mimics the pressor component. These results suggest that vagal mechanosensory afferent fibers mediating the excitatory components of the ECVR terminate in the vicinity of esophageal premotoneurons comprising the NTSc and activate interneurons via glutamate receptors of both the NMDA and non-NMDA subtype. Functionally, these interneurons presumably respond with a tonic firing pattern to esophageal distension, have a wide receptive field, and increase their activity when vagal afferent input is intensified. These presumed physiological properties correspond to those of the type II units recorded in this region.

Originally, the NTSc was defined to be a group of cells that projects to the rostral AMB (Bieger, 1984; Ross et al., 1985; Cunningham and Sawchenko, 1989) where esophageal motoneurons are located (Bieger and Hopkins, 1987), and receives vagal afferent inputs from the esophagus (Altschuler et al., 1989). Anatimically, although the majority of NTSc neurons are believed to be esophageal premotoneurons (Lu and Bieger,

1998a; for review, see Bieger, 1993a), NTSc cells also reportedly project directly to DMV parasympathetic preganglionic neurons and contribute to gastric motility control (Rogers et al., 1999). The present data obtained with glutamate microstimulation suggest that the interneurons mediating the pressor component of the ECVR and esophageal premotoneurons are closely adjacent or even intermingled. Thus, possibly part of these ECVR interneurons are located in the range of the NTSc. More work is needed to determine the detailed distribution of these ECVR interneurons, especially with respect to the location of the esophageal premotoneurons.

On the other hand, while the cardioaccelerator response of the ECVR could not be evoked by application of glutamate in the NTS region, it is still possible that some interneurons located in perhaps more caudal NTS area other than the NTSc region receive esophageal vagal afferent input and contribute to the ECVR.

Finally, the present findings suggest that RVLM bulbospinal neurons are involved in the mediation of the ECVR. Glutamatergic neurotransmission in this region is likely to contribute to the pressor component. Thus, the central pathway differs from that mediating the baroreflex. In the latter, RVLM bulbospinal neurons are inhibited via $GABA_A$ synapses connected to interneurons located at the caudal ventrolateral medulla, and the latter receives projections from NTS interneurons. Although a direct connection between the ECVR interneurons in the NTSc region and RVLM bulbospinal neurons has been demonstrated (Ross et al., 1985), its inferred role in mediating the ECVR needs to be confirmed. As regards the cardiovascular component of the ECVR, its central pathway conceivably impinges on cardiomotor parasympathetic preganglionic neurons located in

the external formation of the AMB (Nosaka et al., 1982; Bieger and Hopkins, 1987; and for review see Hopkins et al., 1996; Loewy and Spyer, 1990). These preganglionic neurons persumably receive direct projections from NTS interneurons (Stuesse and Fish, 1984; Ross et al., 1985). Possibly, these direct projections are involved in the mediation of the cardiomotor component of the ECVR.

6.3 Future directions

The investigations described in this thesis raise a range of issues that merit further study.

1. Esophageal vagal mechanosensory afferent fibers are suggested to comprise different populations. However, the detailed physiological characteristics of each group remain to be carefully determined. Especially, it is important to determine if any vagal mechanosensory afferent fiber has a high threshold. On the other hand, the question of whether vagal C-fiber afferents innervating the esophagus contribute to any component of special or general visceral reflex responses is still open. The question of whether some of these afferents arise from the mucosa layer also remains to be answered. Also, as the distension-evoked discharge rate demonstrated in this work is much lower than that reported for afferents supplying the cervical esophagus (Andrew, 1956), there is a need to compare the characteristics of vagal afferents from different segments of the esophagus. Furthermore, the existence of fast adapting receptors in the esophagus, as suggested by the presence of calcium-binding protein, remains to be confirmed. Combined in vivo vagal single unit recording and neural labeling experiments should help resolve this issue.

Moreover, there is little information about the esophageal vagal sensory receptors other than mechanosensory ones. More studies are needed to identify the characteristics of chemical, temperature, and/or polymodal receptors in the rat esophagus.

2. The present and previous studies suggest that vagal afferent neurons mediating both special and general visceral reflexes use an EAA-like substance as neurotransmitter and activate NTS interneurons via glutamate receptors of both NMDA and non-NMDA subtype; however, receptor subtypes at the postsynaptic membrane need to be further studied. The available data tentatively imply that the NMDA receptor subtype more effectively contributes to special visceral reflex than the non-NMDA subtype, while the non-NMDA subtype makes a larger contribution to general visceral reflex than does the NMDA subtype. Detailed investigation is required to determine the relative contribution of each receptor subtype. Furthermore, the contribution of metabotropic glutamate receptors also needs to be studied.

3. The function of NTS esophageal responsive interneurons, especially the neurons that have the type II and III discharge patterns, needs to be investigated in detail. Since neurons that have type II discharges act like interneurons mediating both the ECVR and distal inhibition, they probably fall into different subtypes and differ in neurotransmitter utilized. The neurons that have the type III discharges are presumably involved in the esophageal motility control; however, this inference needs to be tested further. Combined electrophysiological, anatomical and immuno-histochemical studies will be required to identify the function of these neurons, especially with regard to their anatomical connectivity and neurotransmitter specificity.

4. Whereas detailed information exists concerning the central pathway mediating the esophageal reflex motility control, our knowledge about the central pathway of the ECVR is still limited. For the pressor component, the connection(s) between the NTS interneurons and the RVLM bulbospinal neurons remain to be established. Moreover, little is known about the central pathway of the cardioaccelerator and the inhibitory components of the ECVR. Combined *in vivo* intracellular recording and neurotracing studies should provide more information to answer these questions.

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