BRAINSTEM MECHANISMS SUBSERVING OESOPHAGEAL PERISTALSIS IN THE RAT

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By

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ABSTRACT

Functional organization and neurotransmitter mechanisms of the central oesophagomotor pathway from the subnucleus centralis of the nucleus tractus solitarii (NTS_c) to the compact formation of the nucleus ambiguus (AMB_c) were investigated *in vivo* in the anaesthetized rat and *in vitro* in a rat brainstem slice preparation. Techniques utilized included micropneumophoresis, extra- and intracellular recording, neuronal tracing and immunocytochemistry.

Results from neuropharmacological investigations indicate solitarial GABA neurons exert a tonic inhibition of oesophageal premotor elements in the NTS_c. Moreover, these GABAergic neurons are involved in pharyngoesophageal coupling of primary peristalsis as well as generation of secondary peristalsis.

Activation of muscarinic cholinoceptors in the NTS_c is necessary for generation of both fictive primary and secondary peristalsis. Such activation is functionally confined to ipsilateral AMB_c motoneurons. The rat brainstem appears to lack excitatory commissural connections between the oesophagomotor circuits in each hemi-medulla.

Results from *in vivo* and *in vitro* experiments demonstrate NTS_c premotoneurons utilize an excitatory amino acid (EAA)-like substance, which activates N-methyl-D-aspartate (NMDA) and non-NMDA receptor subtypes on AMB_c neurons. Activation of NMDA receptors is required for functional information transfer in this oesophagomotor pathway and, in turn, this system is dependent on the integrity of somatostatinergic input originating from the NTS_c.

Apart from solitarial input, NTS_c-AMB_c synaptic transmission is subject to modulation by nicotinic cholinoceptor-mediated input originating from the zona intermedialis reticularis parvicellularis (ZIRP). By augmenting the depolarizing response to glutamate and inhibiting nicotinic cholinoceptor-mediated excitation, somatostatin presumably plays a critical role in oesophagomotor control at the AMB_c level.

Taken together, the present work lends strong credence to the hypothesized role of the NTS_c-AMB_c projection as the common premotor substrate of oesophageal peristaltic control. NTS_c premotoneurons are involved in initiating and organising the sequence of peristaltic activity, whereas AMB_c motoneurons are intricately involved in modifying the final oesophagomotor output.

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LIST OF ABBREVIATIONS

ACh	acetylcholine
ACSF	artificial cerebrospinal fluid
AMB	nucleus ambiguus
AMB_c	compact formation of AMB
APV	D,L-2-amino-5-phosphonovaleric acid
AP-7	D,L-2-amino-7-phosphonoheptanoic acid
Bicu	bicuculline
CD	cis-2-methyl-dimethylaminomethyl-1,3-dioxolane methiodide
CE	cervical oesophagus
ChAT	choline acetyltransferase
ChAT-IR	ChAT-immunoreactive
CPG	central pattern generator
CSH	cysteamine
DβE	dihydro-β-erythroidine
DE	distal oesophagus
τ-DGG	τ-D-glutamylglycine
DMPP	1,1-dimethyl-4-phenyl-piperazinium iodide
DMV	dorsal motor nucleus of vagus
E	oesophagus
EAA	excitatory amino acid

ENK	enkephalin
EPSP	excitatory postsynaptic potential
FITC	fluorescein isothiocyanate
GABA	gamma-aminobutyric acid
$GABA_A$	GABA-A receptor
Glu (G)	glutamate
GLY (Gly)	glycine
HTF	high-threshold fast (component of the EPSP)
IPSP	inhibitory postsynaptic potential
KA	kainate, kainic acid
KYN (Kyn)	kynurenic acid
LTS	low-threshold slow (component of the EPSP)
mAChR	muscarinic cholinoceptor
MSCP	methscopolamine
MUSC	muscarine
nAChR	nicotinic cholinoceptor
NMDA	N-methyl-D-aspartate
NPG	network pattern generator
NTS	nucleus tractus solitarii
NTS_c	subnucleus centralis of NTS
NTS_i	subnucleus intermedialis of NTS
NTS_v	subnucleus ventralis of NTS

P	pharynx
PBS	phosphate-buffered saline
PHAL	*phaseolus vulgaris* leucoagglutinin
R (r)	respiration
SCP	scopolamine
SLN	superior laryngeal nerve
SST	somatostatin
STR (Str)	strychnine
TMM	tunica muscularis mucosae
TMP	tunica muscularis propria
TS	solitary tract
TTX	tetrodotoxin
V_m	facial nucleus
XII	twelfth nerve nucleus
ZIRP	zona intermedialis reticularis parvicellularis

Chapter One

INTRODUCTION

1.1 Swallowing and oesophageal peristalsis - an overview

Swallowing in both invertebrates (125,136) and vertebrates (7,21,47,140) is a centrally programmed motor synergy of sequentially co-ordinated contractions and relaxations of muscles in the upper alimentary tract which serve to propel ingested food and drink from the mouth to the stomach (15,21,41,42,47,96,102,119,122,140,142). Swallowing can be divided into two main phases, viz. the buccopharyngeal (linguo-palatal and pharyngeal) and the oesophageal stage (7,96). Though coupled during the normal act of swallowing, these two stages may operate independently of each other under certain conditions, particularly when oesophageal peristalsis is initiated by distention of the oesophagus. They are therefore thought to be controlled by two distinct components of the neural pattern generator for swallowing. Since relaxation of the lower oesophageal sphincter occurs shortly after initiation of the buccopharyngeal but well before the oesophageal stage, it is generally considered an entity separable from the other stages of swallowing and subject to a central control mechanism of its own.

Currently available neuroanatomical and pharmacological evidence, mostly obtained from the rat (2,6,10,28-30,70,72,103-106), suggests that the medullary circuitry controlling the oesophageal stage is less complex than that controlling the buccopharyngeal stage. However, the movements of the oesophagus are actually quite intricate for they must coordinate with movements of both the buccopharyngeal stage and

the lower oesophageal sphincter so as to propel material to the stomach and at the same time prevent reflux of corrosive gastric contents. Thus, the neural network organising oesophageal motility provides a system with a desirable degree of simplicity, yet enough complexity to challenge those neurobiologists who attempt to uncover the mechanism by which networks generate behaviour in mammals. Within the last ten years, substantial progress has been made in elucidating the brainstem network controlling oesophageal motility. These advances have been reviewed in depth by several authors (30,41,42,83,143,161,202). Therefore, this dissertation will not attempt a survey of the extant literature but limit itself to contemporary concepts of brainstem control mechanisms governing oesophageal motility following a brief account of some general features of oesophageal movement.

i. ***Oesophageal peristalsis***: The basic pattern of oesophageal movement is characterized by a propulsive contraction of the oesophageal musculature propagating in the aboral direction, a pattern generally described as peristalsis (41,42,83,140,143,161). Meltzer distinguished primary from secondary peristalsis according to the mode of initiation (134). ***Primary peristalsis*** ensues during the act of swallowing, i.e. represents the oesophageal component of swallowing (41,133,134,143,161). Though triggered by the swallowing act, primary peristalsis does not always follow buccopharyngeal deglutition in a 1:1 ratio. When swallows are elicited in quick succession, oesophageal peristalsis will only occur after the last swallow is completed (133). This phenomenon is known as '***deglutitive inhibition of the oesophagus***' (76,77,192).

Secondary peristalsis is independent of swallowing but elicited by distention of

the oesophageal wall (134). Both primary and secondary peristalsis are dependent on a central control mechanism for both are abolished by bilateral vagotomy (19,41,134,140,158,161).

There is yet another type of oesophageal peristalsis termed '*tertiary*' (19,41,161,162) or '*autonomous*' *peristalsis* (41,161), which occurs only in the smooth muscle containing portion of the oesophagus. Unlike primary and secondary peristalsis, this type of activity is not dependent on a central mechanism, i.e. it can be elicited in the absence of extrinsic innervation.

ii. ***Musculature of the mammalian oesophagus***: The mammalian oesophageal body possesses two main muscle layers comprising the tunica muscularis propria (TMP) and the tunica muscularis mucosae (TMM). The TMP is arranged in two layers containing both striated and smooth muscle. Fibers of the outer layer run predominantly in the longitudinal axis, whereas those of the inner layer have mainly a circular orientation. The proportion of these two muscle types varies widely between species. In the dog, sheep and rat, nearly the entire TMP is made up of striated muscle fibers (41,141,161). In other species such as the cat, opossum and human, striated muscle predominates only in tl e proximal oesophagus. Distalwards this gives way to smooth muscle such that in the thoracic and remaining distal oesophagus, the TMP in the opossum and human is composed entirely of smooth muscle (41,141,161).

Lying between the TMP and mucosa, the TMM is made up of bundles of obliquely oriented smooth muscle fibers throughout the length of the oesophagus. In the rat, the TMM is capable of generating longitudinal and transverse tension and may

therefore maintain intraluminal pressure *in vivo*.

iii. ***Innervation of the oesophagus***: Oesophageal afferents, regardless of their origins from either striated or smooth muscle segments, travel in the cervical vagal trunk and superior laryngeal nerve (SLN) (2,9,30,41,161). In the medulla they terminate in a very discrete portion of the nucleus of the solitary tract (NTS), termed subnucleus centralis (NTS_c) in the rat (2,9). The termination field within the NTS_c has a limited topographic distribution, in that fibers arising from more proximal levels of the oesophagus project to more rostral parts of the subnucleus (2). In addition, a portion of oesophageal afferents terminate in the spinal cord via lower cervical and upper thoracic dorsal root ganglia (24,25,65,73). This non-medullary projection may be involved in mediating pain of oesophageal origin. Its role in the regulation of oesophageal motility remains to be determined.

The efferent motor innervation of the oesophagus differs according to the type of muscle supplied. In the rat, neurons innervating the striated portion have been demonstrated both anatomically (6,10,28) and physiologically (6,105) to occupy the rostral nucleus ambiguus (AMB), representing the compact formation of the nucleus ambiguus (AMB_c) (10). The projection of these AMB_c neurons shows a crude rostro-caudal organotopy (10). Axons of AMB_c oesophagomotor neurons exit through the vagal trunk and two of its major branches, the superior laryngeal and recurrent nerves (10). Their terminals do not synapse on cells of the myenteric plexuses lying between the two muscle layers but end directly on striated muscle cells forming a nicotinic cholinergic synapse similar to the endplate of skeletal muscle fibers (41,140,161). Thus, peristalsis

occurring in this portion is thought to be entirely dependent on the commands programmed within the lower brainstem (41,140,161).

Smooth muscle of the oesophagus, like that elsewhere in the digestive tract, is innervated by the parasympathetic visceromotor pathway. Preganglionic cell bodies presumably lie in the medulla within the dorsal motor nucleus of the vagus (DMV) (41,195,161), whose axons travel to the oesophagus in the vagal trunk. However, they do not synapse directly on the muscle cells but are relayed by neurons of the intrinsic plexuses. In the rat, these parasympathetic postganglionic cells are only present in the myenteric plexus (196). Postganglionic fibers from these plexuses in turn innervate the smooth muscle layers. Peristalsis of the smooth muscle portion of the oesophagus is therefore subject to a mechanism of integrated central and peripheral control (41,140,161). In addition, the oesophagus also receives sympathetic innervation (41) (relatively sparse in the rat (203)). Its origin, distribution and physiological function, however, remain unclear.

Since peristalsis of the striated musculature of the oesophagus, unlike that of the smooth muscle portion of the oesophagus, is entirely dependent on central efferents, it is advantageous to investigate central mechanisms serving oesophageal peristalsis in animals in which peristalsis is effected chiefly by striated muscle. The rat may be such a species. Its oesophageal peristaltic activity is abolished following pharmacological blockade of nicotinic synaptic transmission at the neuromuscular junction, but unaffected by peripheral muscarinic cholinoceptor blockade (Lu and Bieger, personal communication). The following sections will address recent concepts regarding the

central organization of motor output to oesophageal striated muscle. No attempt will be made to deal with the topic of intrinsic neural control of smooth muscle peristalsis.

1.2 Central control of the oesophageal peristalsis

Since the last century, the central control of swallowing in general, and oesophageal peristalsis, in particular, has been the subject of scientific research. These studies have gone through several discernible but overlapping experimental stages.

1.2.1 *Emergence of the neuronal network pattern generator organizing the oesophageal peristalsis*

The initial experimental stage aimed to establish how the nervous system controls the complete swallowing sequence and was dominated by two rival hypotheses. The first postulates a ***reflex chain*** mechanism: "each motion of the pattern stimulates peripheral receptors whose afferent impulses trigger the next motion" (140). The second hypothesis holds that the swallowing sequence is regulated in toto, independent of sensory inputs, by a specific group of neurons termed ***center*** by earlier workers (47), but now more commonly referred to as ***central pattern generator*** (CPG) (21,41,95,96,140,143). Evidence accumulated in recent years favours the latter hypothesis as far as buccopharyngeal swallowing is concerned. During reflex swallowing in the dog, cat (48,139,141) and rabbit (178), the basic sequence of motoneuronal activation remains essentially unchanged following either physically or pharmacologically induced sensory deafferentation. Moreover, swallowing related unit activity of NTS interneurons recorded both in sheep and rats persists after curarization of the animal (94,105).

Evidence supporting the existence of a CPG for oesophageal peristalsis comes largely from oesophageal transection studies. Mosso (cited from ref. 140) and Meltzer (133) initially showed that oesophageal transection did not completely stop the progression of primary peristaltic contractions of the oesophagus in the dog, suggesting that oesophageal peristalsis is due to a central mechanism, which, once initiated, could run its entire course without additional afferent input. This finding was later substantiated by oesophageal transection and bolus deviation at different levels of the oesophagus performed on several species including rabbit, opossum and rhesus monkey (90).

More direct evidence for the existence of an oesophageal CPG was provided by the work of Roman (160), in which the central end of the vagus was used to reinnervate the sterno-mastoid muscles in sheep and monkey. From this study, Roman demonstrated that activity of the vagal efferents to the oesophageal muscles occurred sequentially in both the striated and smooth muscle portions during both primary and secondary peristalsis. Roman and Tieffenbach (162) also demonstrated in the baboon that primary peristalsis of the smooth muscle portion persisted after paralysis of the upper cervical striated muscle portion with curare. In addition, microelectrode recordings from medullary neurons indicate that oesophageal related activity of NTS interneurons persists after curarization of the animal (94,161). Taken together, these findings provide strong evidence supporting the idea that oesophageal peristalsis, like buccopharyngeal swallowing, is governed by a CPG network, which is able to organize the motor sequence of peristalsis in the absence of afferent inputs.

In contrast to the above, work from Longhi and Jordan (120) has challenged the concept of a CPG organizing oesophageal peristalsis. They found that in the dog, the presence of a bolus in the oesophagus is required to maintain primary peristalsis. Deviation of the bolus from the oesophagus by means of an oesophageal cannula or oesophagotomy abolished all oesophageal peristaltic activity below the level of the deviation. Janssens and his colleagues (91,92) confirmed this result and further concluded that afferent feedback due to the presence of a bolus was necessary at the cervical level but not at the thoracic level of the oesophagus.

Thus, while it is generally agreed that oesophageal peristalsis, be it primary or secondary, is centrally mediated, the requirement of peripheral input originating from the oesophagus itself remains equivocal. For this reason, brainstem oesophagomotor circuitry is more suitably referred to as a neuronal ***network pattern generator*** (NPG). This term may be used to denote a neuronal pattern generating network so strongly influenced by afferent feedback that functional unity of the generator is disrupted by the deafferentation.

1.2.2 *Anatomical location of the NPG for oesophageal peristalsis*

The second research phase continues to this day with attempts to determine the location of the oesophageal NPG and to map synaptic connectivity within the circuit. The organization of oesophageal peristalsis is thought to take place within a swallowing center (41,140,143,161). Since the pioneering work of Meltzer in 1899 (133), Ishihara in 1906 (cited in ref. (49)) and Miller and Sherrington in 1916 (138), the search for such a center has intrigued many investigators and has been approached by a variety of

strategies including brain transection, lesioning, electrical and chemical stimulation, pathway tracing and microelectrode recording.

Earlier studies employing brain transections and lesions (46,138) (see also ref. (15)) showed that structures responsible for generating the basic motor pattern of swallowing lie within the rhombencephalon, especially within the medulla (for review see (140)). This assumption has now been generally accepted (7,21,41,42,47,79,96,140,142,161). However, to date, the precise localization of the swallowing center remains unsettled.

On the basis of lesion experiments on the dog, cat and macaque, Doty et al. (49) localized the center to a region of the pontine and medullary reticular formation extending from the rostral pole of the inferior olive to the posterior pole of the facial motor nucleus. Because the lesion itself can cause irritable foci or disrupt connections between components of the circuitry and fibers of passage instead of damaging the "center", results from such studies must be interpreted with caution.

Indeed, systematic exploration of the medulla by means of electrical stimulation both in the sheep (20), dog (146) and rat (105) failed to confirm Doty's interpretation. Instead, through microelectrode recordings of swallowing related unit activity in the medulla and pons of both sheep (94) and rats (105), the NTS has emerged as the principal neural substrate involved in the organisation of swallowing.

Based on experiments in sheep, Jean (94) classifies swallowing related neurons into a dorsal and a ventral group. The former contains neurons located in the NTS and the adjacent reticular formation, whereas the latter includes neurons in the lateral

reticular formation near the AMB. Both groups contain so-called "early" neurons, which fire before or during the buccopharyngeal stage, and "late" and "very late" neurons, which discharge during the cervical and distal oesophageal contractions, respectively. Jean further proposes that the dorsal group of neurons executes overall deglutitive programming. The ventral group performs a 'switching' function, in that its neurons receive inputs from the dorsal group and distribute commands to individual motor nuclei.

With regard to the oesophageal NPG in sheep, oesophageal neurons are located in the medial part of the NTS. Lesioning this region selectively impairs the oesophageal stage of swallowing evoked by electrical stimulation of the ipsilateral SLN (93). Thus, Jean's work provides evidence consistent with the assumption that two NPGs are present within the 'swallowing center', one for the buccopharyngeal, the other for the oesophageal stage of swallowing; the latter within the medial part of the NTS in sheep. Nevertheless, it should be noted that an oesophageal-modulated pattern of unit activity is not sufficient to demonstrate involvement in pattern generation. Conceivably, these neurons may be involved merely in the coordination of the ingestive synergy and other brainstem functions such as breathing, coughing and chewing. Similarly, loss of the oesophageal stage caused by lesioning the medial part of the NTS may not necessarily signify destruction of the oesophageal NPG, but might merely reflect deafferentation.

More recently, Bieger (6,68-70) employed chemical microstimulation and neuronal tracing techniques to explore the NPG for swallowing and oesophageal peristalsis and to elucidate its structural and functional organization in the rat. Progressively refined chemical microstimulation techniques applied in these studies

enabled deglutitive loci to be mapped in the rat medulla oblongata on the scale of 50-70 μm. These investigations furnished new evidence indicating that this nucleus plays an important role in programming motor sequences of swallowing as well as oesophageal peristalsis (7,68). Moreover, these studies have strengthened the view that the NPG for swallowing can be divided into two sub NPGs governing the buccopharyngeal and oesophageal stages. Direct chemical microstimulation with glutamate of structures within the confines of the NTS gives rise to either complete swallows or their constituent stages, depending upon the site of stimulation within this complex.

The first extensive map of medullary substrates which make up the oesophageal NPG in the rat emerged from the work of Bieger (1984) (6). This study demonstrated that pressure ejection of glutamate or ACh in a very circumscribed area in the medial NTS, a region which was subsequently termed the central subnucleus of the NTS (NTS_c) (163), evoked isolated oesophageal responses. Moreover, neurons in this area were shown to project directly to the region of the ventrolateral medulla known to contain oesophageal motor neurons, i.e. the AMB_c (10). This study provided a neuroanatomical frame of reference for further investigations into the central NPG for oesophageal peristalsis and stimulated pharmacological (68-71) and anatomical interest in this region (2,28,29,31,72).

Data accumulated from recent electrophysiological and neuroanatomical studies thus far have supported the hypothesis (6) that NTS_c neurons in the rat are premotoneurons ("interneuronal elements forming an intrinsic part" (7)) of the oesophageal NPG. First, microelectrode recordings in the rat reveal that the NTS_c region

contains neurons whose activity is phase-locked with oesophageal peristalsis (105). Second, the NTS_e not only projects directly to the AMB_e (6,28,163), the oesophagomotor portion of the AMB (10), but also receives oesophageal primary afferent input (2,9). Finally, activation of muscarinic cholinoceptors associated with the NTS_e evokes rhythmic oesophageal contractions resembling secondary peristalsis (6,69,70). Thus, currently available evidence supports the presence of two neuroanatomically defined oesophageal NPGs, one on each side of the brainstem, in which NTS_e neurons receive oesophageal primary afferents and in turn project to AMB_e neurons, the sole group of motoneurons innervating the striated muscle of the oesophagus. Many questions with respect to the functional organization of the oesophageal NPG remain such as how synchronized activity of the left and right NPGs is accomplished and how activity is coordinated with that of the buccopharyngeal NPG.

As regards buccopharyngeal loci within the NTS, chemically excitable substrates are coextensive with the subnuclei intermedialis (NTS_i) and ventralis (NTS_v) in the rat (6,68-70). These subnuclei overlap with regions where early (buccopharyngeal) swallowing related unit discharges are recorded (105). In addition, neuroanatomical tracing studies indicate that the NTS_i overlaps with termination fields of buccopharyngeal afferents (2) and projects directly to all buccopharyngeal motor nuclei (68,72) consistent with work by Travers (190) and Travers and Norgren (191). Since both oesophageal and buccopharyngeal premotoneurons in the NTS appear to project directly to their corresponding motoneurons, the physiological relevance of deglutitive ('switching') interneurons within the ventral reticular formation (94,105) remains questionable, at least

in the rat.

1.2.3 *Neuropharmacology of oesophageal peristalsis*

The most recent phase in the analysis of the NPG for oesophageal peristalsis concerns the characterization of underlying transmitter processes with the use of pharmacological tools. Employing focal chemostimulation, one can induce the complete sequence of swallowing or its component buccopharyngeal and oesophageal stages via activation or inactivation of specific receptor systems in the anaesthetized rat (7). Bieger (7) conceptualized such drug-induced swallowing and oesophageal peristalsis as "***fictive swallowing***" and "***fictive oesophageal peristalsis***", respectively, because of their autorhythmicity and occurrence in the absence of stimulation of sensory input from the upper alimentary tract. Pharmacological selectivity in evoking components of swallowing provides direct clues to the synaptic mediators used by the NPG.

Moreover, since drugs may be applied by bath perfusion to *in vitro* preparations, as well as administered centrally to specific brain regions of intact animals, such studies provide an important bridge between reduced and intact animal preparations. Therefore, a great deal has been learned from the pharmacological study of the NPG for swallowing and oesophageal peristalsis (4-8,68-71,79,103,104,106,183,184). More specifically, these studies provide important clues to the neurotransmitter mechanisms associated with the NPG at both the NTS premotor and the AMB motoneuronal levels.

i. *Putative transmitter substances associated with premotoneurons in the NTS*

A. Excitatory mediators operating at NTS_c oesophageal premotoneurons: Studies of the neuropharmacology of the NPG for oesophageal peristalsis at the NTS

level began with the work of Bieger in 1984 (6). This work revealed the considerable importance of central muscarinic cholinoceptor-mediated processes in the control of oesophageal peristalsis. Thus, surface application of muscarinic agonists to the NTS or ejection of the same agonists from micropipettes at intrasolitary loci overlapping with the NTS_c gives rise to rhythmic peristaltic contractions of oesophageal striated musculature unaccompanied by consistent buccopharyngeal activity (6). Since the overall pattern of the muscarine-induced oesophageal response resembles secondary peristalsis, it may be defined as fictive secondary peristalsis (7). Moreover, oesophageal components of both fictive and reflex swallowing are readily suppressed following intravenous administration of antimuscarinic agents such as scopolamine, a tertiary compound which is capable of crossing the blood-brain barrier (6). The quaternary compound methscopolamine is also effective, but the concentration required is 10 times larger than that of the tertiary parent compound scopolamine (6). These findings provide strong support for the hypothesis that central muscarinic activation is necessary for generating the neural impulse pattern required for oesophageal peristalsis. It is relevant to note that in other species, including cat (158), opossum (155) and human (155), oesophageal activity is also suppressed by systemic administration of muscarinic cholinoceptor antagonists. This effect has been attributed solely to a blockade of peripheral oesophagomotor nerves, although in another human study, the incidence of primary peristalsis is markedly reduced following intravenous injection of atropine (45). It now seems necessary to reevaluate this view, since the contribution of central muscarinic blockade in these studies may have been overlooked.

Although a recent immunohistochemical study reveals that the NTS_c is densely supplied with choline-acetyltransferase (ChAT) immunoreactive terminals (164), the source of this cholinergic input remains unclear. One possibility is that oesophageal primary afferents are themselves cholinergic (55,154,185).

In addition to ACh, the work of Bieger (1984) (6) and that of Hashim and Bieger (1989) (70) suggests that a glutamate-like substance, i.e. an excitatory amino acid (EAA), operates at the premotoneuronal level within the NTS. These studies indicate that the EAA input is mediated through activation of different EAA receptor subtypes at different NTS deglutitive loci. Thus, N-methyl-D-aspartate (NMDA) is more potent than other selective EAA agonists such as kainate and quisqualate when directly ejected at the oesophageal locus in the NTS_c, suggesting the preferential association of the NMDA receptor subtype with oesophageal premotoneurons. Consistent with this observation, the selective NMDA receptor antagonists 2-amino-5-phosphonovaleric acid (APV) and 2-amino-7-phosphonoheptanoic acid (AP-7) reversibly block oesophagomotor response evoked from the NTS_c by glutamate (70).

B. Excitatory mediators operating at NTS pharyngeal loci: In contrast, kainic acid (KA) is most potent at pharyngeal loci in the NTS_i and NTS_v (70). Moreover, the glutamate-evoked pharyngeal response elicited at these loci is blocked by non-selective EAA antagonists, but not APV and AP-7, a finding which has been corroborated by Kessler and Jean (104). Thus, these data are consistent with an association of non-NMDA subtype EAA receptors with the pharyngeal loci within the NTS. It should be noted that the preferential sensitivity of NTS pharyngeal loci to KA does not exclude

involvement of NMDA receptors in generating deglutitive interneuronal excitation directed to pharyngeal motoneurons. In fact, Jean and his co-workers recently suggested that in the NTS NMDA receptors may be critically involved in the rhythmogenesis for buccopharyngeal swallowing (182-184).

Besides ACh and EAA, several other chemical mediators, such as serotonin (5,7,11,12,69), noradrenaline (7,11), dopamine (4,8,11), thyrotropin-releasing hormone (7), vasopressin (7) and oxytocin (7) are implicated as excitatory transmitters associated with the NPG for buccopharyngeal swallowing within the NTS. When applied to the extraventricular surface of the NTS or directly ejected at NTS loci, these substances evoke or facilitate the buccopharyngeal component of swallowing activity in the rat. However, unlike ACh and NMDA, none of these agents exerts preferential action on the oesophageal stage. (for review see (7)).

C. Inhibitory mediators operating at the NTS deglutitive loci: At the present time, very little is known about inhibitory inputs to the oesophageal NPG, though several putative transmitters are implicated as inhibitory mediators of the buccopharyngeal NPG. Preliminary studies from this laboratory suggest GABAergic involvement in the control of buccopharyngeal swallowing. Thus, both fictive and reflex swallowing is completely abolished by application of the $GABA_A$ receptor agonist, muscimol (14,175), to the ventricular surface of the NTS (Bieger, in preparation). Moreover, intravenous injection of diazepam, a drug which enhances $GABA_A$-mediated inhibition (14,175), inhibits reflex swallowing in the cat (Hockman and Bieger, unpublished manuscript). These results suggest that central GABAergic synapses may play important roles in the generation of

swallowing.

In addition, 5-HT and noradrenaline inhibit reflex swallowing when injected directly in the NTS at large doses (30-50 nmol) (103,106). However, as mentioned above, overt stimulation of swallowing activity is observed when these drugs are given topically to the dorsal surface of the NTS (7) or directly to the NTS_i at doses two orders of magnitude lower (69). In addition to differences in methods of drug application and dose, involvement of different receptor subtypes in mediating these deglutitive actions may contribute to the controversy, at least insofar as 5-HT is concerned (12).

Although stimulation of swallowing has been found following direct injection of D-ala^2-met enkephalin-amide into the lateral cerebral ventricle (51), inhibition of swallowing by opioids has also been reported. Thus, following intravenous applications of nonpeptide opioid agonists (135) or topical applications of opioid peptides to the extraventricular surface (7), fictive swallowing is inhibited.

ii. ***Putative transmitter substances acting at oesophageal motoneurons in the AMB***

The study by Bieger in 1984 demonstrates that both ACh and glutamate, when pressure-ejected in the rostral portion of the AMB, are capable of producing short latency oesophagomotor responses, suggesting that these two substances may function as mediators acting on the AMB_e oesophageal motoneurons (6). The ACh-induced response from the AMB_e, unlike that evoked from the NTS_e, is scopolamine-resistant, implying a nicotinic mechanism. The presence of nicotinic cholinoceptors within the AMB (179,197) is consistent with this result. However, ACh does not appear to be the primary transmitter of the NTS_e neurons projecting to the AMB_e since the presence of

the cholinergic marker enzyme in the NTS_c cell bodies can not be demonstrated by ChAT-immunocytochemistry (164,181). Nonetheless, the presence of ACh receptors in the ambigual complex and the ability of ACh to excite AMB_c neurons suggest that ACh functions to modulate the final oesophagomotor output at the AMB_c.

Unlike ACh, glutamate or aspartate is a plausible candidate as primary transmitter mediating the solitario-ambigual oesophagomotor pathway because EAAs are thought to mediate synaptic transmission at most central excitatory synapses (27,130,205). More to the point, EAAs are implicated as transmitters at premotoneuron-motoneuron synapses elsewhere (67,74,101,111,118,151).

In addition to the above-mentioned transmitters, two peptides, somatostatin (SST) and enkephalin, have been identified in NTS_c neurons projecting to AMB_c motoneurons (28,31). The association of SST with this pathway is of particular interest because SST inhibits cholinergic (84,88,145,200), but facilitates glutamate (43,85) responses elsewhere. Conceivably, SST could exert a differential action in modulating excitatory inputs to the AMB_c, thereby playing a major role in generating oesophageal peristalsis. With regard to enkephalin, while most studies in the central nervous system have suggested that it acts to reduce neuronal excitability (148), its role in oesophageal peristalsis remains unclear.

1.3 Rationale and objectives

The foregoing account summarizes current concepts regarding central control of oesophageal peristalsis. At this point, at least two components of the postulated

brainstem NPG for oesophageal peristalsis can be identified anatomically, viz. oesophageal secondary sensory neurons (premotoneurons), constituting the NTS_c and the monosynaptically connected oesophageal motoneurons of the AMB_c innervating striated muscles of the oesophagus.

ACh and an EAA are major transmitter candidates in the NPG. They act both at the level of the NTS_c as well as the AMB_c. Another potentially important putative mediator is SST, which is present in NTS_c neurons projecting to AMB_c motoneurons, where it may interact with the aforementioned transmitters.

There are still wide gaps in our knowledge that obviate a thorough understanding of the chemical signalling processes by which the brainstem NPG initiates oesophageal peristalsis. First, there is no concrete information concerning the role of a central inhibitory process in the generation of oesophageal peristalsis. However, inhibition is considered a prerequisite mechanism for generation of neuronal patterned activity. We know little about mechanisms by which oesophageal peristalsis is coordinated with buccopharyngeal swallowing and is inhibited when swallows occur in quick succession. Second, little is known concerning the manner by which the two oesophageal NPGs, one on each side of the brainstem, generate synchronized bilateral activity in oesophageal motoneurons during peristalsis, and the extent to which activity on one side is dependent on that of the other. Third, solitario-ambigual projection neurons in the NTS_c are thought to be oesophageal premotoneurons involved in generating the motoneuronal firing pattern underlying oesophageal peristalsis. However, how commands generated in NTS_c neurons are relayed to AMB_c motoneurons and what transmitters are involved in

mediating solitario-ambigual synaptic transmission remain unknown. Finally, the multiplicity of inputs to the AMB_c motoneurons implies that complex processing takes place at this level. To what extent oesophageal peristalsis is modified and shaped at the motoneuronal level remains to be determined.

The present investigations are aimed at resolving three basic questions concerning the manner in which oesophageal peristalsis is organized by the brainstem NPG in the rat. (i) Are oesophageal premotoneurons subject to synaptic inhibition and if so, what is the contribution of solitarial GABAergic neurons? (ii) How are premotoneuronal impulses distributed to motoneurons? (iii) What transmitters are utilized by oesophageal premotoneurons? In light of the evidence reviewed above, the following working hypotheses are proposed:

A. Solitarial GABAergic synapses form an intrinsic component of the NPG for oesophageal peristalsis. By interacting with other transmitter-mediated excitatory processes, this GABAergic mechanism is critically involved in the organization of both primary and secondary peristalsis at the NTS_c premotoneuronal level.

B. Central oesophagomotor drive initiated unilaterally from NTS_c is distributed to AMB_c motoneurons bilaterally via commissural or decussating connections between the two halves of the brainstem.

C. NTS_c neurons utilize an EAA-like substance as transmitter acting through EAA receptors, which in turn are modulated by multiple neurochemical inputs from both the solitary complex and the medullary reticular formation.

Specific objectives:

First, a pharmacological approach is used to analyze the role of solitarial GABAergic synapses in primary and secondary peristalsis, with particular emphasis being placed on the contribution of this inhibitory system to pharyngo-esophageal coupling and deglutitive inhibition of the oesophagus.

Second, oesophageal peristalsis triggered by unilateral central chemostimulation is studied to determine if unilateral excitation of oesophageal premotoneurons is distributed to both ipsi- and contralateral motoneurons.

Third, the involvement of an EAA transmitter in mediating NTS_c-AMB_c synaptic transmission is examined *in vivo*. The approach employs micropneumophoresis of EAA receptor antagonists at the level of the AMB_c with a view to inhibiting the oesophagomotor response induced from the NTS.

Fourth, to extend the investigation to the cellular level, an *in vitro* brainstem slice is used to enable AMB_c EPSPs to be recorded intracellularly in response to electrical stimulation of NTS_c efferent fibers, and to be pharmacologically characterized in terms of receptor subtypes involved in generating the EPSP.

The fifth phase of investigations aims to determine the contribution of SST to synaptic transmission from the NTS_c to the AMB_c.

The sixth objective is to characterize the cholinergic mechanisms mediating excitation of AMB_c neurons *in vivo*, and *in vitro*, as well as to determine the source of cholinergic afferents.

Finally, interactions between SST, EAA and ACh receptor-mediated inputs at the

AMB_e level are studied in the brainstem slice preparation and then corroborated in the whole animal.

Chapter Two

Role of solitarial GABAergic mechanisms in swallowing

2.1 Introduction:

As centrally-programmed motor acts, oesophageal peristalsis and swallowing presumably require both synaptic excitation and inhibition to ensure the timing and coordination of motoneuron outputs. To date, several putative transmitters are implicated in excitatory synaptic transmission at buccopharyngeal and oesophageal premotoneurons (reviewed in ref (7)). In particular, glutamate activates both the buccopharyngeal and oesophageal stages of swallowing (70), whereas, ACh exerts a selective action on the oesophageal stage (6,70). In contrast, little is known about inhibitory processes operating at both stages, although inhibitory mechanisms can be assumed to play an important role in (i) the coordination of deglutitive motoneurons within each stage; (ii) the linkage between the two stages and (iii) deglutitive inhibition of the oesophagus.

GABA is the principal inhibitory neurotransmitter at supraspinal levels of the mammalian neuraxis (40,175) and is involved in the central control of locomotion (67,176), respiration (56) and cardiovascular functions (113). Evidence for involvement of GABA in deglutitive control is still fragmentary and in part based on pilot data from this laboratory suggesting that muscimol, a $GABA_A$ receptor agonist (40,168,175), inhibits both fictive and reflex swallowing, whereas picrotoxin produces the opposite effect. Moreover, diazepam, which enhances $GABA_A$-mediated inhibition (14,40,175), inhibits the swallowing reflex in the cat (Hockman and Bieger, unpublished manuscript).

Similar observations have also been reported in the dog (48). Thus, the threshold for evoking reflex swallowing by electric stimulation of the SLN is substantially increased following low dose intravenous application of pentobarbital, which enhances $GABA_A$ function. All these results suggest that the NPG for swallowing is subject to inhibition by a $GABA_A$ receptor-mediated mechanism. In light of numerous immunochemical (99,123) and physiological (113) studies on the presence of GABA neurons in the NTS, further investigation into the role of solitarial GABA-ergic inhibitory mechanisms in deglutitive control is clearly warranted.

In the present study, the selective GABA receptor agonist, muscimol and the selective antagonists, bicuculline and picrotoxin (14,40,175), were used as tools to analyze the role of solitarial GABAergic synapses in deglutition, with particular emphasis on their contributions to the coordination of the buccopharyngeal and oesophageal stages and the deglutitive inhibition of oesophagus.

2.2 Methods:

Experiments were done on male Sprague-Dawley (Charles Rivers, Montreal) rats weighing 250-450 g. Rats were anaesthetized with urethane (1.2 g/kg, i.p.) and then tracheotomized. The right external jugular vein was cannulated for intravenous infusion of drugs. Submandibular glands were removed in ten of the animals to minimize salivation. The animals were then mounted in a stereotaxic frame.

Changes in intraluminal pressure within the pharynx and the oesophagus were recorded by using miniature balloon-tipped catheters constructed with PE-90 polyethylene tubing (6,69). These catheters were filled with water and each connected to a Statham pressure transducer. Balloons were thickly coated with 2% xylocaine before being inserted into the upper alimentary tract. As shown in Fig. 1, to record the pharyngeal swallowing response (P), one of the balloons was placed in the oral cavity, between the palate and the dorsal aspect of the posterior tongue. To record oesophageal responses, two balloons were inserted into the oesophagus, one in the cervical oesophagus (CE), placed at a position just below the pharyngo-oesophageal junction and one in the distal oesophagus, positioned at the level of the diaphragm. Correct placement of the balloons was ascertained by evoking reflex swallowing by intratracheal tactile stimulation, a manoeuvre empirically used in this laboratory, although the neural mechanism subserving this reflex remains unclear. Corresponding intraluminal pressures were recorded on a 6-channel-recorder (Grass polygraph). Respiratory rate was derived from tidal pressure fluctuations recorded by an air-filled catheter inserted into the tracheal cannula. Rectal temperature was maintained between 37.5-38°C by means of radiant heat.

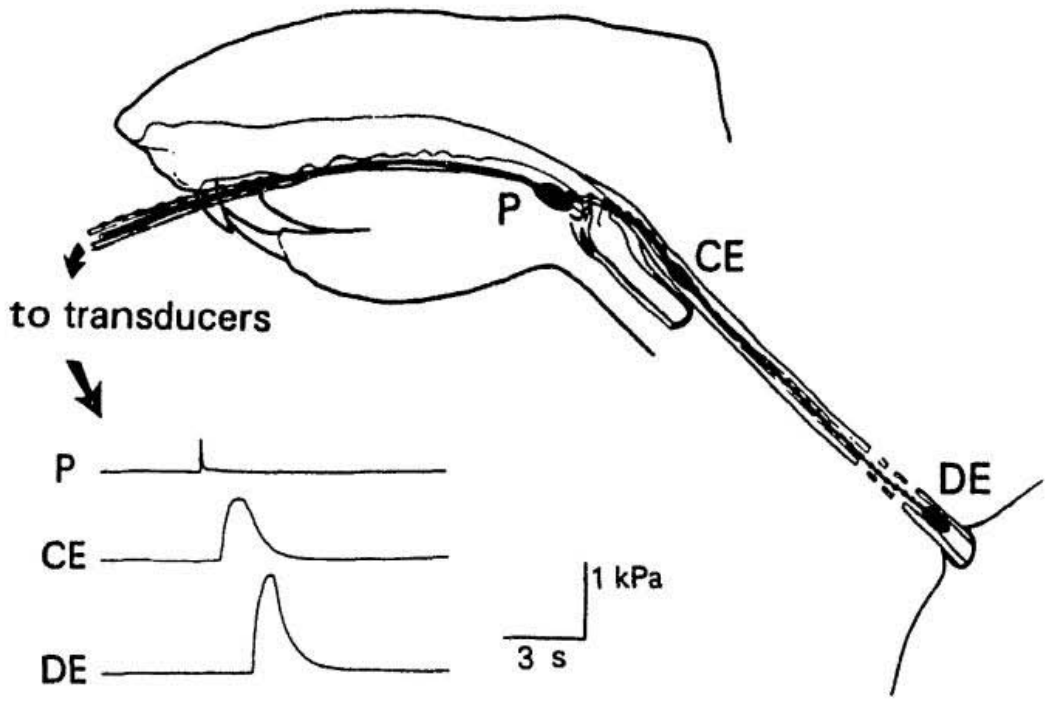


Figure 1. Diagram showing methods used to record both spontaneous and evoked swallowing and oesophageal responses. The anaesthetized rat is held in a stereotaxic frame. Three water-filled balloons connected to pressure transducers are inserted into the pharynx (P), the cervical oesophagus (CE) and distal oesophagus (DE), respectively. Corresponding intraluminal pressures are recorded on a polygraph.

The caudal roof of the fourth ventricle and the surrounding structures of the dorsal medulla were exposed through a suboccipital craniectomy and incision of the atlanto-occipital membrane. Cerebrospinal fluid was drained continuously with a wick.

For electrical stimulation of the NTS, an insulated stainless steel monopolar electrode with an exposed tip length of less than 0.3 mm was used. The electrode was placed in the NTS with reference to previously determined coordinates and landmarks on the dorsal medullary surface under visual control through an operating stereomicroscope (6). Stimuli consisted of repetitive square wave pulses of 0.3 ms duration, 1-8 Hz frequency and 1-3 V amplitude.

For chemical stimulation of swallowing loci in the NTS, both topical and micropneumophoretic application of drugs were used.

For topical application of drugs, aqueous drug solutions were ejected onto the dorsal extraventricular surface of the NTS from a microliter syringe fitted with a thin flexible polyethylene catheter on the needle tip. The volume applied was kept within 0.2 μl, in order to minimize drug spread to the contralateral NTS.

For the pneumophoretic application of drugs, 4-barrel glass pipettes (tip o.d. 2-5 μm) were positioned in the NTS by a 3-dimensional hydraulic micromanipulator (Narishige MO-203). Drugs were ejected by means of a nitrogen-pressured 'Picospritzer' pneumophoresis pump (General Valve Corporation). The volume ejected with each pressure pulse was determined by measuring the diameter of the ejected droplet with a microscope and kept in the range of 20-100 pl.

Swallowing loci were located by glutamate pulses (6-8 pmol) delivered from one

barrel of the pipette. This protocol permits accurate localization of swallowing substrates associated with both the NTS and AMB (6,69,70). With respect to NTS loci, extensive characterization of stimulation loci within the nucleus has previously been carried out in this laboratory (6,69-71) and has established that: (i) the buccopharyngeal and oesophageal sites are coextensive with the NTS subnuclei intermedialis and centralis, respectively and (ii) with the S-glutamate concentration, ejectate volumes and pipette tip diameters used, the elicitation of short-latency pharyngeal or oesophageal responses requires the tip to be within 50 μm of these subnuclei. Furthermore, since the NTS subnuclei lie less than 500 μm below the NTS dorsal surface, they are also accessible for drugs applied to the extraventricular NTS surface.

Drugs were purchased from the following sources: DL-2-amino-7-phosphonoheptanoic acid (AP-7), (-)-bicuculline methiodide, sodium s-glutamate, kainic acid (kainate), methscopolamine bromide, DL-muscarine hydrochloride, muscimol, noradrenaline and picrotoxin (Sigma); cis-2-methyl-dimethylaminomethyl-1,3-dioxolane methiodide (CD; Research Biochemicals Inc.).

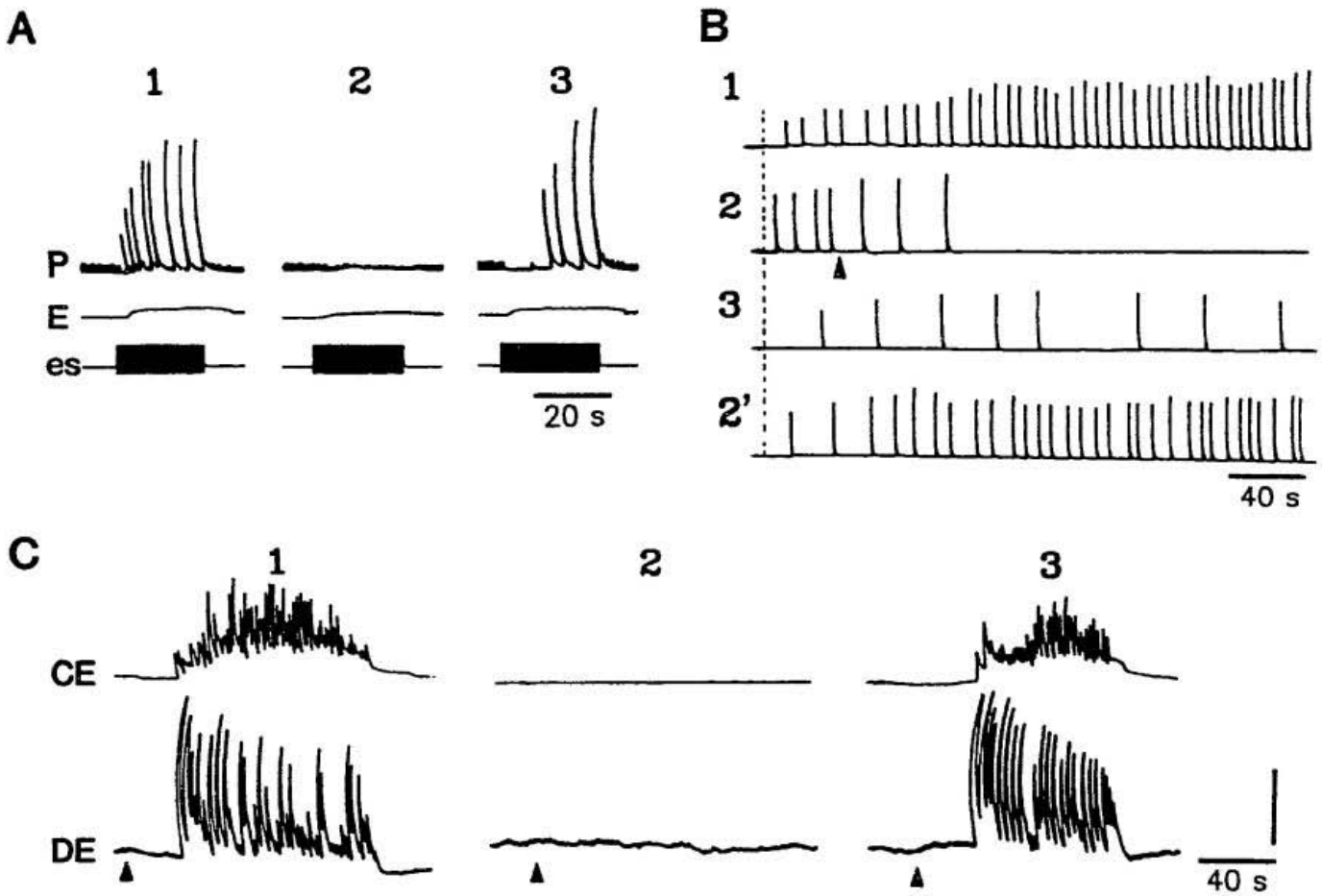
2.3 Results:

2.3.1 *Muscimol*

Repetitive swallowing was induced by electrical stimulation of the NTS at the level of the fovea inferior (n=4). Responses consisted of a buccopharyngeal component which attained rates between 10-50/min at stimulation frequencies of 1.5-6 Hz and intensities of 1-3 V. The deglutitive response was reversibly abolished by ipsilateral application of muscimol (0.1 nmol), a $GABA_A$ receptor agonist, onto the extraventricular surface of the NTS. This inhibitory effect had a rapid onset, i.e. developed in less than 1 min, and lasted from 30 min to 1 h. However, in one experiment recovery was incomplete even after more than 2 h. The inhibition could be partially overcome by a large dose of bicuculline (2 nmol) applied at the same site (Fig. 2A).

The question of whether muscimol exerted a differential action on the buccopharyngeal and the oesophageal stage of swallowing was investigated next. In order to dissociate the two stages, either kainate (1 nmol) or the muscarinic cholinoceptor agonists, muscarine (0.1 nmol) or a synthetic analogue, CD (0.1 nmol), were applied unilaterally to the extraventricular surface of the NTS. In keeping with previous work, kainate (n=8) produced pure repetitive buccopharyngeal responses (Fig. 2B), whereas muscarine or CD (n=5) elicited rhythmic peristalsis-like oesophageal responses(Fig. 2C). Both types of evoked deglutitive responses were effectively inhibited by muscimol (0.01-2 nmol) applied at the same site (Fig. 2B and 2C). In the kainate group, complete ipsilateral inhibition was evident at all muscimol doses tested (0.01 nmol, n=2; 0.1 nmol, n=4; 2 nmol, n=2). Contralateral inhibition was obtained in one of the 4 animals

Figure 2. Muscimol inhibits swallowing activity evoked from the solitary complex (NTS). In this and the following figures, traces represent intraluminal pressure within the pharynx (P), the oesophagus (E), the cervical oesophagus (CE), the distal oesophagus (DE), and electrical stimulation of the NTS (es). **A:** Pharyngeal deglutitive response to electrical stimulation (3 Hz, 0.3 ms and 3 V) of the intermediate NTS is reversibly inhibited by muscimol 0.1 nmol, applied to surface of ipsilateral NTS. (1) Control; (2) 5 min after muscimol; (3) partial reversal by bicuculline 2 nmol, applied to the same site 20 min after muscimol. **B:** Pharyngeal fictive swallowing elicited by kainate 1 nmol applied to dorsal surface of the NTS at dashed line is inhibited by muscimol. (1) control response; (2) inhibitory effect of muscimol 0.1 nmol applied at same site as in A (triangle); (3) kainate effect partially recovered 20 min after muscimol application; (2') muscimol fails to abolish kainate response elicited from the contralateral NTS (6 min after muscimol application). **C:** Oesophageal response elicited by muscarine 0.1 nmol applied to NTS surface is inhibited by muscimol. (1) control response; (2) response 5 min after muscimol 0.1 nmol applied at same site; (3) recovery of muscarine effect 1 h after muscimol application. Calibration: vertical bar represents 2.6 and 1.3 kPa for pharyngeal and oesophageal traces, respectively.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treated with muscimol 0.1 nmol and in both animals treated with the highest dose. Similarly, in the muscarine group, complete ipsilateral inhibition was present after muscimol 0.1 nmol (n=3) and bilateral inhibition after muscimol 2 nmol (n=2). This inhibition was reversible and followed a time course similar to that described above. Besides its deglutitive effects, kainate applied to the NTS surface induced respiratory stimulation, indicated by an increase in both volume and rate of breathing. Interestingly, following application of muscimol to the ipsilateral NTS, kainate gave rise to a marked respiratory inhibition, both volume and rate being decreased.

To test the possibility that muscimol inhibited swallowing by diffusing to GABA receptors in structures remote from the NTS, muscimol 0.1 nmol was applied in two animals to the laterodorsal surface of the spinal trigeminal tract, about 2.5 mm lateral to the midline at the level of the fovea inferior. Kainate, applied to the surface of the NTS within 2 min following muscimol application, elicited a strong swallowing response, not different from that of controls. However, when the same amount of muscimol was then applied to the dorsal surface of the NTS in these two animals, the kainate-evoked response was abolished.

2.3.2 *$GABA_A$ antagonists*

The above results suggest that activation of $GABA_A$ receptors in the NTS is capable of inhibiting swallowing and oesophageal activity. The role of these $GABA_A$ receptors in swallowing was next investigated by applying antagonists.

i. **Topical application of bicuculline and picrotoxin:** Unilateral application of bicuculline to the NTS surface in doses ranging between 5 and 200 pmol resulted in

vigorous deglutitive activity, which started within 1 to 2 min after application, peaked within 2 to 4 min and lasted for approximately 5 to 20 min. This response was dose-dependent in terms of latency, duration and number of evoked swallows (Table I). Unlike other deglutitive excitants such as kainate (70) and serotoninergic (69) agonists, which predominantly produce buccopharyngeal activity, bicuculline invariably gave rise to repetitive complete swallows (Fig. 3), i.e. buccopharyngeal pressure waves were followed by propulsive oesophageal contractions. This swallowing effect persisted when submandibular salivary glands had been extirpated. Moreover, increased salivation was not observed following applications of bicuculline in intact animals. After cutting the IXth and Xth nerves bilaterally close to their exit from the skull, deglutitive stimulation by bicuculline was still evident (n=2). Thus, phasic wave activity, albeit of reduced amplitude, persisted with a similar rhythm in the posterior oral cavity. Applications of bicuculline (5-200 pmol) to the laterodorsal surface of the medulla failed to elicit any deglutitive effect (n=2).

Picrotoxin (50-500 pmol, n=4), a blocker of the $GABA_A$ receptor Cl^- channel (14,175), mimicked bicuculline in eliciting a pharyngo-oesophageal response when applied to the surface of the NTS (data not illustrated).

ii. **Microinjections of bicuculline into NTS deglutitive loci**: Pressure ejection of glutamate (10-20 pmol) at pharyngeal sites, corresponding to the region of the subnucleus ventralis and intermedialis of the NTS, yielded a pharyngeal pressure wave at short latency. Prepulses of bicuculline (0.05-0.1 pmol) applied at these sites via an adjacent barrel of the pipette, facilitated the glutamate response as evidenced by an increase in

Table I. Dose-effect relationship of deglutitive responses evoked by topical application of bicuculline to the solitary complex of the rat*

Dose (pmol)	Latency (s)†	Duration (s)‡	Swallows (No.)§
1000 (4)	43 ± 6	765 ± 86	157 ± 42
100-200 (8)	42 ± 4	578 ± 45	105 ± 13
50 (8)	61 ± 6	353 ± 24	56 ± 13
5-10 (5)	73 ± 3	298 ± 33	20 ± 3

*: Data were obtained from pharyngeal and esophageal pressure waves. Bicuculline was applied unilaterally to the extraventricular surface of the solitary complex (NTS) in a volume of 0.05-0.2 μl. Numbers of experiments are shown in parentheses. All numerical values were expressed as mean ± SE.

†: Latency corresponds to the interval between drug application and the appearance of the first pharyngeal pressure wave.

‡: Duration, the interval between start of the first and the last pharyngeal pressure waves.

§: Number of swallows, the total number of pharyngeal pressure waves produced by a single dose of bicuculline.

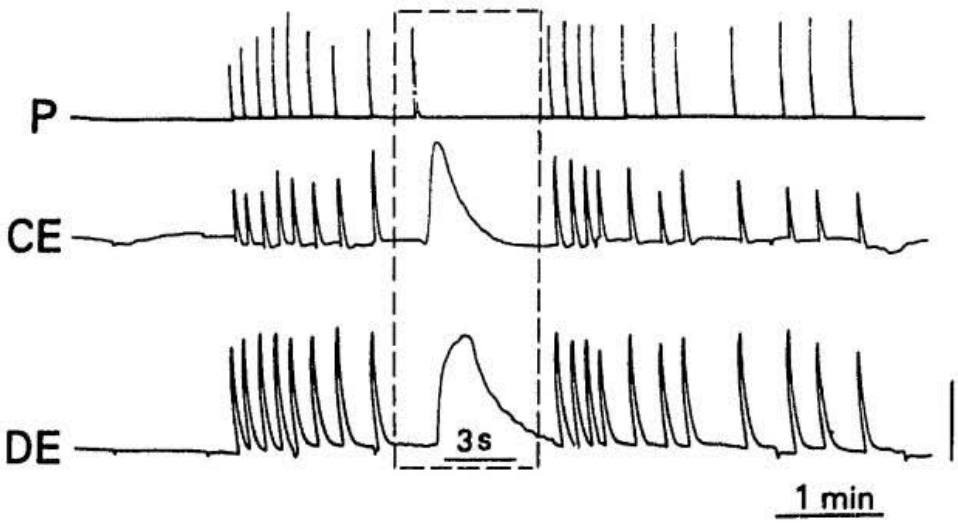


Figure 3. Repetitive pharyngo-oesophageal swallowing response to bicuculline. Topical application of bicuculline to the dorsal surface of the left side NTS evokes a repetitive swallowing response consisting of buccopharyngeal and oesophageal components coupled in a 1:1 ratio. The inset shows, at high chart speed, the sequential progression of pharyngo-oesophageal pressure waves. Vertical bar represents 1.3 and 0.6 kPa for pharyngeal and oesophageal traces, respectively.

amplitude and number of swallows (Fig. 4A). At higher doses (0.5-1 pmol), bicuculline on its own evoked either pure pharyngeal (n=4), or repetitive complete swallows (Fig. 4B, n=5). At oesophageal sites, corresponding to the subnucleus centralis of the NTS, pulses of glutamate (10-20 pmol) gave rise to monophasic oesophageal contractions. Prepulses of bicuculline 0.05-0.2 pmol not only enhanced the amplitude of the glutamate response, but also converted the monophasic pressure wave into rhythmic repetitive activity (Fig. 5, n=6). At the highest dose tested (1 pmol), bicuculline produced rhythmic peristalsis-like response on its own (Fig. 5, n=5).

2.3.3 *Bicuculline and pharyngo-oesophageal coupling*

As described above, bicuculline, unlike kainate, gave rise to complete deglutitive sequences. These results invited examination of the mechanism responsible for coupling pharyngeal and oesophageal stages. In these experiments, repetitive buccopharyngeal swallowing was induced by kainate (1 nmol, n=4) and noradrenaline (1 nmol, n=2) applied unilaterally to the extraventricular surface of the NTS. Subthreshold pulses of bicuculline (0.1-0.5 pmol) were then pneumophoresed at glutamate-responsive oesophageal loci in the ipsilateral NTS. As shown in Fig. 6, the GABA antagonist not only acutely enhanced the kainate or noradrenaline evoked buccopharyngeal swallowing activity, but also caused the appearance of coupled propulsive oesophageal contractions. The number of evoked peristaltic waves ranged from 4 to 16, varying between individual trials and animals (6.7 ± 0.7; 20 trials in 6 animals). This effect could be repeated up to 4 times without obvious loss of effect in each individual trial of kainate or noradrenaline.

The 'coupling' effect of bicuculline was also observed in rats subjected to

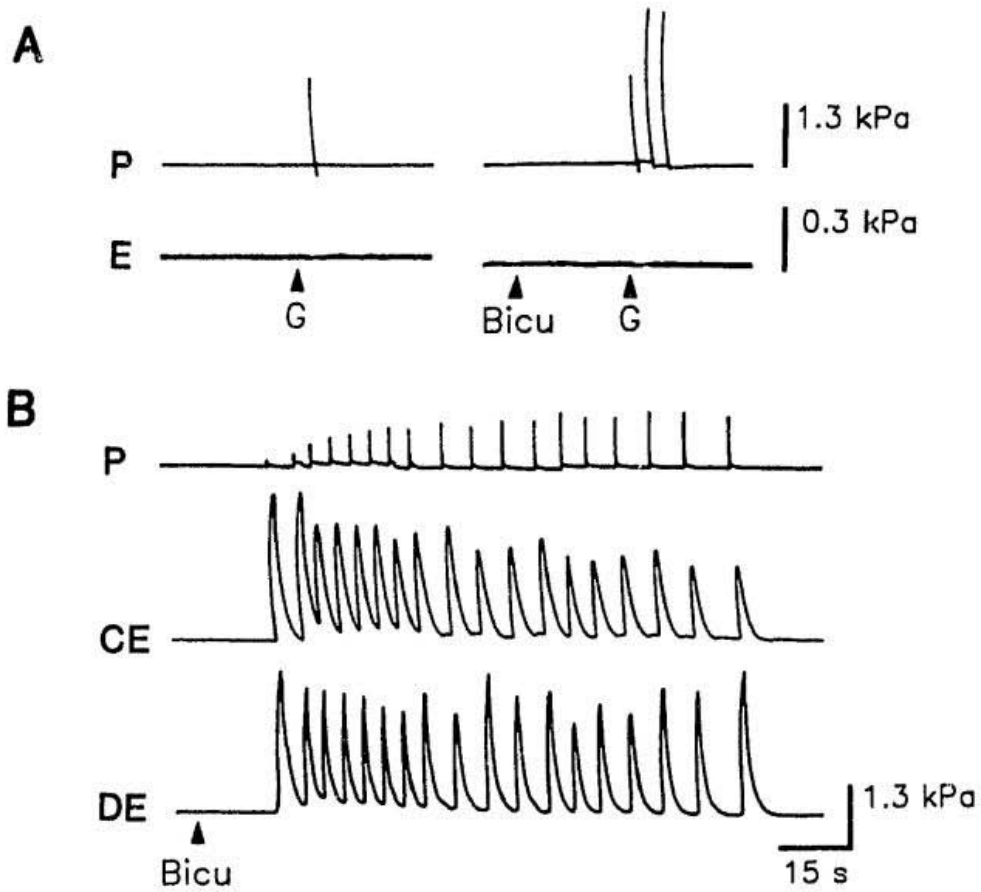


Figure 4. Effects of bicuculline at a pharyngeal site in the solitary complex. **A.** Facilitation of glutamate response by bicuculline. **Left:** Control pharyngeal response to glutamate (G) 15 pmol ejected into the intermediate subnucleus of the NTS. **Right:** Facilitation of the glutamate response by a pulse of bicuculline (0.1 pmol) 15 s prior to glutamate from an adjacent barrel of the pipette. **B.** Bicuculline at a larger dose (0.5 pmol), ejected at same site evokes repetitive complete swallows.

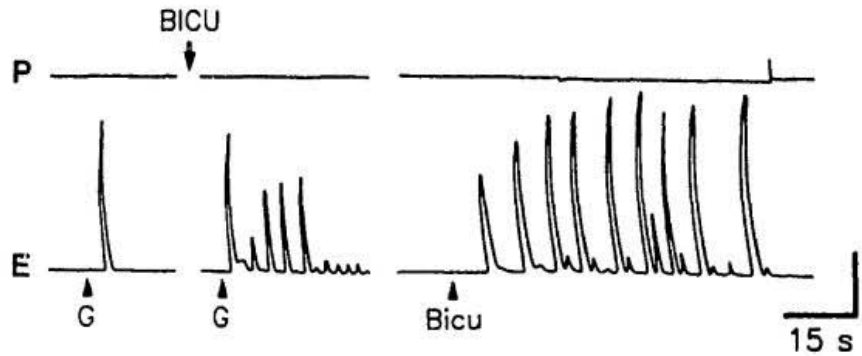


Figure 5. Effects of bicuculline at an oesophageal site in the solitary complex. **Left:** control response elicited by a pulse of glutamate (12 pmol) ejected in the central subnucleus of the NTS. **Middle:** Facilitation of the glutamate response 20 s after a pulse of bicuculline (0.05 pmol) from an adjacent barrel of the pipette. **Right:** Rhythmic peristalsis evoked by a larger dose of bicuculline (1 pmol) ejected at same site. Vertical bar represents 2.6 and 0.6 kPa for pharyngeal and oesophageal traces, respectively.

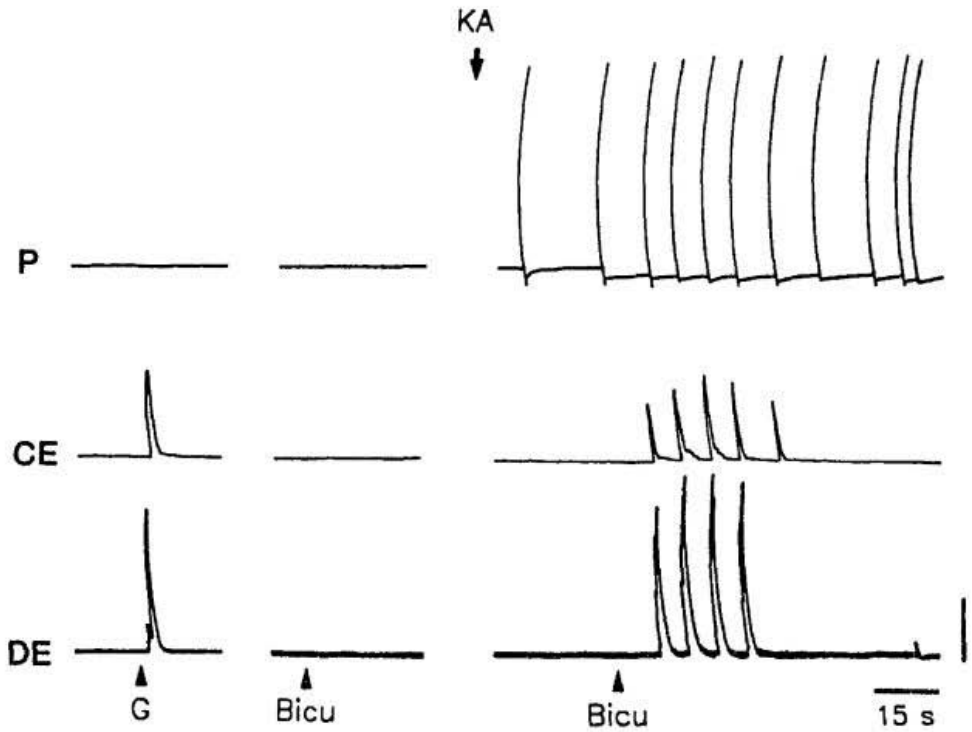


Figure 6. Coupling effect of bicuculline pressure-ejected at an oesophageal site in the NTS_c. **Left:** an oesophageal site located by a pulse of glutamate (12 pmol), where it produces propulsive oesophageal contractions. **Middle:** Lack of effect of subthreshold dose of bicuculline (0.2 pmol) ejected from an adjacent barrel of the pipette. **Right:** Same dose of bicuculline ejected at same site produces coupled oesophageal response, i.e. deglutitive (primary) peristalsis, during ongoing pharyngeal swallows evoked by kainic acid (KA) applied to the dorsal surface of the ipsilateral NTS. Note that the last cervical pressure wave fails to progress to the distal oesophagus, indicating that the injection site is a cervical oesophageal locus. Vertical bar represents 1.3 and 0.6 kPa for pharyngeal and oesophageal traces, respectively.

electrical stimulation of the NTS (n=4, Fig. 7). As described above, electrical stimulation of the NTS, with appropriate parameters, produced repetitive buccopharyngeal swallows unaccompanied by oesophageal activity. Prior application of subthreshold doses of bicuculline (0.5-1 pmol) to the extraventricular surface of the stimulated NTS augmented the swallowing response evoked by electrical stimulation. Thus, not only was there increased pharyngeal swallowing activity, but oesophageal activity coupled in a 1:1 ratio with pharyngeal swallowing, provided the rate of pharyngeal swallowing did not exceed 10 to 12 per min. Compared with bicuculline, kainate (0.05 nmol) applied at the same site also enhanced the pharyngeal swallowing evoked by electrical stimulation of NTS, but did not yield oesophageal activity (Fig. 7).

2.3.4 *Bicuculline induced primary peristalsis - relation to NMDA and muscarinic receptor activation*

As both muscarinic cholinoceptors and NMDA receptors have been implicated in oesophagomotor control at the level of the NTS, experiments were next undertaken to determine the involvement of these receptors in the production of primary oesophageal peristalsis induced by topical application of bicuculline (5 pmol) to the dorsal surface of the NTS. As shown in Fig. 8, swallowing responses evoked by bicuculline were fully and reversibly inhibited by AP-7, a NMDA receptor antagonist, applied at the same site (n=6). This blockade lasted 10-20 min and was dose dependent within the range of 5 to 500 pmol. However, a preferential effect of AP-7 on the oesophageal component was not evident even at the lower dose tested, at which two or three pharyngo-oesophageal swallows persisted. Application of the muscarinic cholinoceptor antagonist,

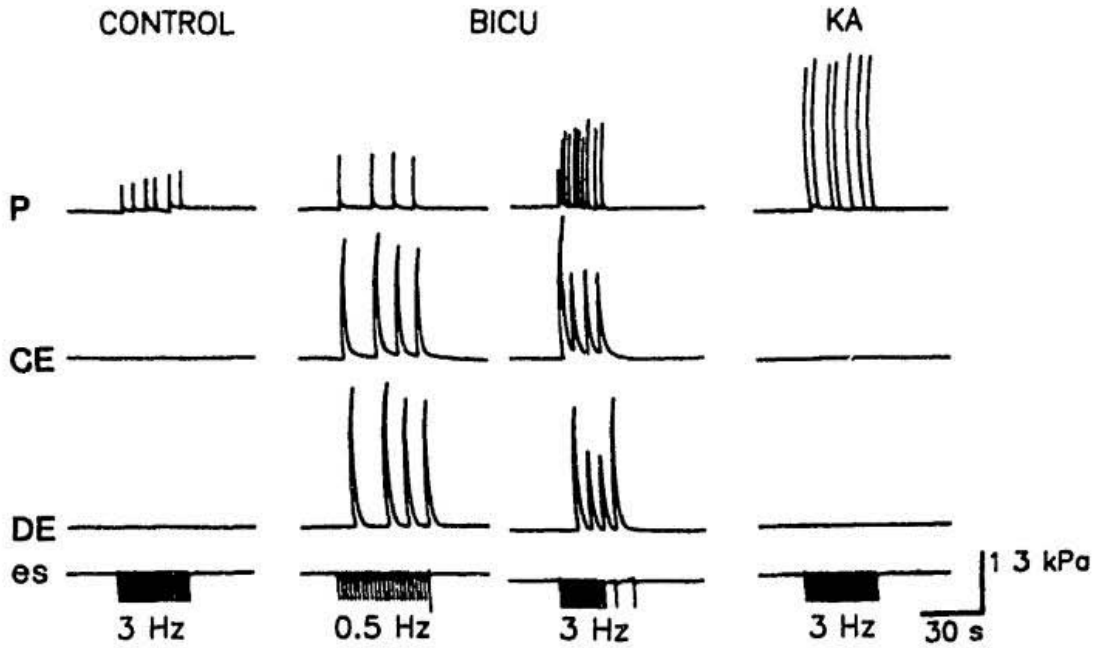


Figure 7. Effect of bicuculline applied to the dorsal surface of the solitary complex on pharyngo-esophageal coupling. Panels show, from left to right, the control pharyngeal response to electrical stimulation (0.3 ms, 3 V) of left NTS; coupled pharyngo-oesophageal response following application of bicuculline 1 pmol to the dorsal surface of the ipsilateral NTS (two middle panels) and lack of coupling effect of kainate (KA) 0.2 nmol applied at the same site (right). Note the facilitation of pharyngeal activity by both bicuculline and kainate.

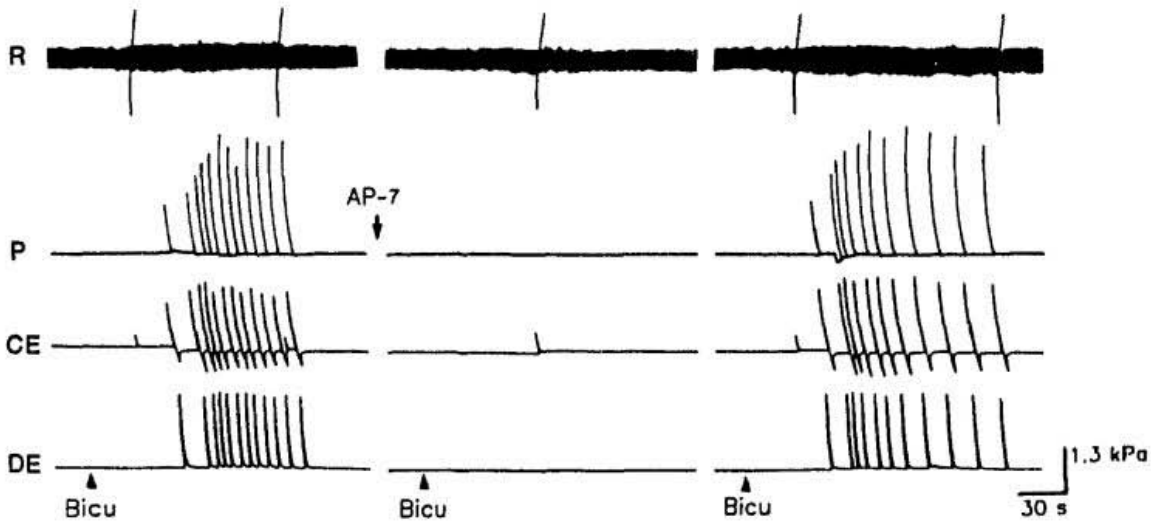


Figure 8. AP-7 blocks repetitive pharyngo-oesophageal swallowing response evoked by bicuculline. **Left:** Control response, evoked by bicuculline (Bicu. 5 pmol) applied to the extraventricular surface of the NTS. **Middle:** Complete blockade of the response by AP-7 (5 pmol, bold arrow) applied to the same site 1 min prior to bicuculline. **Right:** Partial recovery 15 min later. Note the weak effect of bicuculline on respiration (top trace R).

methscopolamine (5-50 pmol), selectively abolished the oesophageal component of the bicuculline-evoked response, whereas the pharyngeal component persisted (Fig. 9, n=6). Blockade of the oesophageal component was restricted to the ipsilateral medulla. Recovery of the response was not evident during a 2 h observation period and was not studied further.

2.3.5 *Muscarinic antagonists and fictive secondary oesophageal peristalsis*

As shown in Fig. 5, bicuculline, when injected within the NTS_c at a large dose, evoked a rhythmic oesophageal response. The question thus arose as to whether this bicuculline evoked response was also susceptible to inhibition by muscarinic antagonists. As illustrated in Fig. 10, ejection of bicuculline (1 pmol), at glutamate-responsive loci within the NTS_c, gave rise to rhythmic, multiple-wave activity (see also Fig. 5). This activity was not regularly associated with buccopharyngeal activity. Topical application of methscopolamine 100 pmol to the extraventricular surface of the ipsilateral NTS completely blocked the bicuculline-evoked fictive secondary oesophageal response (n=2). No recovery was obtained over a 4 h observation period following applications of methscopolamine in these two experiments.

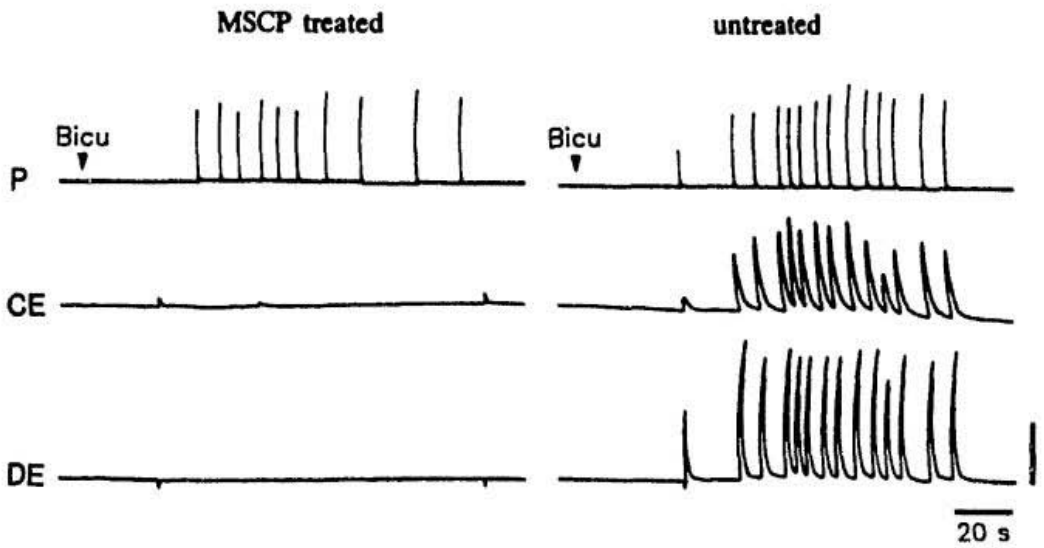


Figure 9. Effect of muscarinic receptor blockade on bicuculline-evoked primary oesophageal peristalsis. **Left:** Oesophageal activity evoked by bicuculline (5 pmol) applied to the extraventricular surface of the left NTS is blocked by methscopolamine (MSCP, 10 pmol), applied to the same site 2 min prior to bicuculline. The blockade persists for longer than 2 hours (not illustrated). **Right:** Lack of blocking effect on deglutitive oesophageal peristalsis evoked by same dose of bicuculline applied to contralateral NTS surface 20 min after MSCP application on the left side. Calibration: vertical bar represents 3 and 1.3 kPa for pharyngeal and oesophageal traces, respectively.

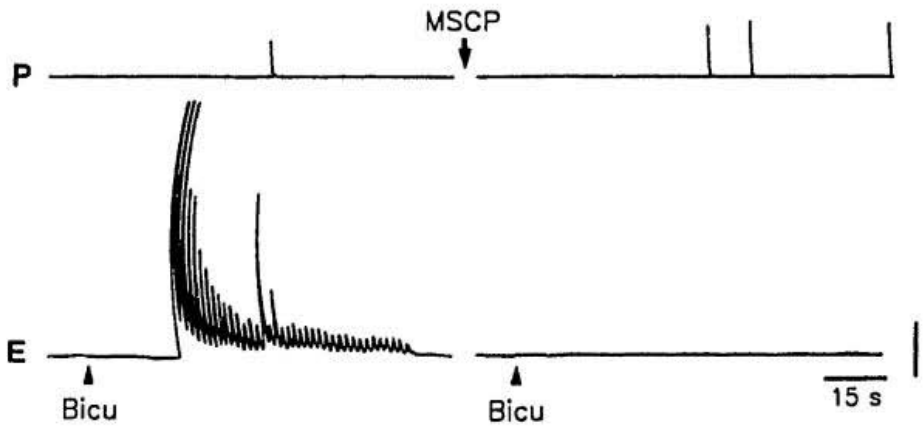


Figure 10. Effect of muscarinic receptor blockade on bicuculline-evoked fictive secondary peristalsis. Bicuculline 1 pmol pressure-ejected at an oesophageal site within the NTS_c elicits oesophageal peristalsis independent of buccopharyngeal swallowing (left) which is blocked by methscopolamine 100 pmol applied to the dorsal surface of the ipsilateral NTS (right). Calibrations: Vertical bar represents 2.6 and 0.6 kPa for pharyngeal and oesophageal traces, respectively.

2.4 Discussion:

The present study demonstrates the importance of GABAergic mechanisms in the premotoneuronal control of oesophageal peristalsis in particular and swallowing in general. These results prompt several conclusions: i) a $GABA_A$ receptor-mediated inhibitory mechanism in the NTS maintains the NPG for swallowing and oesophageal peristaltic activity in a quiescent state; ii) release from this inhibition results in rhythmic swallowing and/or oesophageal peristalsis; iii) GABAergic inhibition operates to counteract excitatory inputs acting at NMDA and muscarinic receptors on pharyngeal and oesophageal premotoneurons, respectively; iv) a $GABA_A$-ergic mechanism is involved in inhibiting coupling between the two stages of the swallowing, and could, therefore, in part contribute to deglutitive inhibition of the oesophagus; v) bicuculline can be used as a versatile pharmacological tool for studies on swallowing and oesophageal peristalsis.

2.4.1 *$GABA_A$ receptor-mediated mechanisms in swallowing*

The present work provides strong evidence that GABAergic inhibition is an intrinsic component of deglutitive control. Specifically, the present findings implicate $GABA_A$ receptors, since centrally-evoked deglutitive responses were reversibly inhibited by muscimol, a $GABA_A$ receptor agonist (14,175), whereas both bicuculline, a $GABA_A$ receptor antagonist (14,32,33,61) and picrotoxin, a blocker of $GABA_A$ Cl^- channel (14,175), elicited complete swallowing responses consisting of a buccopharyngeal and oesophageal stage.

Bicuculline was first shown to have potent and selective antagonistic effects on GABA-mediated postsynaptic responses in the spinal cord of the cat by Curtis and his

colleagues (32,33). Since then, this agent has been widely accepted and used as a selective $GABA_A$ receptor antagonist (14,56,152,175,176). However, other effects of bicuculline have been observed that are apparently unrelated to GABA receptors. In particular, inhibition of acetylcholinesterase (152) seems relevant, in light of the stimulant effects on swallowing of acetylcholinesterase inhibition revealed in previous work (6). However, it is unlikely that the deglutitive effects of bicuculline observed in this study resulted from its anticholinesterase activity instead of blockade of GABA receptors. First, the stimulating effects of the cholinesterase inhibitor, eserine, are restricted to the oesophageal stage and the increased oesophageal activity appears to be associated with an inhibition of pharyngeal activity (6). However, bicuculline displayed similar potency in activating both stages of swallowing, as demonstrated by robust pharyngo-oesophageal responses. Second, picrotoxin, a blocker of the GABA receptor-gated chloride channel (14,175), produced similar deglutitive stimulation.

Another pertinent property of bicuculline is its convulsant activity (152). Along with other convulsants, such as picrotoxin, bicuculline could exert wide-spread stimulant actions on other medullary functions, including respiration, emesis, and salivation. The latter might secondarily (reflexly) stimulate swallowing (8,100). However, this possibility can also be discarded since: (i) the swallowing effects of bicuculline were not impaired by extirpation of salivary glands and (ii) intact animals failed to show increased salivation following application of bicuculline. Hence, the stimulating effects on swallowing by bicuculline observed in the present work are most likely due to inhibition of $GABA_A$ synapses on deglutitive interneurons.

The discrete localization of solitary sites responsive to bicuculline corroborates the close anatomical association of the NPG for swallowing with the solitary complex (7,21,70,96,140). Furthermore, it supports the idea that endogenous GABA release within the solitarial complex exerts a powerful, tonic inhibition of premotor elements forming part of the NPG. Pharmacological blockade of this inhibition appears to be sufficient to trigger oscillatory activity of the NPG i.e. rhythmic fictive swallowing and oesophageal peristalsis. At present, it is difficult to pinpoint the precise anatomical substrate of GABAergic inhibition of the NPG. The effects of muscimol and bicuculline on swallowing were evident only when drugs were applied onto the surface of the NTS or ejected into swallowing loci within NTS. Moreover, GABA neurons are present within NTS (99,123). Accordingly, one may reasonably suppose that local GABA neurons within NTS, especially those within or nearby swallowing loci, are the source of this GABAergic inhibition. However, involvement of extrasolitary GABAergic inputs to the NTS cannot be ruled out at this time. Clearly, the synaptic organization of GABAergic afferents concerned in deglutitive functions requires further investigation.

2.4.2 *GABAergic interactions with NMDA- and a muscarinic-mediated excitatory processes*

It is thought that the medullary NPG for swallowing comprises at least two subcircuits governing the buccopharyngeal and oesophageal stages (6,70,96), which have distinct neuroanatomical and pharmacological substrates and are capable of functioning independently (see below). Ejections of bicuculline into the subnucleus intermedialis and subnucleus centralis of the NTS gave rise to isolated buccopharyngeal and oesophageal

responses, respectively. Tonic GABAergic inhibition of the NPG therefore appears to be directed separately to each stage.

Among other putative excitatory transmitters, EAA have received increasing attention. Both NMDA and non-NMDA- receptor mediated swallowing responses have recently been described (70,71,104,176). The enhancement of glutamate-evoked responses by bicuculline points to an interaction between EAA- and GABA-mediated mechanisms in the generation of swallowing activity. Arguably, both excitatory and inhibitory processes may be required to generate patterned, oscillatory neuronal activity. In particular, this may apply to pharyngeal premotor control, as evidenced by the effectiveness of AP-7 in blocking bicuculline-evoked responses. This antagonist blocks NMDA-, but not glutamate-evoked buccopharyngeal responses or cholinoceptor-mediated oesophageal activity (70). Thus, inhibition by AP-7 of bicuculline-induced swallowing can safely be attributed to antagonism at the NMDA receptor. These results strengthen the view that both excitatory, NMDA receptor-mediated, and inhibitory processes are critical elements in motor pattern generation (67). The techniques used in the present study do not permit further insight into the precise manner in which these two proposed mechanisms interact at the cellular level. If elements of the pharyngeal NPG in the solitarius complex were to receive convergent input via $GABA_A$ and NMDA receptors, the GABAergic input would normally predominate so as to keep the NPG silent. Removal of inhibition by blocking $GABA_A$ receptors, as demonstrated by the present work, or surmounting it by applying exogenous glutamate or NMDA agonists (6,70,104), would permit oscillation of the NPG.

The present finding that NMDA receptors play a pivotal role in the bicuculline effect is intriguing because questions have been raised as to whether these receptors participate in fast synaptic information transfer, given the voltage-dependent blockade by Mg^{2+} of the NMDA receptor/channel complex (186). However, there is support for the contribution of NMDA receptors to the generation of excitatory postsynaptic potentials both *in vivo* and *in vitro* (186). The present work adds weight to the growing body of evidence for activation of NMDA receptors in synaptic transmission under physiological conditions and points to a primary role of these receptors in excitatory synaptic transmission at NTS deglutitive interneurons.

Studies on the locomotor generator in the lamprey suggest that NMDA receptor activation generates pacemaker-like activity (67). The basis of such NMDA-activated rhythmic activity is the voltage-dependent blockade of the channel by Mg^{2+} and the permissive entry of Ca^{2+} through the channel. A similar mechanism may form the basis of rhythmic oscillation leading to the buccopharyngeal swallowing as suggested by the AP-7 data. This hypothesis is corroborated by recent experiments performed on NTS slices which demonstrate that application of NMDA induces rhythmic bursting activity similar to the rhythmic swallowing activity of the NTS neurons recorded *in vivo* (182,183). Although non-NMDA excitatory amino acid receptors are also present on deglutitive premotoneurons (70,104) they do not appear to play a critical role in the bicuculline effect. Work in progress in this laboratory suggests that bicuculline responses are relatively resistant to 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), a selective blocker of kainate and quisqualate receptors (80). On the other hand, it has been

suggested that among EAA receptors, it is the kainate receptor that predominates within the NTS subnuclei associated with the buccopharyngeal stage of swallowing (70). Thus, further study is needed to determine the physiological relevance of this subtype of EAA receptor in generating the buccopharyngeal stage of swallowing.

The oesophageal stage in the rat depends on activation of a muscarinic cholinoceptor link (6). Because fictive secondary peristalsis could be evoked by injection into the NTS_c of bicuculline and since this bicuculline-evoked response was fully blocked by methscopolamine, a muscarinic cholinoceptor antagonist, pharyngeal and oesophageal sub-NPGs probably share a common inhibitory mechanism. However, the oesophageal NPG is probably primed by activation of a muscarinic rather than an NMDA receptor. Like the NMDA receptor, activation of neuronal muscarinic receptors is capable of activating rhythmic bursting in central pattern generators, for instance the one involved in generating the pyloric rhythm in lobsters (126,127).

The mechanism by which GABAergic and cholinergic inputs interact remains to be determined. One possibility is that GABA input may impinge on cholinoceptive neurons within the NTS_c rather than on cholinergic cell bodies. In this case, GABA, by reducing the excitability of the postsynaptic elements, would act to decrease their responsiveness to ACh input. Alternatively, GABA may inhibit ACh input directly via a presynaptic mechanism. GABA has long been known to depolarize primary afferent terminals in the dorsal horn of the spinal cord, where it plays a major role in presynaptic inhibition (33,117). A similar $GABA_A$-mediated presynaptic inhibition of peripheral afferent input to the NTS has also been suggested (57,144). Particularly interesting in

this regard is that oesophageal vagal afferents may be cholinergic (154,185). Whether GABAergic axons synapse with these oesophageal cholinergic afferent terminals within the NTS_c remains to be determined.

2.4.3 *GABAergic coordination of two swallowing stages*

As discussed above, pharyngeal and oesophageal NPGs are neuroanatomically and functionally distinct. The nature of the central mechanism coordinating these two stages has been a matter of debate. Some investigators have proposed that oesophageal motor activity occurs primarily in response to distension; in other words, the coordination between the two stages is primarily dependent on peripheral sensory input rather than on a central mechanism. Specifically, oesophageal peristalsis following a pharyngeal swallow is explained as being due to oesophageal distension secondary to the passive opening of the upper oesophageal sphincter (30). One argument in support of this concept is the lack of demonstrable neuroanatomical connections between the two NPGs at the premotoneuron level (30). However, the present results militate against this view. Specifically, application of kainic acid or noradrenaline to the NTS surface elicited vigorous pharyngeal swallowing not progressing to the oesophageal stage; however, the concurrent application of subthreshold doses of bicuculline, delivered to the NTS subnucleus centralis during an ongoing buccopharyngeal swallowing response, was effective in triggering coupled oesophageal peristalsis. These data therefore provide strong support for the existence of an intrinsic central mechanism coordinating the two NPGs, i.e. a "coupling" mechanism, through which the rhythm generated by the pharyngeal NPG controls that of the oesophageal NPG with the appropriate phase delay,

enabling the full deglutitive motor program to be executed.

This coupling mechanism presumably involves two parallel connections linking the pharyngeal and oesophageal NPGs. One connection operates via $GABA_A$-mediated inhibition, whereas the other operates via muscarinic cholinoceptor-mediated excitation, as evidenced by the effectiveness of bicuculline in eliciting primary oesophageal peristalsis and the blockade of this effect by methscopolamine. The location of the propriobulbar cholinergic neurons responsible for the excitatory muscarinic link remains to be determined. However, it is reasonable to propose that these neurons receive their inputs from the buccopharyngeal NPG and in turn project to the oesophageal NPG, ensuring that the buccopharyngeal progresses to the oesophageal stage. The inhibitory link would normally ensure functional independence of the two stages by counteracting the muscarinic cholinoceptor-mediated coupling process. At present it is not clear whether the GABA neurons in question are associated with the NTS_i, the NTS_c, or both. However, it may be reasonably hypothesized that the GABA neurons, like the cholinergic neurons, receive their excitatory inputs from the buccopharyngeal premotoneurons in the NTS_i and project to the NTS_c, where they counteract the cholinergic input to inhibit oesophageal activity. This mechanism may in part account for the well documented "deglutitive inhibition", a physiological process triggered at high rates of swallowing (41,47). However, it should be noted that even in the presence of bicuculline, oesophageal peristalsis failed to follow the pharyngeal responses at rates $>$ 12/min during electrical stimulation of the NTS (Figure 7, middle). This result suggests that other mechanisms contribute to deglutitive inhibition of oesophageal peristalsis.

Taken together the present work demonstrates that solitarial GABAergic transmission exerts a tonic inhibition on the NPGs for buccopharyngeal swallowing and oesophageal peristalsis and prevents them from expressing their potential rhythmic motor output at rest. Release from GABAergic inhibition results in oscillatory activity, i.e. rhythmic fictive swallowing or peristalsis. Accordingly, this work demonstrates the utility of bicuculline as a tool for studying deglutitive neurotransmission, especially as regards the central control of the oesophageal stage of swallowing. Its effectiveness in inducing fictive primary peristalsis proved to be critically important in the following investigations.

Chapter Three

Unilateral control of primary and secondary oesophageal peristalsis

3.1 Introduction:

The oesophagus is capable of producing two types of centrally-mediated movement, namely primary peristalsis induced by swallowing and secondary peristalsis in response to distention of the oesophagus (41,83,134,140,143,161). These two types of peristalsis are thought to involve similar control mechanisms (41,58,161,172). In agreement with this suggestion, neuroanatomical (2,6,10,28,68) and physiological studies (6,68,93,94) have provided evidence that premotoneurons and motoneurons involved in both types of peristalsis are the same and the NTS_c-AMB_c central oesophagomotor pathway is the final common pathway. More significantly, available pharmacological observations (6) (chapter 2 of this thesis) suggest that a muscarinic cholinergic mechanism is required for both primary and secondary peristalsis. For instance, muscarine, when injected into the NTS_c, produces an oesophageal response resembling secondary peristalsis (6), i.e. fictive secondary peristalsis (7). Moreover, both fictive primary and secondary peristalsis evoked by bicuculline from the NTS are blocked by topical application of a muscarinic cholinoceptor antagonist to the dorsal surface of the ipsilateral NTS (chapter 2). The question therefore arises as to whether the muscarinic cholinoceptors associated with NTS_c interneurons are the common substrate through which ACh generates both primary and secondary oesophageal peristalsis. In this regard, it is necessary to determine if both types of oesophageal activity are abolished by

muscarinic cholinoceptor blockade restricted to the NTS_c.

A related issue with respect to the final common pathway for oesophageal peristalsis is whether unilateral activation of NTS_c is distributed bilaterally to the AMB_c motoneurons. A recent neuroanatomical study reveals that although the bulk of the NTS_c-AMB_c project is ipsilateral, a small proportion of these fibers projects to the contralateral NTS_c and AMB_c (68). Given that oesophageal peristalsis is a non-lateralized movement, one may hypothesize that NTS_c-AMB_c pathways on each side of the brainstem are normally synchronized via commissural interconnections so as to enable bilateral oesophageal motor output to be generated during peristalsis. This arrangement would be analogous to the one proposed for buccopharyngeal swallowing (47,49) and mastication (22).

The present work was undertaken to test two hypotheses: 1) activation of muscarinic cholinoceptors associated with NTS_c neurons is the common step in generation of both primary and secondary peristalsis; 2) oesophagomotor drive initiated by activation of NTS_c neurons on one side of the medulla is distributed to the AMB_c bilaterally.

3.2 Methods:

Experiments were performed on male Sprague-Dawley rats (Charles River, Montreal) weighing 250-450 g. General surgery and procedures for recording swallowing responses are described in section 2.2.

Acute or subchronic unilateral vagotomy was performed in 6 animals. Either the right or the left cervical vagal trunk was cut. To obtain complete interruption of ipsilateral oesophageal motor output, the ipsilateral superior laryngeal nerve (SLN) was also cut as it has been shown to contain motor fibers innervating the striated muscle of the uppermost cervical oesophagus (10). Acute vagotomy was done on three animals anesthetized with urethane (1.2 g/kg, i.p.). For subchronic vagotomy, operations were performed on the other three animals under i.p. sodium pentobarbital anaesthesia (45mg/kg). After a survival period of 7-10 days, the animals were reanaesthetized with urethane (1.2 g/kg, i.p.) and then subjected to the same experimental protocol that was employed in the acutely vagotomized animals.

Intra-NTS_c injection of methscopolamine was made by means of a two barrel pipette containing glutamate (0.2 M) and methscopolamine (10^{-3} M), respectively. As described in section 2.2, the NTS_c was located by test pulses of glutamate and then methscopolamine was delivered to these glutamate responsive sites from the other barrel of the pipette. Fluorogold (0.05%) or bis-benzimide (0.03%) mixed with methscopolamine solution was used as a marker to permit identification of the ejection sites by fluorescence microscopy.

Following the recording session, the rat was transcardially perfused with

heparinized saline containing 1% magnesium chloride followed by a phosphate-buffered (0.1 M) paraformaldehyde (4%). Serial transverse vibratome sections (40 μm) of the medulla oblongata were cut and examined with an UV fluorescence microscope.

For extracellular unit recording and chemostimulation of NTS_e oesophageal premotoneurons, two-barrelled glass micropipettes were used. The recording barrel of the pipette contained a carbon fibre (8 μm in diameter) which was electro-etched with chromic acid after the barrel was filled with 4 M NaCl, leaving 10-25 μm of exposed carbon fibre. The other barrel was filled with 0.2 M glutamate and used to locate the NTS_e oesophageal neurons. Extracellular unit activity was window-discriminated with a spike trigger. Standard pulses from the spike trigger, along with buccopharyngeal and oesophageal pressure waves, were recorded on a 4-channel instrumentation tape recorder for later analysis.

3.3 Results:

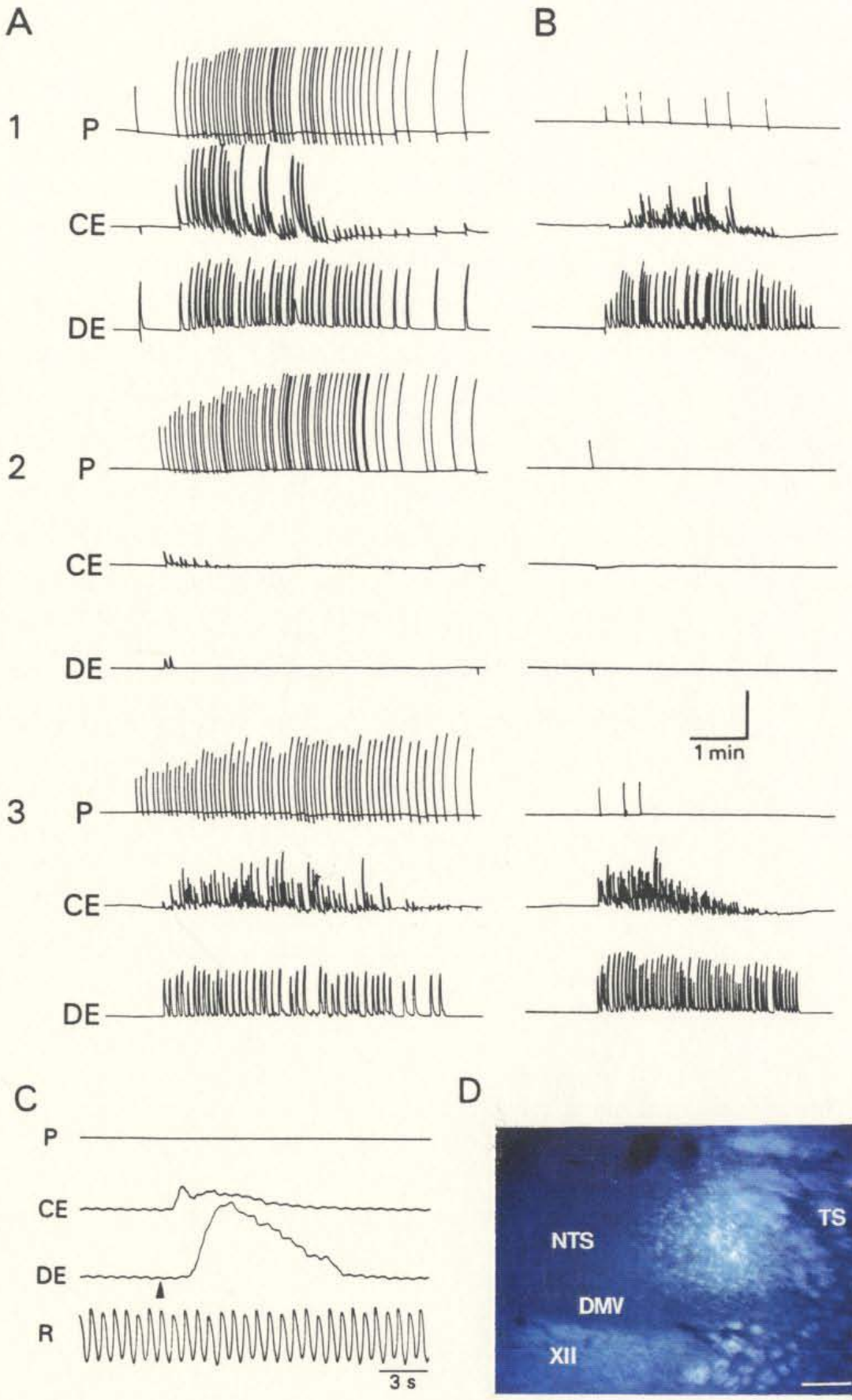
3.3.1 *Effects of muscarinic cholinoceptor blockade within the NTS_c on fictive primary and secondary oesophageal peristalsis*

Oesophageal peristalsis was induced in seven animals by topical application of bicuculline (5 pmol) and muscarine (100 pmol) to the dorsal surface of the NTS. As expected, peristalsis was completely blocked in all cases by injection of methscopolamine (< 0.3 pmol) into glutamate-responsive oesophageal sites within the ipsilateral NTS_c (Fig. 11). Recovery was not observed within the subsequent 4 h observation period. In contrast, neither primary nor secondary peristalsis evoked from the contralateral NTS was affected (Fig. 11).

3.3.2 *Extracelluar recording of NTS_c unit activity*

The question of whether the same population of NTS_c neurons is involved in the generation of both primary and secondary peristalsis was next investigated by means of extracellular unit recording from NTS_c oesophageal neurons. Oesophageal premotoneurons were defined by two criteria: i) a location coextensive with the functionally identified oesophageal loci within the NTS_c; ii) increased unit activity in response to glutamate (6 pmol) phase-locked with and preceding an evoked short-latency oesophageal pressure wave. Six neurons meeting the above criteria were obtained in 4 animals. These neurons exhibited irregular tonic discharges at a rate ranging from 2-10 Hz. In response to a glutamate pulse, they gave rise to burst discharges reaching 50-80 Hz, which preceded the oesophageal pressure wave by 0.2-1 s (Fig. 12A). Application of either muscarine (100 pmol) or bicuculline (10 pmol) to the extraventricular surface

Figure 11. Inhibition of both fictive primary and secondary oesophageal peristalsis by intra NTS_c muscarinic cholinoceptor blockade. Primary **(A)** and secondary **(B)** fictive oesophageal peristalsis are induced by bicuculline 5 pmol and by muscarine 100 pmol topically applied to the dorsal surface of the NTS, respectively. **1.** control responses. Note that the buccopharyngeal activity shown in B is independent of rhythmic oesophageal activity, probably due to a lingering effect of bicuculline applied in A; **2.** Oesophageal activity is blocked 3 min (A) and 15 min (B) following ejection of methscopolamine (0.2 pmol) at the glutamate-responsive oesophageal locus within the NTS_c shown in C and D; **3.** Undiminished responses are evoked from the contralateral NTS 30 min (A) and 45 min (B) after the application of methscopolamine. **C:** Oesophageal response evoked by glutamate at the methscopolamine injection site of the NTS_c shown in panel D. Pulse of glutamate (8 pmol) delivered from the other barrel of the pipette evokes a propulsive oesophageal response with a latency less than 1 s. Note the lack of effect on respiration following the glutamate pulse (bottom trace, R). D: fluorescence darkfield-photomicrograph of the injection site. The injection site is marked by fluorogold (0.05%) contained in methscopolamine solution and shown in a transverse section through medulla oblongata. The site is identified at the center of the area corresponding to the NTS_c (2,163). Abbreviations: NTS, nucleus of the solitary tract; TS, solitary tract; DMV, dorsal motor nucleus of vagus and XII, twelfth nerve nucleus.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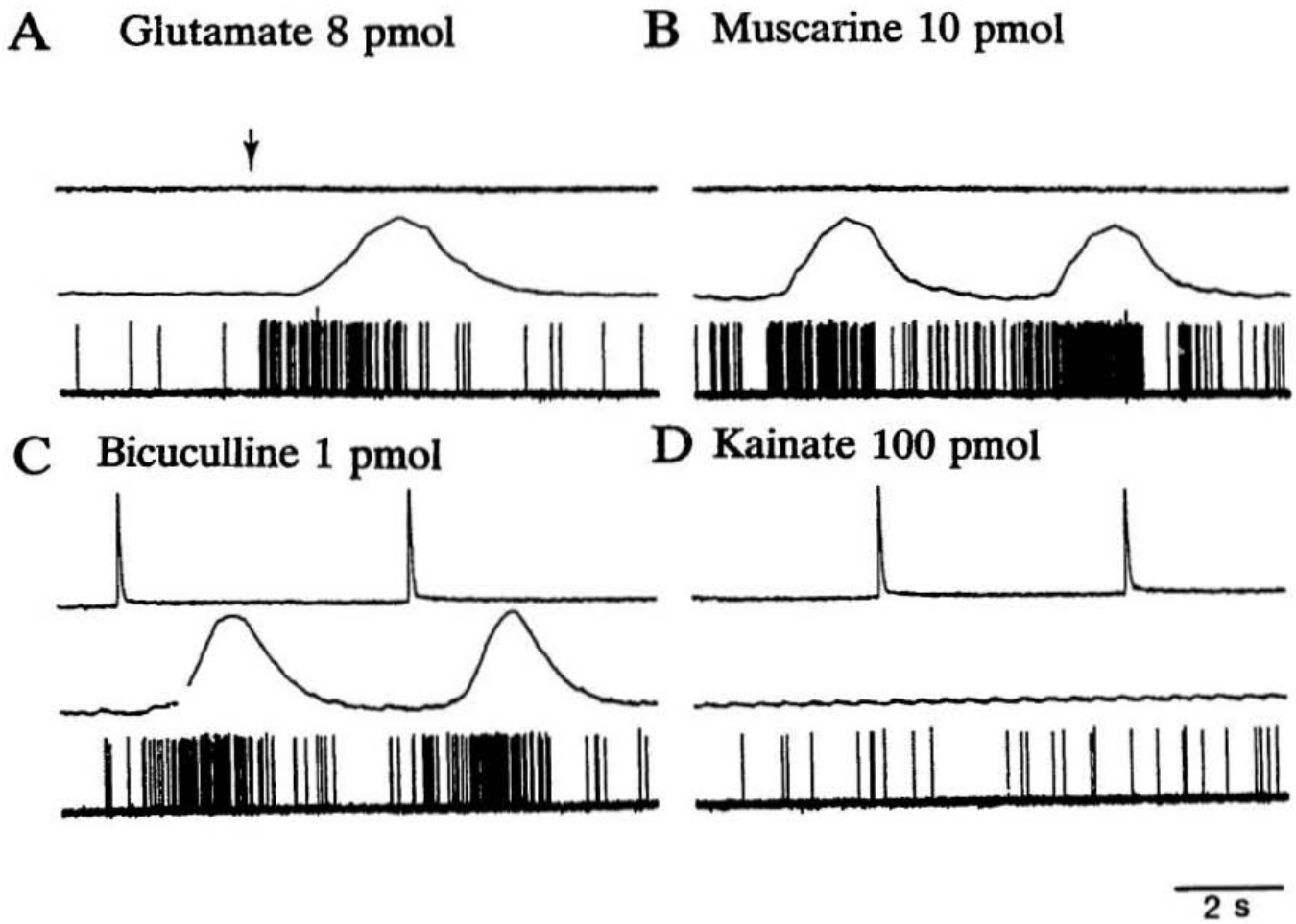
DMV
XII

Figure 12. Extracellular recording of NTS_e oesophageal premotor neuronal unit activity. In each panel, top two traces represent buccopharyngeal and oesophageal pressure waves, respectively, lower trace is window-discriminated discharge from the same unit. The NTS_e is located by a pulse of glutamate pressure-ejected from the other barrel of the recording pipette. **A:** Pulse of glutamate (downward arrow) produces a burst of unit activity which precedes the oesophageal pressure wave. **B** and **C:** Unit discharges are phase-locked with oesophageal pressure waves of both secondary and primary peristalsis evoked by topical applications of muscarine (100 pmol) and bicuculline (5 pmol) to the dorsal surface of the ipsilateral NTS, respectively. **D:** Buccopharyngeal swallowing induced by topical application of kainate (100 pmol) to the same site lacks an oesophageal component and NTS_e rhythmic unit activity.


A Glutamate 8 pmol
B Muscarine 10 pmol
C Bicuculline 1 pmol
D Kainate 100 pmol
2 s

of the ipsilateral NTS converted these irregular discharges to rhythmic bursting, which was phase-locked with the oesophageal waves of the corresponding peristalsis (Fig. 12B and 12C). In contrast, kainate (100 pmol) applied at the same site, though producing fictive buccopharyngeal swallowing activity, neither altered the unit activity of these oesophageal neurons nor induced any change in oesophageal intraluminal pressure (Fig. 12D).

3.3.3 *Unilateral vagotomy*

The effects of unilateral vagotomy on fictive primary peristalsis induced by bicuculline (10 pmol), applied to the extraventricular surface of the NTS, were examined first. As illustrated in Fig. 13, contralateral application of bicuculline induced rhythmic swallowing consisting of a buccopharyngeal component followed by oesophageal peristalsis. However, bicuculline applied ipsilaterally to the vagotomy failed to produce peristaltic oesophageal activity although buccopharyngeal activity was evident in all the animals (Fig. 13A, left). Results obtained with acute or subchronic unilateral vagotomy were qualitatively similar.

With regard to fictive secondary peristalsis, unilateral vagotomy resulted in the failure of oesophageal activity in response to ipsilateral application of muscarine (100 pmol). However, muscarine remained effective when applied contralaterally (Fig. 13B, right).

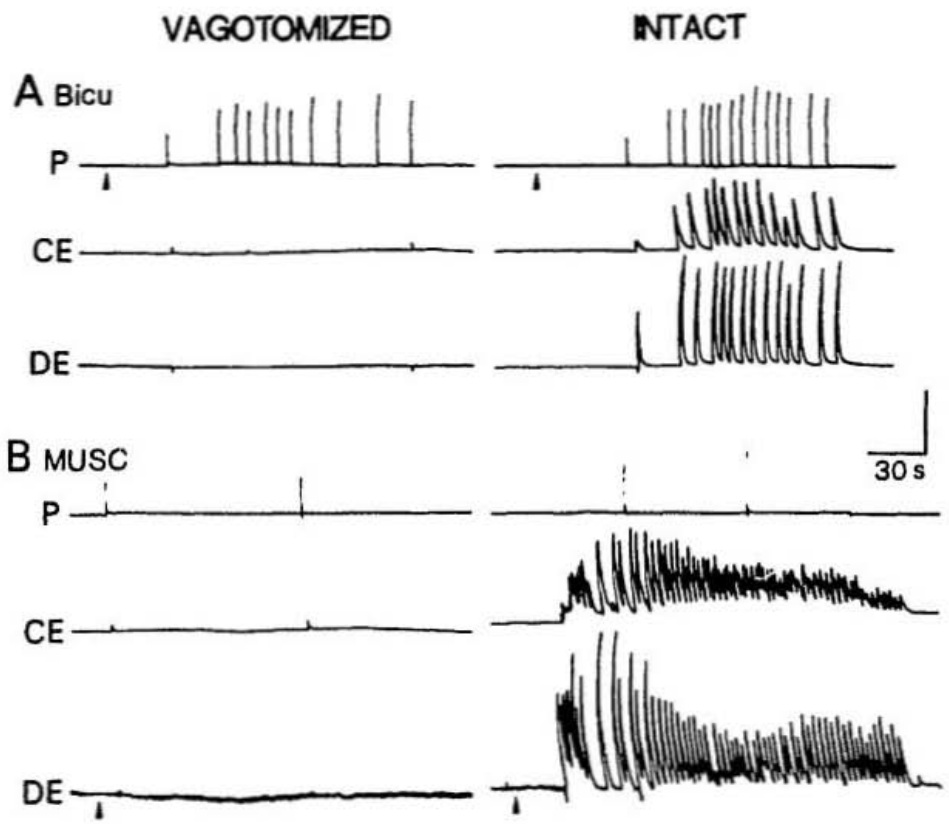


Figure 13. Effect of subchronic unilateral vagotomy on fictive oesophageal peristalsis. **A:** Bicuculline (5 pmol), topically applied to the dorsal surface of the NTS on the vagotomized side, only induces rhythmic buccopharyngeal without accompanying oesophageal activity. However, when applied to the dorsal surface of the NTS on the intact side, it produces propulsive oesophageal contractions coupled with buccopharyngeal swallows. **B:** Oesophageal peristaltic response to unilateral application of muscarine (100 μM) to the dorsal surface of the NTS is also abolished on the vagotomized side but remains unchanged contralaterally.

3.4 Discussion:

The two major findings in the present study are: (i) activation of muscarinic receptors associated with the NTS_e neurons is required for the generation of both primary and secondary fictive peristalsis and (ii) unilateral activation of the NTS_e is functionally confined to ipsilateral motoneurons.

3.4.1 *NTS_e-AMB_e projection - the common final pathway for both primary and secondary fictive peristalsis*

Based on the experiments in the dog by Meltzer (134), the mechanism of secondary peristalsis, unlike that of primary peristalsis, would largely depend on a process consisting of a succession of vago-vagal reflexes originating in oesophageal receptors sequentially stimulated by a moving bolus. However, preliminary study on the rat has revealed that secondary peristalsis can be induced by inflation of a balloon positioned at any level of the oesophagus (Lu, Neuman and Bieger, in preparation). Similar results were observed in the cat (158) and human (58,172). More significantly, Janssens noted in the monkey and opossum that secondary peristalsis induced by balloon distension below the transection of the oesophagus could begin above the transection (90). These findings argue against the suggestion that a moving bolus in the oesophagus is a prerequisite for maintaining secondary peristalsis. Rather, it would appear that the central mechanism for secondary peristalsis should be similar to that for primary oesophageal peristalsis.

Recent anatomical (2,6,10,28), and functional (6,70,105) studies have produced evidence suggesting that NTS_e neurons are oesophageal premotoneurons. However,

whether the same population of NTS_e neurons are involved in both cases remains to be determined. Pharmacological studies (6,68) demonstrated that fictive primary peristalsis is abolished by muscarinic antagonist applied systemically and muscarinic agonists, when directly ejected at NTS_e, evoke fictive secondary peristalsis, suggesting that both types of peristalsis depend on a central muscarinic link. However, one cannot conclude from these studies that muscarinic cholinoceptors mediating primary peristalsis are the same as those involved with secondary peristalsis.

The present study confirms the findings of earlier studies and extends them in several ways. First, the combination of extracellular recording and focal chemostimulation produced data suggesting that NTS_e neurons represent oesophageal premotoneurons, excitation of which elicits an oesophageal motor response. Second, the same population of NTS_e neurons that is involved in generating primary peristalsis is also engaged in programming secondary peristalsis, since its electrical activity is phase-locked with oesophageal pressure waves during both types of fictive peristalsis. Third, it is the activation of muscarinic receptors associated with NTS_e neurons that is required for the generation of both types of peristalsis, as evidenced by the effect of intra-NTS_e muscarinic cholinoceptor blockade. Thus, in the rat both primary and secondary peristalsis are governed by a common central mechanism. Under normal conditions, the brainstem oesophageal NPG is responsible for organizing sequential excitation of motoneurons innervating the oesophagus during both primary and secondary peristalsis. What might differ between these two types of centrally-mediated peristalsis, however, is the trigger to set off the full program; primary peristalsis results from input originating

in the buccopharyngeal NPG, and secondary peristalsis from excitation of the oesophageal NPG via oesophageal reflex afferents. In both cases, concurrent activation of muscarinic cholinoceptors associated with the NTS_c appears to be required.

3.4.2 *Independence of 'half-centers' organizing oesophageal peristalsis*

It has been proposed for some time that the central NPG for swallowing actually consists of two paired functionally complete NPGs (47,49). These are kept in perfect synchrony by a system of cross-connections or interconnections between the bilateral structures which constitute a functionally single NPG. This 'half-center' concept was originally proposed by Ishihara (cited in ref. (47)) and later elaborated by Doty (49). However, their experiments were confined to the buccopharyngeal stage. Whether the same also holds for the oesophageal stage has remained an open question.

Recent neuroanatomical studies in the rat (2,6,10,28) and electrophysiological studies in the sheep and rat (92,93,105) provide evidence supporting the existence of two NPGs, one on each side of the brainstem, controlling oesophageal motility. As a non-lateralized movement, oesophageal peristalsis might be presumed to require an especially tight coordination via interconnections between the two hypothetical NPGs. So far only one study has addressed this issue. Jean demonstrated in sheep that a unilateral lesion placed between the solitary tract and the DMV suppressed the oesophageal stage of swallowing elicited by electrical stimulation of the ipsilateral SLN without affecting that induced by contralateral SLN stimulation. However, it is difficult to rule out the possibility that unilateral loss of oesophageal activity observed in this study is secondary to deafferentation. This is of particular relevance in view of the finding that vagal

oesophageal afferent fibers projecting to the NTS_c are uncrossed and unilateral (Bieger, unpublished observation).

The present work clearly shows that central oesophagomotor excitation initiated unilaterally from the NTS_c is not distributed to motoneurons bilaterally. Thus, selective blockade of muscarinic receptors within the NTS_c or unilateral vagotomy abolished oesophageal activity evoked from the ipsilateral NTS, leaving unaffected that induced by contralateral chemostimulation. These results argue against the existence of functionally significant excitatory commissural or decussating connections between the two oesophageal NPGs.

It is relevant to note that recent neuroanatomical work employing anterograde tracing with PHAL revealed the presence of crossing fibers originating from the NTS_c and terminating in the contralateral NTS_c and AMB_c (68). Observations from the present study suggest these crossing fibers are either not excitatory or alternatively do not have sufficient excitatory drive to synchronize activities of the two NPGs. While supporting the existence of bilateral NPGs, the present work suggests a different mode of operation. Accordingly, synchronization of the two NPGs does not depend on intact interneuronal connections crossing the midline of the medulla. This is fundamentally different from the organization proposed for buccopharyngeal swallowing (47,49) and mastication (22), where an independent operation of each 'half-center' can only be demonstrable after a midline transection.

The apparent independence of each oesophageal NPG raises the question as to how bilateral synchrony is achieved. Since peripheral fibers from the rat oesophagus

probably project unilaterally to the NTS_c, synchrony of afferent input to NPGs in each hemi-medulla may be of primary importance.

In conclusion, the present work provides corroboratory evidence that: (i) primary and secondary peristalsis are subject to similar central control; (ii) activation of muscarinic cholinoceptors associated with NTS_c neurons is a common step in generating oesophageal peristalsis and (iii) oesophageal premotor control is effected by a predominantly unilateral pathway from the NTS_c to the AMB_c. These findings provide the basis for the following investigations directed toward unravelling the physiological and pharmacological mechanisms governing synaptic transmission from NTS_c to AMB_c.

Chapter Four

Excitatory amino acid receptors mediate solitario-ambigual synaptic transmission

4.1 Introduction:

The experiments described above have confirmed and further defined the critical role of the NTS_c-AMB_c pathway in the generation of oesophageal peristalsis. It appeared logical therefore to investigate the identity of the neurotransmitters involved in transmitting the input from the NTS_c to the AMB_c. Neurochemical and pharmacological studies have already established a diversity of neurochemical inputs converging on the AMB_c (see chapter one). As mentioned previously, an EAA-like substance may be considered a plausible candidate for primary transmitter of the solitaro-ambigual oesophagomotor pathway.

Glutamate acts through both NMDA and non-NMDA (kainate/quisqualate) receptors in mediating synaptic transmission (27,130,205). The non-NMDA subtype is generally considered to principally contribute to generating fast EPSPs in the mammalian CNS (130). With regard to the NMDA subtype, the view has been expressed that the Mg^{2+}-mediated voltage dependence of these receptors (98,131) precludes their involvement in low frequency synaptic transmission (26). On the other hand, there is evidence from both *in vivo*, and *in vitro* experiments to suggest NMDA receptors play an important role in fast synaptic information transfer (18,60,75,151,188,189). Moreover, NMDA receptors have been implicated in central rhythmogenesis during locomotion (67), swallowing (70,104), mastication (101), and in spinal interneuron-motoneuron synaptic transmission (74,111,151).

The present study was designed to examine the hypothesis that NTS_e neurons employ a glutamate-like substance as transmitter. In addition, efforts were made to investigate the role played by EAA receptor subtypes in mediating NTS_e-AMB_e synaptic transmission with an emphasis on that played by the NMDA receptor. The contribution of EAA receptors to the solitario-ambigual synaptic transmission was first assessed through ejecting EAA receptor antagonists into the ipsilateral AMB_e with a view to inhibiting oesophageal peristalsis *in vivo* in the anaesthetized animal. Due to the difficulty in achieving well-defined drug concentrations, interpretation was complicated by issues of antagonist selectivity, localization and mechanisms of action. In an effort to overcome these problems, synaptic responses of AMB_e neurons to electrical activation of solitario-ambigual fibres were examined in an *in vitro* sagittal brainstem slice. This preparation permitted stable intracellular recording of AMB_e neurons responses while allowing local application of agonist or antagonist in known concentrations.

4.2 Methods:

Surgical procedures, techniques for recording swallowing and oesophageal peristaltic activity are described in chapter two and three. Fig. 14 diagrammatically illustrates methods used to activate oesophageal premotoneurons in the NTS, localize oesophagomotor sites in the AMB and pressure-eject EAA antagonists at these sites.

For *in vitro* studies, rats weighing 80-200 g were anaesthetized with urethane (1g/kg, i.p.). The skull was rapidly opened and the brain removed. The brainstem was hemisected and blocked in an oblique sagittal plane based on anterograde tracing of the solitario-ambigual projection (68). This preserved the solitario-ambigual pathway. Slices (350 μm) were cut on a vibratome at 0-4°C in modified artificial cerebrospinal fluid (ACSF). Following 1 h recovery at room temperature in modified ACSF, slices were transferred to a submerged type recording chamber and perfused with normal ACSF at a flow rate of 2 ml/min and a temperature of 33-34°C. Normal ACSF consisted of (mM) NaCl 126; KCl 3; $CaCl_2$ 2; $MgCl_2$ 2; KH_2PO_4 1.2, $NaHCO_3$ 26 and glucose 10 bubbled with 95% O_2/5% CO_2 to maintain pH 7.3-7.4. In modified ACSF, NaCl was replaced by iso-osmolar sucrose (1). Sharpened tungsten wires were used for bipolar stimulation (monophasic square wave pulses of 0.05-0.1 ms duration) of solitario-ambigual fibres.

For intracellular recordings, glass microelectrodes filled with 3 M KCl or 2 M K-methyl-sulphate (80-140 MΩ) were used. Intracellular recording and dye labelling were made with an Axoclamp II amplifier. Neurons were voltage-clamped by means of a single electrode voltage clamp at a sample switching frequency of 3-4 kHz, 30% duty

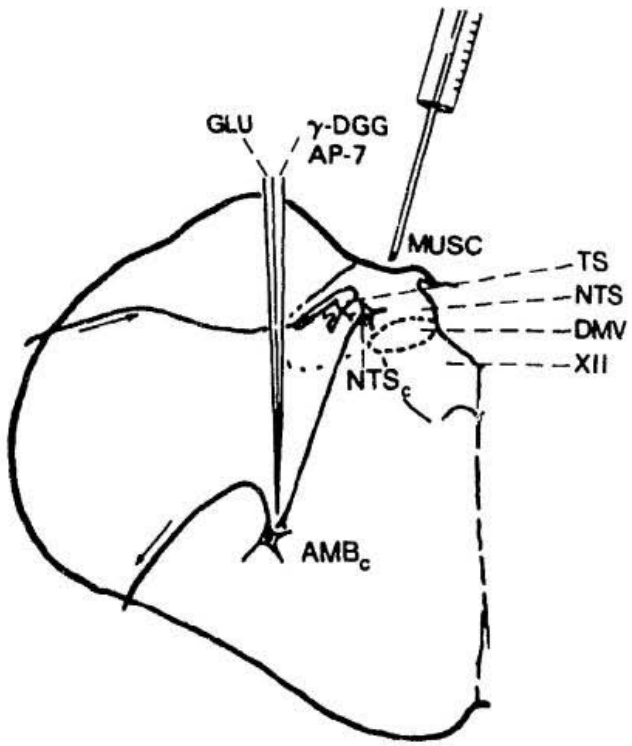


Figure 14. Diagram showing methods used for *in vivo* experiments. Oesophageal premotoneurons are activated by topical application of muscarine (MUSC) with a microsyringe to the extraventricular surface of the NTS. Test pulses of glutamate from a two-barrel micropipette are used to locate the AMB_c, where glutamate evokes a short latency oesophagomotor response (shown in Fig. 15 A). An EAA antagonist, γ-DGG or AP-7, is then ejected at the glutamate responsive site from the second barrel of the pipette in order to examine the involvement of an EAA transmitter in mediating the oesophagomotor response induced by activation of NTS_c muscarinic cholinoceptors. Abbreviations: NTS, nucleus of the solitary tract; TS, solitary tract; DMV, dorsal motor nucleus of vagus and XII, twelfth nerve nucleus.

cycle. The voltage at the head stage amplifier was monitored continuously on another oscilloscope to test the characteristics of the recording electrode. Membrane potential and current were displayed and captured on a Nicolet 310 digital oscilloscope and saved on a computer. Data were averaged and analyzed using a suite of software routines written in Assyst (Assyst Lab. Technology, Rochester, N.Y.).

For intracellular labelling, electrodes were filled with 2 M LiCl containing 1% Lucifer yellow. After a stable recording was obtained, 2 nA negative DC current was delivered through the recording electrode for 5-10 min. The slice was kept in the recording chamber for at least 1 h to permit the dye to diffuse before immersion in 4% paraformaldehyde for 24 h. Slices were cut on a vibratome in 40 μm sections for subsequent examination under UV illumination. Sections were mounted on a gel-coated slide and coverslipped with mounting medium containing 80% glycerol in phosphate-buffered saline (PBS).

Drugs were either pressure-ejected in volumes of 10-50 pl from a multibarreled pipette positioned within 100 μm from the recording electrode or applied by bath perfusion.

Drugs were obtained from Sigma Chem. Co. with the exception of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, gift from Dr. T. Honoré).

4.3 Results:

4.3.1 *Functional localization of the AMB_c*

Test pulses of glutamate (6-10 pmol) delivered at 50 μm steps were used initially to locate oesophagomotor loci in the ventrolateral tegmentum of the medulla. As shown previously (6), ejection of glutamate at sites in the ventrolateral tegmentum of the medulla was effective in eliciting monophasic oesophageal contractions (Fig. 15, left). Histological examinations of 25 injection sites from 18 rats confirmed that all ejection loci were confined to the AMB_c (Fig. 15, right). Oesophageal responses were elicited only if the tip of the pipette was within 50 μm of the AMB_c. Accordingly, this method was employed to locate the AMB_c in the following investigations.

4.3.2 *Ambigual EAA receptor blockade and fictive oesophageal peristalsis in-vivo*

As shown previously (chapters 2 and 3), application of muscarine (50-100 pmol) to the dorsal surface of the NTS induced fictive oesophageal peristalsis (Fig. 16 A and B). When τ-DGG (0.5-1.5 nmol; n=7), a broad spectrum EAA receptor antagonist (130), was ejected in the ipsilateral AMB_c, there was a nearly complete inhibition of the oesophageal response in 5 animals and a partial inhibition in the other two. In the latter cases, both the number and amplitude of contractions were reduced in the cervical oesophagus, whereas in the distal oesophagus the frequency was significantly more affected than the amplitude (Fig. 16A). Recovery from τ-DGG was complete within 30 to 60 min after drug application. Application of AP-7 (0.5-1.5 nmol; n=6), a specific NMDA receptor antagonist, quantitatively and qualitatively mimicked the effect of τ-DGG in blocking the muscarinic-evoked oesophageal response (Fig. 16B), albeit with a

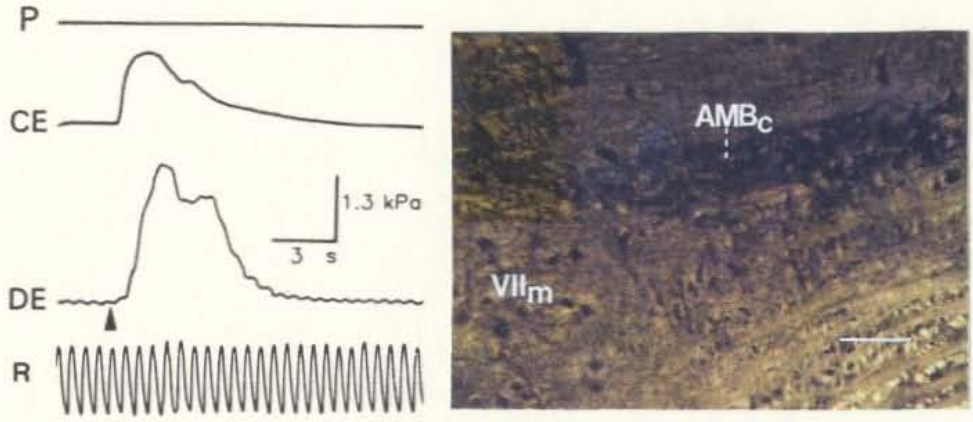


Figure 15. Oesophagomotor response to glutamate. Left: Single pulse of glutamate (8 pmol) ejected at the tip of the AMB_c (right) evokes a propulsive oesophageal contraction. **Right:** Fluorescence darkfield-photomicrograph of the injection site marked by bis-benzimide (0.03%) mixed with the glutamate solution, as seen in a sagittal section through the nucleus ambiguus. The caudal third of the AMB_c blends into the semicompact formation of the nucleus ambiguus. VII_m: facial motor nucleus.

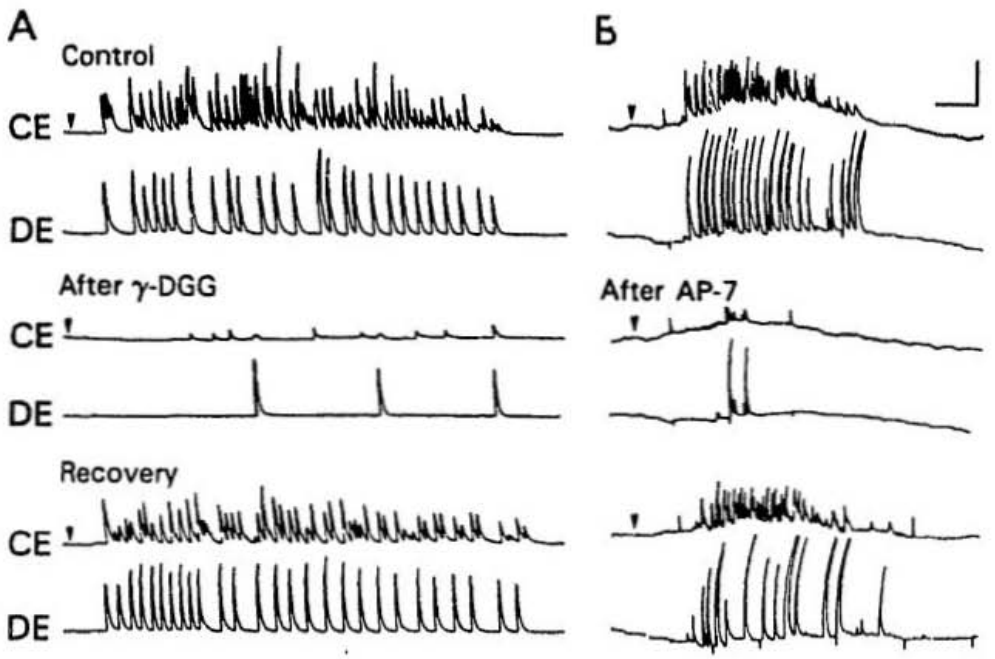


Figure 16. Blockade of fictive oesophageal peristalsis by intraambigual EAA antagonists. Records from two animals. The panels from top to bottom are the control response to muscarine (0.1 nmol; downward triangles), applied to surface of the ipsilateral solitarial complex; blockade of the response 5 min after τ-DGG (2 nmol, left) and AP-7 (2 nmol, right) injected into the AMB_c; and recovery of the response 40 min later. Calibrations: vertical bar represents 1.3 pKa, horizontal 40 s for A and 1 min for B, respectively.

shorter duration (20-30 min). Full blockade of the muscarine-evoked oesophageal response was evident in 4 animals and partial blockade in the other two after ejection of AP-7. Neither γ-DGG nor AP-7 altered basal oesophageal pressure or respiration. Ejections of an equal volume of vehicle (ACSF) into the AMB_c had no detectable effect either on oesophageal pressure or muscarine-induced oesophageal peristalsis (see also Figure 28B in chapter 6).

4.3.3 *Synaptic responses of AMB_c neurons in vitro*

Synaptic responses of ambigual neurons were evoked by electrical stimulation of solitary afferents (Fig. 17A). Intracellular recordings were obtained from 70 AMB_c neurons in slice preparations which met the following criteria: (i) resting membrane potential greater than -55 mV (-66±5 mV; mean±SD); (ii) spike (≥ 75 mV) in response to depolarizing current injection (0.6 to 1.2 nA).

Sixty-nine of 70 neurons generated an EPSP as judged by: 1) amplitude varying with stimulus intensity; 2) an all-or-none action potential superimposed on the EPSP evoked by suprathreshold stimuli and 3) blockade by tetrodotoxin (TTX, 1 μM) or $Mn^{2}+$ (5 mM). In only 1 of 10 cells recorded with a K-methyl SO_4 electrode was there any evidence for an IPSP. Most EPSPs had two (rarely three) discernible peaks (Fig. 17B). At low stimulus intensity, the EPSP consisted of a monophasic wave with a relatively slow rate of rise and smooth decay. At intermediate intensities, the EPSP occasionally displayed a second delayed peak. Since the latter was evident with both chloride or methylsulfate-filled electrodes, it was not considered an IPSP. This component was not investigated further. At high intensities but still below spike threshold, the EPSP

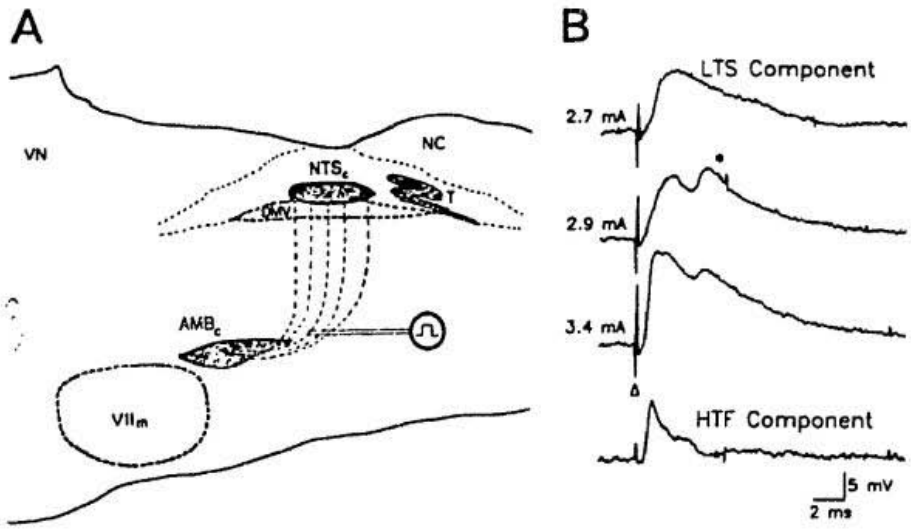


Figure 17. Intracellular recording of ambigual EPSP *in vitro*. **A:** Schematic diagram showing pathway from the NTS_c to the AMB_c. The topographic trajectory of the NTS_c afferents to the AMB_c is based on anterograde tracing experiments (68). In slices, the pathway was stimulated as shown. DMV: dorsal motor nucleus of vagus; VN: vestibular nuclei; NC: cuneate nucleus; VII_m: facial motor nucleus; T: solitary tract. **B:** Variation of EPSP in AMB_c neuron with stimulus intensity. Stimulation of the solitario-ambigual pathway produces a multi-component EPSP. At the low intensity only the low-threshold slow (LTS) component is present. A delayed component, found in a minority of cells at intermediate stimulus intensities, is indicated by the '*'. At high stimulus intensities a rapidly rising component is present. Subtracting the EPSP elicited at 2.9 mA from that evoked at 3.4 mA reveals the high-threshold fast (HTF) component shown at bottom.

displayed a larger amplitude with a greater rate of rise. In the following, the two components of interest are designated low-threshold slow (LTS) and high-threshold fast (HTF). The HTF component as revealed by digital subtraction is illustrated in Fig. 17B and can be seen to consist of a large early component and a small tail. Both LTS and HTF components had a similar latency of onset although in some cases the latter appeared 0.5 ms before the former. With a sufficiently strong stimulus, the evoked EPSP triggered an action potential. The synaptically activated spike was initiated at the peak or on the falling phase of the HTF component (Fig. 18B). Irrespective of stimulus intensity, no more than a single spike could be elicited. During late repolarization of the spike, the LTS component was attenuated (Fig. 18B).

4.3.4 *EAA receptor antagonists and the ambigual EPSP*

The contribution of EAA receptors in mediating the EPSP was examined by using EAA receptor antagonists. Stimulus intensity was below the threshold for spike initiation, but sufficient to evoke the HTF component. In 8 out of 10 cells, bath perfusion with AP-7 (10-50) μM reduced the peak amplitude of the EPSP by 8 to 30% ($25 \pm 7.4\%$). Digital subtraction revealed that AP-7 completely blocked the first peak corresponding to the HTF component, including its tail (Fig. 18A). The EPSP recovered within 10 min upon washing. In the remaining two cells with slower rising EPSPs, AP-7 did not have an appreciable effect. Kynurenate (1 mM) added to the perfusate reduced the peak amplitude of EPSPs in 11 out of 14 cell tested by $72 \pm 22\%$. Both the HTF and LTS components were depressed as revealed by subtraction (Fig. 18A). The EPSPs evoked from the remaining three cells (including the two AP-7 insensitive ones) were

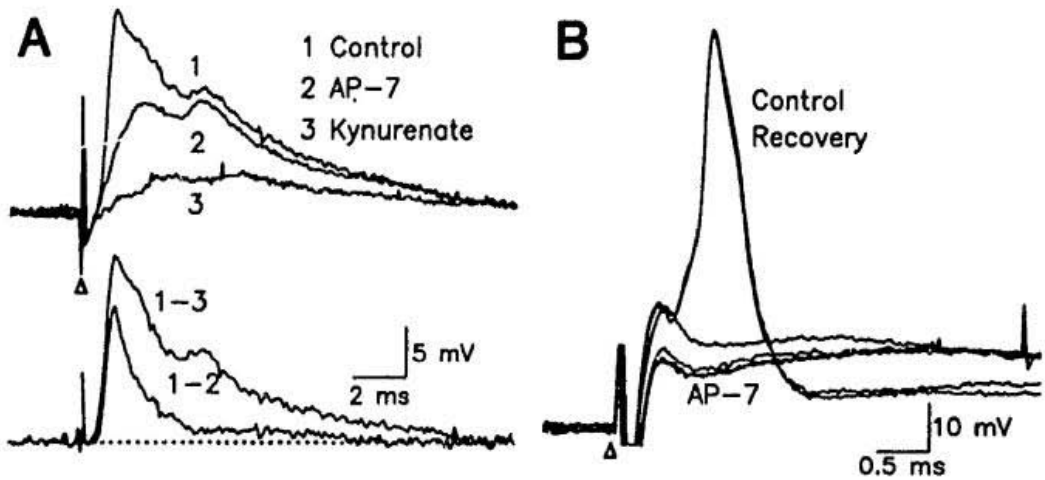


Figure 18. EAA antagonists inhibit the evoked EPSP and synaptically generated action potential. **A:** AP-7 (30 μM) selectively reduces the HTF component while kynurenate (1 mM) reduces all components of the EPSP. Lower panel shows the AP-7 sensitive component obtained by subtracting trace 2 from trace 1 and the kynurenate sensitive component obtained by subtracting trace 3 from trace 1. Note low amplitude tail of the AP-7 sensitive component. **B.** Stimulation (9 mA) evokes a spike arising from falling phase of the HTF component (Control). Application of AP-7 (50 μM) blocked spike generation along with the HTF component (AP-7). During wash the EPSP recovered with intermittent spike failure. Complete recovery was obtained within 10 min upon washing (Recovery). Stimulus artifact was attenuated for clarity. A and B are from different cells.

resistant to kyurenate. The effect of bath applied CNQX (5-10 μM; n=11) was qualitatively similar to that of kynurenate in that both components of the EPSP were suppressed. However, in some cells, CNQX was less effective than kynurenate. Reduction of the EPSP amplitude by CNQX was 45.6 $\pm$ 7.15%. In contrast, the EPSP was resistant to bath application of dihydro-ß-erythroidine (100 μM; n=5, not shown), a nicotinic cholinoceptor antagonist (37,194).

The effect of AP-7 on spike initiation was also investigated. AP-7 (50 μM) either completely blocked the spike (Fig. 18B; n=2) or increased its threshold (n=2; increase by 10 and 50%, respectively).

4.3.5 *Glycine and the ambigual EPSP*

Blockade of the NMDA receptor-mediated component by both kynurenate and CNQX might result directly from competition at the glycine site or indirectly from blockade of a "priming" non-NMDA receptor-mediated postsynaptic membrane depolarization. To pursue the first possibility, protection experiments with glycine were carried out in an attempt to reverse the blockade of NMDA receptor-mediated component by these two antagonists (98,186). As illustrated in Figure 19, in all cells tested glycine (50-1000 μM) in the presence of strychnine (1-100 μM) failed to reverse the antagonist action of kynurenate (n=5) or CNQX (n=2). In fact, glycine enhanced kynurenate (Fig. 19A) inhibition of the EPSP. In the absence of kynurenate, glycine plus strychnine (1-100 μM each, n=4) or glycine alone (100 μM, n=3) selectively and reversibly inhibited the EPSP including the AP-7 sensitive component, without altering the resting membrane potential (Fig. 19B). The residual EPSP was resistant to AP-7 (50 μM, not shown) and

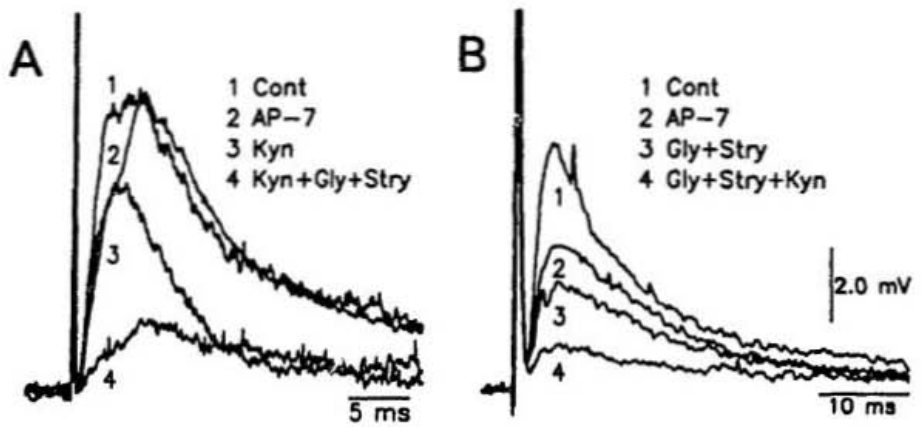


Figure 19. Unexpected depression of the solitario-ambigual EPSP by glycine. EPSPs (A and B) evoked by stimulation of solitario-ambigual fibres recorded from two AMBc motoneurons (RMP -70 and -71 mV respectively). EPSPs from the same neuron are superimposed for comparison. **A:** Failure of glycine to protect the NMDA-mediated component from antagonism by kynurenate. Note the slower rising phase of the EPSP with AP-7, indicative of an early NMDA component. In the presence of glycine, depression of the NMDA component by kynurenate is not reversed. Instead, the inhibition is enhanced. Drug concentrations used: 50 μM AP-7, 1 mM kynurenate, glycine plus strychnine (100 μM each). **B:** Glycine plus strychnine (50 μM each) significantly inhibit the EPSP without altering the RMP. Note that glycine suppresses the AP-7 component as indicated by the slower rate of rise and reduced amplitude of the residual EPSP. The remaining EPSP is further reduced by addition of 1 mM kynurenate.

was further reduced by addition of kyurenate (1 mM, Fig. 19B). This glycine mediated inhibition was dose dependent (over the range of 1 to 1000 μM) and reversible within 20 min of washing.

4.3.6 *Voltage dependence of NMDA currents*

The unusual features of the HTF component described above prompted investigation of the properties of NMDA-mediated inward current at the resting membrane potential. Five AMB_e cells were voltage-clamped at their resting membrane potential (-57 to -68 mV; -62$\pm$5 mV) in the presence of 1 μM TTX. Bath application of 20 μM NMDA resulted in a net inward current which varied from 80 to 500 pA (269$\pm$189 pA). The voltage dependence of the NMDA-induced current from these cells was determined by applying a computer-driven slow voltage ramp starting 30 mV negative to the holding potential (range 60 mV; 2 mV/s). As shown in Fig. 20, the NMDA-induced inward current revealed a negative slope region between -90 to -40 mV.

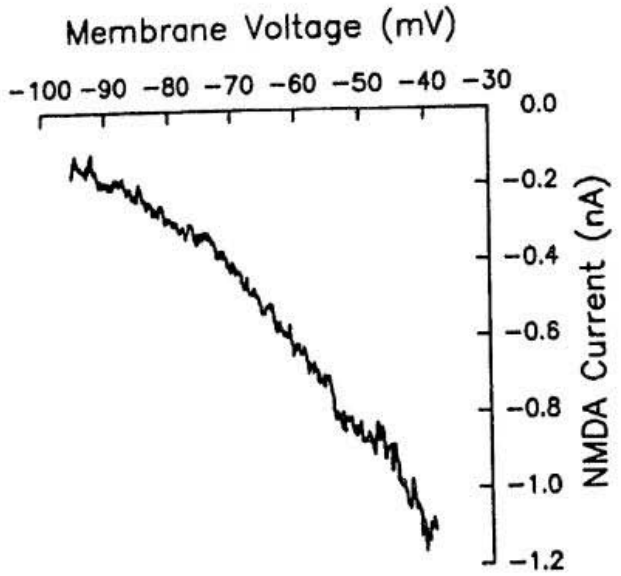


Figure 20. Current-voltage relationship of NMDA-induced current from a AMB_c neuron. NMDA current is obtained by subtracting the control current generated by a slow potential ramp (2 mV/s) in TTX (1 μM) from that recorded in the presence of 20 μM NMDA and 1 μM TTX. Note the negative slope over the range of -90 to -40 mV. The resting as well as the holding membrane potential is -65 mV. At this membrane potential the NMDA induced current is about 450 pA.

4.4 Discussion:

The main finding of this study is that EAA receptors are involved in synaptic transmission at the level of brainstem vagal motoneurons. While both NMDA and non-NMDA receptors contribute to ambigual EPSP generation, it may be the activation of the former that is required for an integrated motoneuronal response. Since EPSPs of rat AMB motoneurons have not previously been described in a slice preparation, separate comment may be appropriate before discussing the specific role of the NMDA receptors.

4.4.1 *Ambigual EPSPs in vitro*

The sagittal slice preparation developed for the present study preserves the major afferent input to AMB_c, i.e. the projection from the NTS_c (6,28,68). This dense and circumscribed projection connects the afferent and efferent limbs of the oesophagomotor reflex arc (28). The topography of stimulation sites from which robust EPSPs were obtained was in register with the trajectory of these solitario-ambigual fiber bundles. As noted previously the AMB complex may receive a cholinergic input from a source other than the NTS_c. However, involvement of an extrasolitary cholinergic pathway in the synaptic responses under study can be ruled out because the EPSP was inhibited by EAA antagonists, but not by dihydro-ß-erythroidine, which blocks nicotinic cholinoceptor-mediated excitation of ABM_c neurons (see section 6.3).

The variation of EPSP configuration with stimulus intensity, as well as its pharmacological features, demonstrate the complexity of underlying synaptic events. For convenience, two major components were distinguished, although this is obviously an oversimplification. In keeping with the *in vivo* observations, the present analysis focused

on the AP-7 sensitive EPSP component.

4.4.2 *NMDA mediated component of EPSP*

Based on the available data, the HTF seems identical with the AP-7 sensitive component. Since the HTF component was also eliminated by kynurenate, an endogenous high-affinity competitive antagonist at the strychnine-insensitive glycine site of the NMDA receptor (13,108), it may be attributed to activation of NMDA receptors. This notion is also supported by the results obtained in the present study that AMB_c neurons possess a significant voltage-dependent NMDA-mediated inward current.

The novel feature of the NMDA component is its rapid rate of rise, which is at variance with observations in most other areas of the CNS (3,59,78,111,114,170) where NMDA components reportedly have a slow rate of rise and slow decay. The only other region to date in which a similar EPSP has been described is the granular layer of the rat cerebellum (38) (but see also (173)). In light of binding (153), conductance and kinetic measurements (89), there are no longer any reasons for excluding NMDA receptors from contributing to the rising phase of the EPSP. In the present work, the only slow NMDA component observed was of low amplitude.

Several factors may account for the high activation threshold of the NMDA component. First, NMDA and non NMDA receptors could be activated via different fibres, the ones innervating NMDA receptors having a higher threshold. At present, there is no evidence to support this conjecture. On the contrary, NMDA and non-NMDA receptors are reportedly co-localized and co-activated (3,111,188). Second, a non-NMDA EAA receptor-mediated postsynaptic membrane depolarization of sufficient

magnitude may be required to remove the Mg^{2+} blockade of the NMDA receptor-gated channel (131,150). Although this explanation is difficult to reconcile with the short latency of the NMDA/HTF component compared with the non-NMDA/LTS component, it would be premature to rule out the contribution of such a mechanism at this time. Finally, the possibility also exists that this pathway may use another mediator beside an EAA-like substance. If this were the case, this substance would play an important role in expression of the NMDA component. With bath application of NMDA, the currents recorded in AMB_c neurons were insufficient to generate the full amplitude of the HTF (NMDA) component. Given an average NMDA current of 270 pA at the resting membrane potential and an input resistance of 40 MΩ (see chapter 6), less than half of the HTF component would be accounted for. However, this could be a gross underestimation due to factors such as desensitization of the NMDA receptor (115,129) and the possibility that the endogenous agonist may attain a very high intrasynaptic concentration and possess higher efficacy.

A significant feature of the NMDA component is its relationship to spike production. The synaptically-driven spike was typically evoked at or immediately following the peak of the NMDA component. Moreover, it was inhibited by AP-7. Interestingly, AMB_c neurons exhibit a relatively high threshold for spike initiation. Substantial depolarization is required to elicit spikes either synaptically or by intracellular current injection. It appears that only the NMDA component has the requisite rapid rate of rise and induces sufficient membrane depolarization to bring cells to their firing level. This accords well with the *in vivo* results demonstrating that rhythmic oesophageal

activity evoked from the NTS_c could be completely abolished by AP-7.

4.4.3 *Physiological function of ambigual NMDA receptors*

The parallelism between *in vivo* and *in vitro* findings strongly suggests a role of NMDA receptors in the generation of oesophagomotor output from the nucleus ambiguus. The synaptic mechanism implicated in spike generation *in vitro* provides a basis on which to explain the *in vivo* blockade of oesophageal peristalsis by the NMDA antagonist AP-7. However, a hiatus still remains between the present *in vivo* and *in vitro* data. In slice preparations, rhythmic oscillatory activity was neither present nor could it be induced by pharmacological manipulations. Nevertheless intrinsic rhythmogenic properties have been attributed by others to AMB_c neurons (39). Moreover, the apparently selective effect of EAA antagonists on oesophageal contractile rhythm, rather than amplitude, would support the notion that ambigual motoneurons play an active part in oesophagomotor rhythmogenesis. The possible advantage of an NMDA receptor-operated input into what appear to be rather sluggish, i.e. difficult to excite, motoneurons can only be conjectured. This receptor-ionophore complex is however noted for its plasticity and susceptibility to modulation.

4.4.4 *Non NMDA-mediated component of the EPSP*

Non-NMDA/EAA receptors appeared to mediate the LTS component, as shown by sensitivity to CNQX and kynurenate, but not AP-7. Although reportedly selective for non-NMDA receptors (3,78,80), CNQX failed to discriminate between the AP-7 sensitive and the AP-7 insensitive components when tested at concentrations in the range of 5-10 μM. This may reflect an antagonist action of this drug at the glycine modulatory site that

is linked to the NMDA receptor (107,187). Alternatively, the non-NMDA induced depolarisation may be required for removal of the $Mg^{2}+$ blockade (186).

Protection experiments with glycine should have distinguished between these two possibilities. Glycine, a co-agonist acting at the strychnine-insensitive site on the NMDA receptor (98,193), has been shown to potentiate NMDA-mediated currents (98,115) and EPSP (189), and reverse inhibition of NMDA responses by both kynurenate (13,108) and CNQX (107,187). It is intriguing that glycine not only failed to reverse the antagonism but actually enhanced the antagonistic action of kynurenate. Besides its effect on the NMDA receptor, the only other electrophysiologically characterized action of glycine is as an inhibitory agonist at the strychnine-sensitive Cl^- channel (34,98). Neither of these glycine effects can account for the present results. Among many explanations, the simplest one is that glycine has yet another modulatory role. As a working hypothesis it is proposed that another transmitter besides an EAA may contribute to the generation of AMB_c EPSP. Integrity of the unknown transmitter-mediated component is required for expression of the NMDA-dependent component. Glycine could modulate the actions of the unknown substance and thereby exert its inhibitory effect on the EPSP. As regards the unknown transmitter, somatostatin appears to be a logical candidate because of its presence in NTS_c cell bodies and their terminal axons in the AMB_c (28) and its synergistic interaction with glutamate (43,85). Thus, further effort was made in the following investigation to determine if SST contributes to the generation of the EPSP and if glycine, synergizing with kynurenate, modulates the action of somatostatin on AMB_c neurons (Chapter 5).

In conclusion, the results obtained provide evidence supporting the following hypotheses: (i) the primary transmitter at the solitario-ambigual synapse is an EAA-like substance; (ii) activation of both NMDA and non- NMDA excitatory amino acid receptor subtypes is involved in mediating synaptic transmission at this site; (iii) activation of NMDA receptors is necessary for functional information transfer in the solitario-ambigual pathway and (iv) another transmitter substance besides an EAA may contribute to the generation of the ambigual EPSP.

Chapter Five

Somatostatin contribution to the ambigual EPSP

5.1 Introduction:

Somatostatin (SST), first isolated from bovine hypothalamic extracts as a "somatotropin-release inhibiting factor" (SRIF) (17), is now regarded as a brain neurotransmitter/neuromodulator (54) in keeping with its presence in nerve endings (28), calcium-dependent release (87) and high affinity binding to neuronal membranes (177). Disparate excitatory and inhibitory effects of this peptide have been reported in hippocampal cells (44,145,156,169). However, postsynaptic potentials attributable to SST have yet to be identified.

AMB_c motoneurons of the rat receive a dense projection of SST-containing fibers from the NTS_c (28,31). The availability of a brainstem slice containing this functionally-defined pathway (see previous section) thus affords an excellent opportunity for investigating the problem of whether this neuropeptide contributes to the generation of synaptic potentials.

As shown in the previous chapter, use of this preparation revealed a complex EPSP, in which NMDA receptors play a pivotal role and contribute to the rising phase. Surprisingly, the NMDA co-agonist glycine, in contrast to its action at other NMDA receptors (13,98,108,187,189,193), neither enhanced the NMDA component of the EPSP nor reversed kynurenate inhibition of the component. Instead, it exerted a synergistic effect with kynurenate in suppressing the EPSP by acting at a strychnine-resistant site. This unusual action of glycine suggested involvement of a transmitter besides an EAA.

In this regard, SST appeared to be a logical candidate because of its presence AMB_c afferents. Moreover, one postulated function of peptides is to modulate actions of transmitters. Therefore, the working hypothesis was that SST may function as a transmitter and/or modulator regulating the expression of the NMDA component of the EPSP. Accordingly, glycine would exert an indirect inhibitory effect on NMDA component via modulating the action of SST. To test this hypothesis, use was made of the SST depletor cysteamine (165,180,204).

5.2 Methods:

Preparations of sagittal brainstem slices and intracellular recordings of AMB_c EPSPs *in vitro* and the techniques used to record and evoke swallowing *in vivo* was as described in previous chapters.

To deplete SST content of the tissues *in vitro*, slices were pretreated with cysteamine (CSH, 200 μM) at least for one h and then recordings were carried out in the presence of CSH 200 μM in the bathing medium. Somatostatin-14 (SST; Sigma, Lot No. 77F-00572) was pressure ejected from a pipette within 100 μm of the recording site.

In vivo, NTS_c neurons were activated by topical application of muscarine (100 pmol) to the dorsal surface of the NTS. The AMB_c was located by test pulses of glutamate (6-8 pmol) pressure-ejected from a two-barrelled glass pipette (see section 4.2) followed by 3-6 ejections at 30 min intervals of CSH (0.5-1 nmol) from the other barrel of the pipette to deplete SST content in the AMB_c.

For SST immunohistochemical staining, 40 μm thick vibratome sections of the paraformaldehyde-fixed brainstem (section 3.2) were stained for SST like immunoreactivity employing the peroxidase-anti-peroxidase method. Antiserum to SST was obtained from Incstar Corp. and was used in 1:5000 dilution.

5.3 Results:

5.3.1 *SST depletion and the ambigual EPSP*

The involvement of SST in generating the ambigual EPSP was first examined by depleting the SST content of the slice with CSH. The working hypothesis predicts loss of the NMDA component of the EPSP and the ability of glycine to suppress the EPSP following depletion of SST. Results shown in Fig. 21, support this prediction. Thus, treatment of slices with CSH (200 μM) from 1 to 4 h had no effect on membrane potential (-69$\pm$7 mV, n=12) when compared with untreated slices (-70$\pm$6 mV; n=10), nor did it alter the amplitude and duration of current-evoked spikes in these neurons. However, the NMDA component of the EPSP was no longer present in any of the cells tested as shown by the ineffectiveness of AP-7 to reduce the EPSP. (Fig. 21A; n=4). Moreover, glycine (100-200 μM) failed to suppress the EPSP in 9 cells from 7 slices (Fig. 21B), although it did so invariably in untreated slices (see Fig. 19 in section 4.3). This was not due to chemical interaction between CSH and AP-7 or glycine because in two slices, CSH was washed out for up to 4 h and under these conditions in 3 out of 4 cells, both AP-7 and glycine again did not alter the EPSP. In the remaining cell, the NMDA component recovered after washing CSH and glycine was again capable of reducing the amplitude of the EPSP, including the AP-7 sensitive component.

5.3.2 *AMB_c response to exogenous SST*

To further test the possibility that SST functions as a transmitter in the generation of the AMB_c EPSP, postsynaptic responses of AMB_c neurons to exogenous SST were investigated. In about half of the cells tested, SST (0.1-0.3 pmol) evoked either a fast

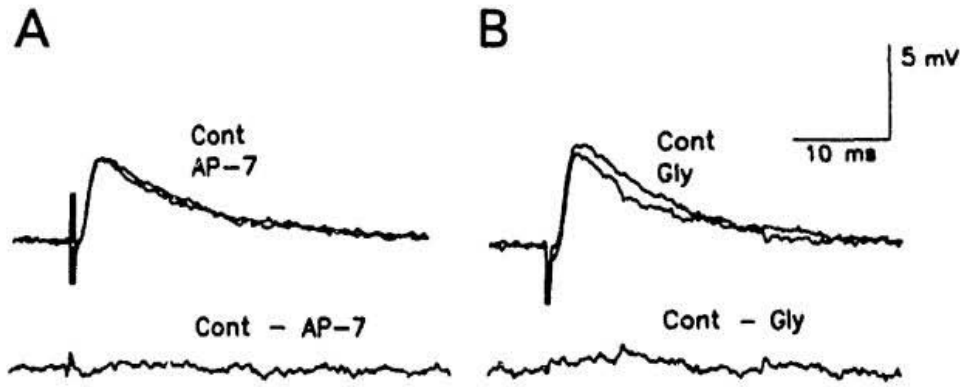


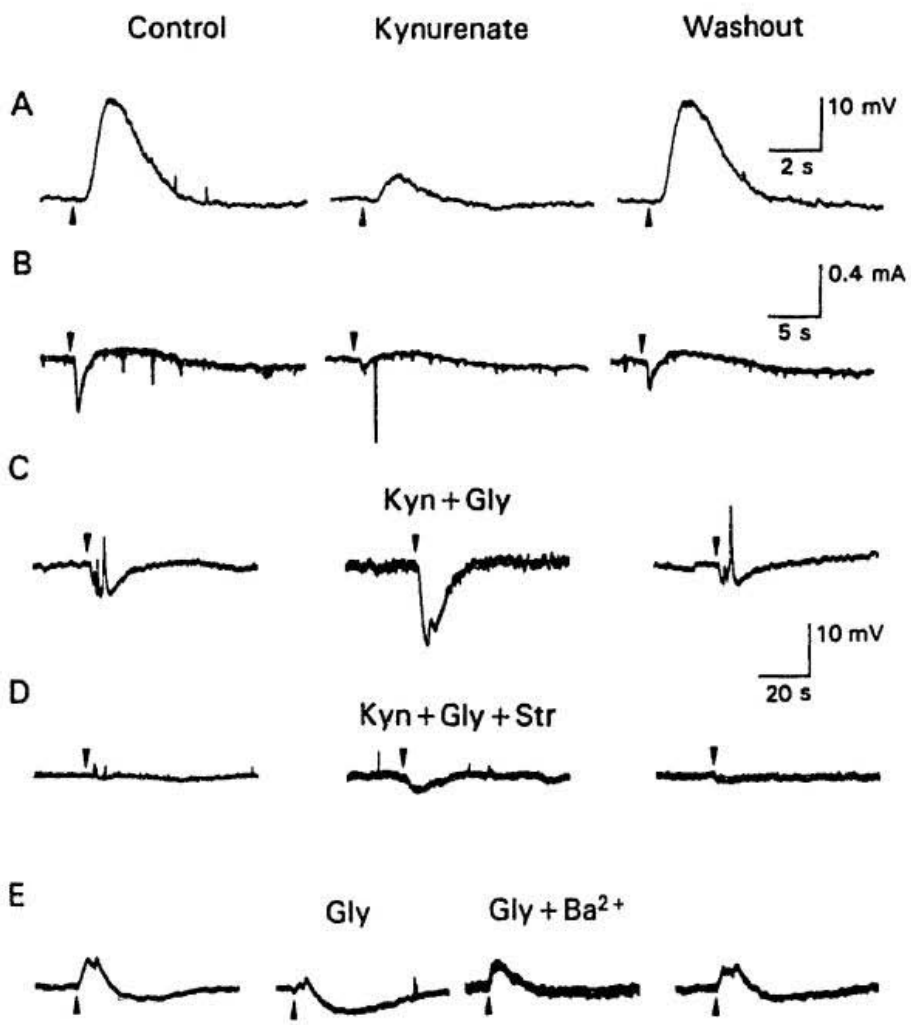
Figure 21. The effect of depleting SST content by cysteamine (CSH) on the solitario-ambigual e.p.s.p. Upper traces are averaged e.p.s.p.s of a single AMB_c neuron recorded from a CSH (200 μM, for more than 1 h) treated slice. Lower traces were obtained by subtractions as indicated. Both glycine (200 μM, A) and AP-7 (50 μM, B) fail to suppress the e.p.s.p.

(time to peak 1 s, duration 2-4 s; n=10; Fig. 22A) or a slow (time to peak 3-10 s, 40-120 s duration; n=20; Fig. 22E) membrane depolarization with or without a following hyperpolarizing component. Voltage clamp experiments revealed a corresponding fast or slow inward current with a time course matching that of the membrane depolarization (Fig. 22B). In nine cells, SST only produced a predominant hyperpolarizing response which was usually accompanied by rapid membrane potential fluctuations as shown in Fig. 22C. It should be noted that all these effects were resistant to TTX (1 μM). In the remaining cells (n=24), SST had no detectable effect on resting membrane potential or current on its own (Fig. 22D).

5.3.3 *Modulation of SST actions by glycine and kynurenate*

In light of the synergistic inhibition of the EPSP by glycine and kynurenate, their effects on AMB_c neuron responses to exogenous SST were investigated next. Bath application of kynurenate (1 mM) in the presence of TTX (1 μM) reversibly inhibited both the SST-induced fast membrane depolarization and inward current (n=5) without markedly affecting hyperpolarizing and/or slow depolarizing responses (n=13; Fig 22A and 22B). However, in the presence of glycine plus strychnine (10-1000 μM each) kynurenate not only effectively suppressed both SST-induced fast and slow depolarizing responses, but also enhanced the hyperpolarizing response (Fig. 22C; n=11). Moreover, in 3 of 4 cells on which it had no effect initially, SST produced a pronounced membrane hyperpolarization in the presence of kynurenate (1 mM) and glycine plus strychnine (50-1000 μM each; Fig. 22D). Glycine alone (10-100 μM) enhanced the SST-induced hyperpolarization and slightly inhibited the slow depolarizing response (Fig. 22E). The

Figure 22. Modification of the response to exogenous SST by kynurenate and glycine. Neurons had resting membrane potentials (RMP) ranging from -66 to -71 mV and were recorded in the presence of TTX (1 μM). **A:** Pressure ejection of SST (0.05 pmol, arrowhead) evokes a fast membrane depolarization followed by a small hyperpolarization. Bath application of kynurenate (1 mM) selectively inhibits the initial fast depolarization without significant effect on the hyperpolarizing response. Full recovery within 10 min of wash. **B:** Current response to SST under voltage clamp at a holding potential of -69 mV, I_m=0. SST evoked a fast inward current and a slow outward current followed by a late inward current. The major effect of kynurenate is suppression of the fast inward current. Partial recovery after 5 min wash. **C:** SST (0.1 pmol) produces a membrane hyperpolarization accompanied by brief depolarizing fluctuations. The combination of kynurenate and glycine (1 mM each) dramatically enhances the SST-induced hyperpolarization and reduces the depolarizing fluctuations. Recovery within 10 min of wash. **D:** Minimal response to SST becomes hyperpolarizing in the presence of kynurenate (1 mM) and glycine plus strychnine (50 μM each). Recovery after 10 min of wash. **E:** SST evokes a slow depolarization followed by a membrane hyperpolarization. Glycine (40 μM) inhibits the former and enhances the latter. Addition of 1 mM Ba^{2+} restores the depolarization and reduces the hyperpolarization. Partial recovery of the SST response after 20 min wash.

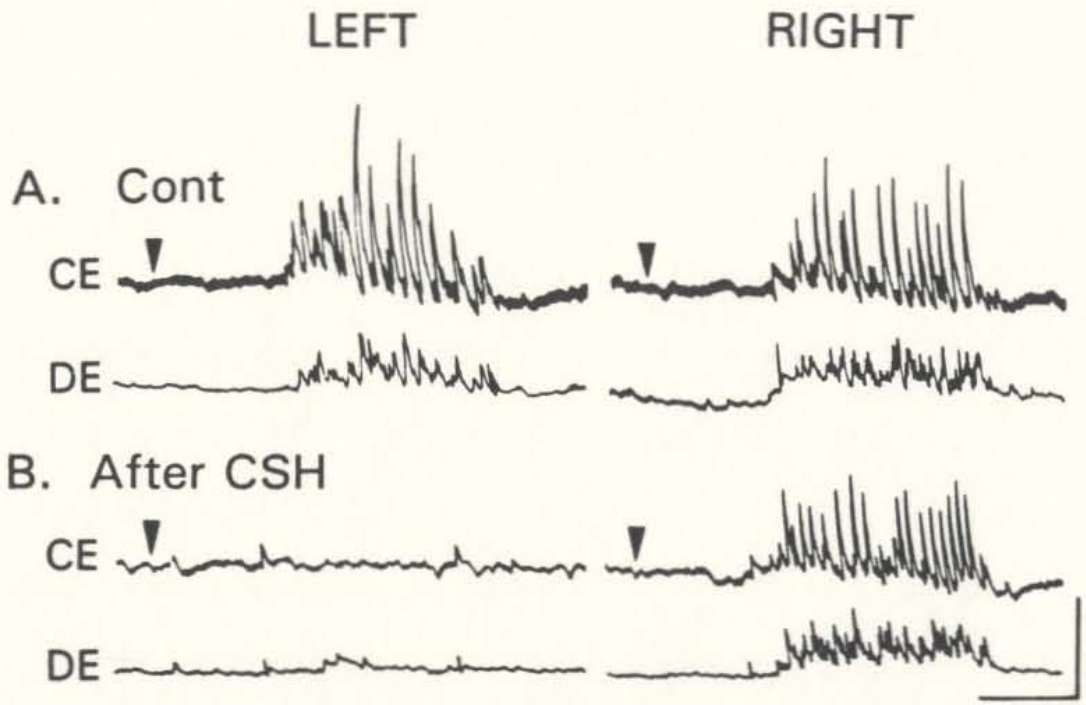

Control
Kynurenate
Washout
A
10 mV
2 s
B
0.4 mA
5 s
C
Kyn + Gly
10 mV
20 s
D
Kyn + Gly + Str
E
Gly
Gly + Ba2+

SST induced hyperpolarization, particularly its enhancement by glycine, was reduced by bath perfusion with 1-5 mM Ba^{2+} (Fig. 22E; n=3).

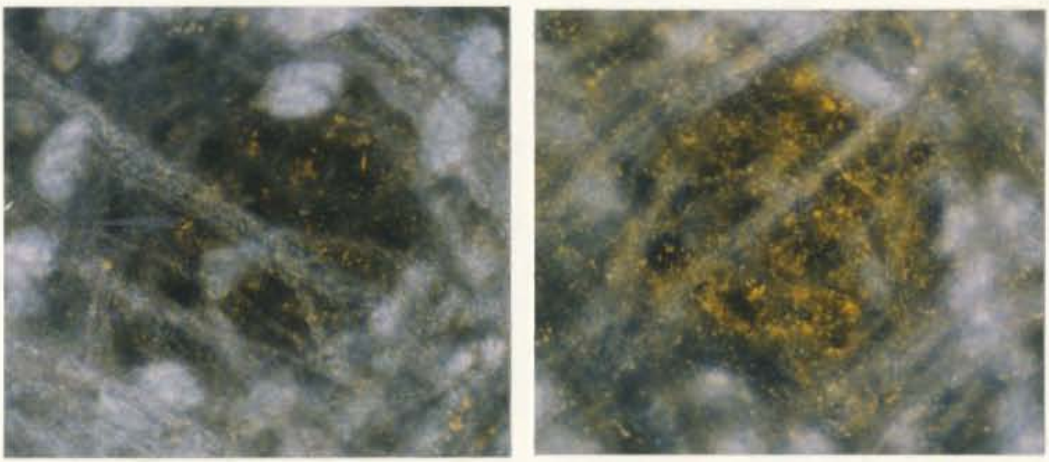
5.3.4 *SST depletion and fictive oesophageal peristalsis in vivo*

Since SST appears essential for expression of the NMDA response, which in turn is required for oesophagomotor activation (section 4.3), loss of SST input to AMB_c neurons should mimic the action of NMDA antagonists *in vivo*. This was tested by means of depleting intra-ambigual SST content by CSH *in vivo*. As shown in Fig. 23A, topical application of muscarine (50-100 pmol; n=6) to the dorsal surface of the NTS induced rhythmic oesophageal activity. Repeated unilateral injections of CSH (0.5-1 nmol) into the AMB_c did not alter basal oesophageal pressure. However, there was a progressive impairment of muscarine-evoked peristaltic activity in 5 out of the 6 animals, as evidenced by a decreased amplitude and frequency of oesophageal pressure waves and the duration of peristalsis (Fig. 23B, left). This effect was evident ipsilaterally within one h and peaked within 2-3 h, at which time there was a nearly complete loss of the oesophageal response in 4 animals and a partial loss in one. In all cases, recovery was not seen during the remaining period of observation (up to 6 h). Oesophageal peristalsis evoked from the NTS contralateral to the CSH injection was unaltered (Fig. 23B, right). SST immunohistochemistry in two of the animals revealed a marked loss of immunoreactive terminals in the injected AMB_c (Fig. 23C, left).

Figure 23. Effect of depleting ambigual SST on fictive oesophageal peristalsis. **A:** Control (CONT) responses evoked by topical application of muscarine (0.1 nmol, downward triangles) to the dorsal surface of the left and right solitary complex (NTS), respectively. **B:** Oesophageal response evoked from the left NTS is severely impaired after the third application of CSH (0.5 -1 nmol), pressure-ejected in the left AMB_c at intervals of 30 min. Note the unaffected contralateral response. **C:** Immunohistochemical staining of sections from the same animal shows a marked loss of SST immunoreactive terminals in the injected AMB_c (dark area). CE and DE are cervical and distal oesophageal pressure waves, respectively. Vertical bar represents 1.3 kPa. Upper horizontal bar represents 50 s for A and B. Calibration is 80 μm for C.



C. SST immunoreactivity in AMB_c



5.4 Discussion:

The present work supports the hypothesis that SST functions as one of the transmitters and/or modulators participating in synaptic transmission in the NTS_c-AMB_c oesophagomotor pathway. Specifically, the data presented demonstrate that SST plays an indispensable role in expressing the NMDA component of the AMB_c EPSP and this action of SST is functionally antagonized by glycine acting at a strychnine-resistant and non-NMDA receptor site. These observations, therefore, provide evidence for peptide participation in generating an EPSP in the mammalian C.N.S.

5.4.1 *SST-NMDA interaction*

One of the critical roles played by SST in solitaro-ambigual synaptic transmission is its permissive effect on expression of the NMDA-mediated component of the EPSP, as evidenced by the loss of this component following depletion of SST. This effect may explain why oesophageal peristalsis is abolished after depletion of SST in the AMB_c *in vivo*. As noted previously, activation of NMDA receptors is necessary for a functional information transfer in this oesophagomotor pathway (see section 4.3).

Several possible mechanisms may account for the permissive effect of SST. First, one of the unique characteristics of NMDA receptors is the voltage-dependent blockade by Mg^{2+} of the NMDA-activated channels, activation of NMDA receptors requiring a prior depolarizing postsynaptic potential to remove the Mg^{2+} blockade (98,131,186). SST may enable the NMDA receptor to be activated by providing such a postsynaptic membrane depolarization. Second, in analogy with the $GABA_A$ receptor, the NMDA receptor is known to have several discrete binding sites, through which ligands alter the

activity of the receptor. In this regard, SST may, like glycine, enhance the NMDA component by binding to an allosteric site on the NMDA receptor complex. Third, SST may indirectly modulate NMDA channels via a second messenger, as has been proposed for ACh modulation (128). Obviously, the exact mechanism underlying the interaction between SST and NMDA merits further study.

5.4.2 *Glycine and kynurenate modulate AMB_e responses to SST*

The present findings provide evidence for complex interactions between SST and glycine as well as kynurenate, under conditions where the latter two agents had no overt effect on resting membrane potential or current. These interactions involve both excitatory and inhibitory effects of SST on AMB_e neurons, and their persistence in the presence of TTX implicates a postsynaptic site of action.

Kynurenate, as a broad-spectrum EAA antagonist, could attenuate responsiveness to SST by withdrawal of background EAA receptor activity (166). With respect to the NMDA receptor at least, glycine should have reversed the antagonism by kynurenate (13,108,186) and partially restored the response to SST which it failed to do. Furthermore, there is no evidence that glycine modulates non-NMDA EAA receptor-gated ionic channels (98,129,189). Thus, withdrawal of background excitation does not provide an adequate explanation.

Glycine differed qualitatively from kynurenate in that it reduced the slow depolarization and enhanced the hyperpolarization induced by SST. Observations with Ba^{2+} suggest that glycine might modulate one or more SST receptor regulated potassium channels to enhance the hyperpolarizing response to SST. Accordingly, such

enhancement of the hyperpolarizing response could mask the slow depolarization. In other words, glycine may induce enhancement of the hyperpolarizing at the expense of the depolarizing component. Besides its effect on the NMDA receptor, the only other electrophysiologically characterized action of glycine is as an agonist at the strychnine-sensitive Cl^- channel (34,98). Neither of these glycine effects can account for the present findings. The hypothesis is thus proposed that glycine has yet another modulatory role, as evidenced by its interaction with SST. An intriguing feature of this modulation is the synergism with kynurenate, which operates to shift the SST excitation toward inhibition. These actions may account for the unexpected depression of the EPSP by glycine and, in particular, its synergism with kynurenate. Both glycine and kynurenate are endogenous brain constituents and along with ACh (124) may well contribute to the reported dichotomous actions of SST (44,145,156,169). Admittedly, a presynaptic action remains to be ruled out.

5.4.3 *SST Contribution to the ambigual EPSP*

Despite what might appear to be a dissociable SST component (c.f. Fig. 19), it is not yet possible to characterize a specific portion of the EPSP as somatostatinergic. SST facilitates glutamate activity. Hence loss of the SST input may well lead to indirect effects, such as loss of the NMDA component, which is in fact observed in the presence of glycine. Furthermore, glycine by enhancing the hyperpolarizing response to SST may distort remaining components of the EPSP.

Taken together, the present observations suggest that SST co-mediates, along with an EAA, the EPSP in the NTS_c-AMB_c pathway and that integrity of the SST-mediated

component is important in the production of oesophageal peristalsis. In the pathway studied, SST appears to act as both transmitter and modulator blurring the distinction between these concepts. With regard to modulation, the demonstrated interaction between SST, glycine and kynurenate may have broad physiological significance beyond the present findings.

Chapter Six

Nicotinic cholinoceptors on oesophageal motoneurons

6.1 Introduction:

Acetylcholine (ACh) applied to oesophagomotor neurons of the AMB_c produces either synchronous or propulsive contractions of the oesophageal striated muscle tunic (6). This oesophagomotor effect is resistant to scopolamine raising the possibility that ambigual motoneurons possess nicotinic cholinoceptors. To date, there is no clear evidence to support such a hypothesis. Feline spinal motoneurons have been reported to be either insensitive to ACh (35) or muscarine-sensitive (112,206), except for one observation suggesting the presence of nicotinic cholinoceptors on immature rat spinal motoneurons (97). As regards brainstem motoneurons innervating striated muscle, electrophysiological or pharmacological evidence for functional nicotinic cholinoceptors has not previously emerged. Recent immunohistochemical and *in situ* hybridization studies have shown that some cranial nerve motor nuclei, including the nucleus ambiguus (AMB), do contain nicotinic cholinoceptor-related protein (179) and mRNAs encoding different subunits of nicotinic cholinoceptors (197). Thus, branchiomotor neurons either contain or are capable of producing nicotinic cholinoceptors.

In addition, the presence of cholinoceptors in the AMB_c and ability of ACh to excite AMB_c neurons raises the question as to the source of cholinergic afferents to the AMB_c. However, as yet there is no neuroanatomical evidence demonstrating any particular group of propriobulbar cholinergic neurons projecting to this subnucleus. The same is also true for the NTS_c, an area densely supplied with choline-acetyltransferase

(ChAT)-immunoreactive terminals (164), although a peripheral cholinergic afferent input to this nucleus has recently been suggested (55,154,185).

The present experiments were undertaken to investigate nicotinic cholinoceptor-mediated synaptic transmission in the AMB_c both *in vivo* and *in vitro*. In addition, possible propriobulbar cholinergic inputs to both AMB_c and NTS_c were studied by combining retrograde tracing and ChAT immunohistochemistry.

6.2 Methods:

6.2.1 *In vivo experiments*

In vivo experiments were done on male Sprague-Dawley rats weighing 250-450 g. Procedures for recording pharyngo-oesophageal activity and chemical microstimulation of brainstem deglutitive structures were as described in previous chapters.

6.2.2 *Neuronal tracing and ChAT immunohistochemistry*

Experiments were carried out on 4 Sprague-Dawley rats weighing 250-400g. Under anaesthesia with sodium pentobarbital (40 mg/kg; ip), the animal was held in a stereotaxic frame and then intubated endotracheally (68). Swallowing and oesophageal responses were recorded as described previously (see section 2.2). NTS_c and AMB_c were located by pulses of glutamate (5-10 pmol) pressure-ejected from a two-barrelled glass micropipette (see sections 2.2 and 4.2, respectively). Fluorogold (10%) was ejected at the functionally-identified site from the other barrel of the pipette in pulses of 0.2-0.5 nl for a total of about 5-10 nl.

After a survival period of 6-10 days, the animals were anaesthetized with an overdose of urethane (> 1.2g/kg) and perfused transcardially at 120 mm Hg with heparinized saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.2. The brain was dissected out and post-fixed in the same fixative for 12-16 hrs.

The brainstem was sectioned (40 μM) in the horizontal plane on a vibratome. Sections were then processed for ChAT-immunoreactivity with the indirect immunofluorescence method. Sections were sequentially incubated in: (i) 10% normal

goat serum for 40 min at room temperature; (ii) rabbit antiserum to ChAT (Chemico, 1:1000 dilution), 24 hrs at 4° C and (iii) goat anti-rabbit serum conjugated to fluorescein isothiocyanate (FITC, Sigma, 1:100 dilution), 2 hrs at room temperature. Sections were washed 4x10 min in PBS between incubations.

Fluorogold was visualized with the aid of a Zeiss microscope equipped with ultraviolet filter set - G365 (excitation-365nm, emission 420nm). The FITC was visualized by means of a blue excitation filter set (excitation 450-490nm, emission 510nm).

6.2.3 *In vitro experiments*

For *in vitro* studies, rats weighing 80-200 g were anaesthetized with urethane (1g/kg, i.p.). The skull was rapidly opened and the brain removed. In order to preserve cholinergic afferents to the AMB_c (see neuronal tracing results described in the following section), the brainstem was blocked in a transverse plan and sliced on a vibratome containing modified artificial cerebrospinal fluid (ACSF) at 0-4°C. One or two transverse slices, 400 μm thick, containing the AMB_c were obtained. Methods used to intracellularly record AMB_c and electrically evoke ambigual EPSP are described in section 4.2.

Drugs were either pressure-ejected in volumes of 10-50 pl from a multibarreled pipette positioned within 50 μm of the recording electrode or applied by bath perfusion.

Drugs were obtained from the following sources: acetylcholine chloride, sodium S-glutamate, DL-muscarine hydrochloride, physostigmine and tetrodotoxin (Sigma); 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP) and hexamethonium (Aldrich); D-

tubocurarine chloride (CalBiochem). Dihydro-ß-erythroidine (gift from Merck Sharp & Dohme Res. Lab.). Solutions of these compounds were made in ACSF. The concentrations were as follows: glutamate, 0.2 M; ACh, 0.1-0.5 M; and 0.01 M for the remaining agents.

6.3 Results:

6.3.1 *In vivo cholinoceptor-mediated oesophagomotor effects*

Ejection of ACh (20-50 pmol) at glutamate-responsive sites consistently produced synchronous oesophageal contractions involving both the cervical and distal oesophagus, with or without a small contraction or relaxation of the pharynx (Fig. 24 and Table II). These responses were qualitatively similar to those induced by glutamate. In 29% of the sites, the cervical was larger than the distal component and in 58% of sites the distal larger than the cervical. In the remaining cases, contractions were restricted to either the cervical or distal oesophagus, or were propulsive with the cervical segment leading the distal by 1-3 s. Contraction of the oesophagus resulted in pressure waves from 0.5 to 4 kPa in amplitude, which was relatively weaker than that seen during normal swallowing (from 3 to 10 kPa). The pattern and magnitude of each contraction was relatively constant for a given site, but varied according to the position of the pipette tip within the AMB_c. The topography of motoneurons innervating different oesophageal segments within the AMB_c was not systematically studied. However, it was noted that moving the ejection site from rostral to caudal and dorsal to ventral tended to shift contractions from cervical to distal levels.

The oesophageal motor response to ACh was fully and reversibly blocked by prior ejection of dihydro-ß-erythroidine (DßE, 8-10 pmol, n=6; Fig. 24B). The blockade lasted 5 to 20 min and was repeatable. Prepulses of either D-tubocurarine (10-20 pmol, n=4) or hexamethonium (10-20 pmol, n=3) failed to depress the ACh-evoked response. DMPP (8-10 pmol; n=6) mimicked the effects of ACh, albeit with a delayed onset and

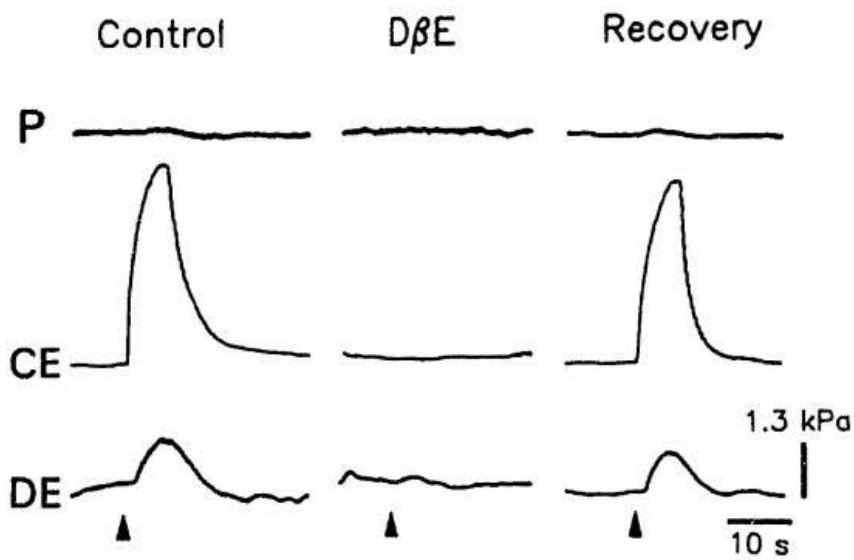


Figure 24. Oesophagomotor response to ACh. **Left:** Single pulse of ACh (40 pmol, triangle) ejected in the AMB_c evoked synchronous oesophageal activity in cervical and distal oesophagus with a small pressure wave recorded in pharynx. **Middle:** Pulses of DβE (8 pmol) 30 s prior to ACh from an adjacent barrel of the micropipette blocks the ACh-evoked response. **Right:** Recovery 25 min after DβE application.

Table II. Oesophageal responses to glutamate and cholinergic agonists

Agonists*	Dosage (pmol)	Latency (s)†	Duration (s)‡
Glutamate (8)	6-8	0.5 ± 0.05	8 ± 0.8
ACh (8)	20-50	1.3 ± 0.2	13.8 ± 1.7
DMPP (6)	6-10	6 ± 0.07	35.3 ± 4.8

*: Data were obtained either from cervical or distal pressure wave. Agonists were ejected from adjacent barrels of a micropipette inserted into compact formation of the nucleus ambiguus. Number of experiments shown in parentheses.

†: Latency corresponds to interval between ejection pulse and the foot of the oesophageal pressure wave.

‡: Interval from foot of oesophageal pressure wave to return to baseline.

prolonged duration of action (Table II). Ejections of muscarine (10-20 pmol; n=5) failed to elicit an oesophageal response.

6.3.2 *Facilitation of glutamate-evoked responses by ACh*

As both glutamate and ACh elicited similar oesophageal responses (6) it seemed pertinent to compare both agonists in more detail and to investigate possible interactions between them. The results are summarized in Table II and Fig. 25. Ejection of glutamate (6-8 pmol) in the AMB_c yielded short latency monophasic oesophageal contractions, which were qualitatively similar to those evoked by ACh except that their latencies and durations were shorter (Fig. 25). A pulse of ACh (15-40 pmol) 15-30 s prior to glutamate facilitated the glutamate-evoked contractions (Fig. 25). These facilitatory effects were dose-dependent within the dose range tested (Fig. 26A) and lasted for 1 to 3 min. It should be noted that in cases where a suprathreshold dose of ACh was used, the glutamate pulse was given at least 5 s after the ACh-evoked oesophageal pressure wave returned baseline. Prior ejection of DßE (8-10 pmol) did not alter the glutamate-induced response on its own. However, when ejected 10-15s prior to ACh, DßE significantly reduced facilitation of the glutamate-evoked response by ACh (Fig. 26B). A similar facilitation was obtained with DMPP, but not with muscarine (5-8 pmol; Fig. 26C). Ejection of an equivalent volume of ACSF at the same sites neither elicited an effect on its own nor enhanced glutamate-evoked responses. ACSF did, however, occasionally result in a small transient depression of the glutamate response.

6.3.3 *Effects of physostigmine*

The results described above are consistent with the existence of nicotinic

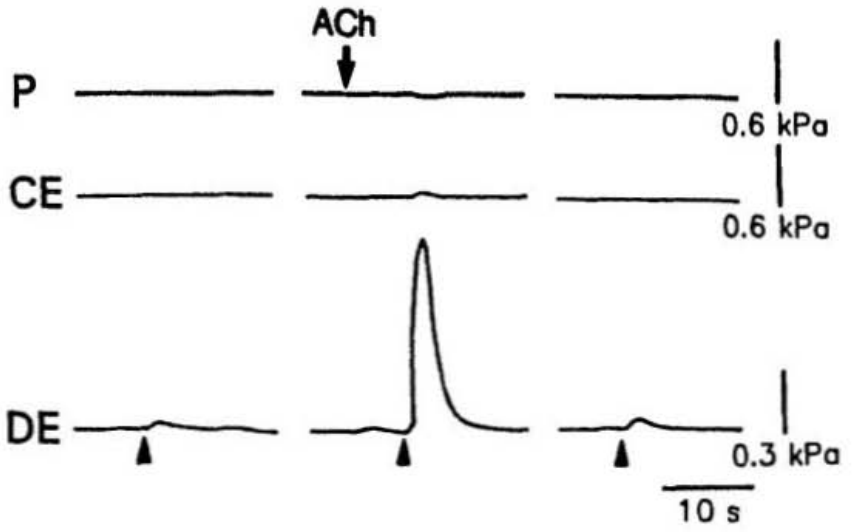


Figure 25. Facilitatory effect of ACh on glutamate-evoked response. Oesophagomotor responses evoked by glutamate (8 pmol) ejected in the AMB_c. Shown are from left to right: control response, enhanced response 7 s after application of 30 pmol ACh, and recovery 2 min later.

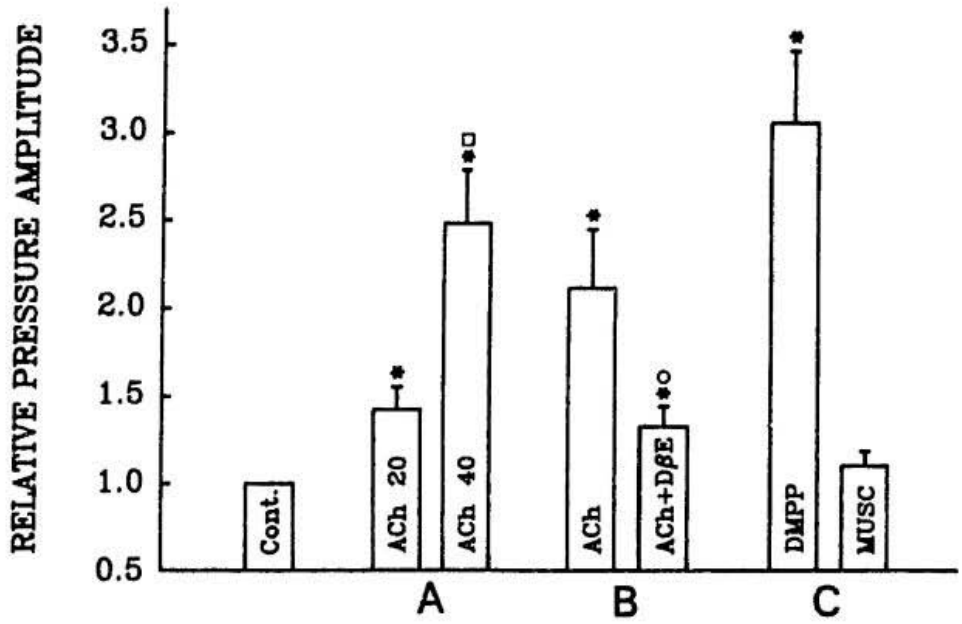


Figure 26. Enhancement by nicotinic cholinoceptor activation of glutamate-evoked oesophageal response. For each comparison (A, B, C) drugs were ejected in the ABM_c from 4-barrelled pipettes. Control response (Cont) evoked by glutamate (6-8 pmol) has been normalized. **A:** dose-dependence of the facilitatory effect of ACh (n=5). **B:** inhibitory effect of DβE (8-10 pmol) on the ACh (30 pmol) facilitatory effect (n=8). **C:** effect of dimethyl-4-phenyl-piperazinium iodide (DMPP, 5-8 pmol; n=9) and muscarine (MUSC, 5-8 pmol; n=9). *, □ and ○ denote significant difference from control ($p < 0.05$), from ACh 20 pmol, and from ACh 30 pmol, respectively.

cholinoceptors in the AMB_c region, and thus prompt the question of an endogenous source of ACh acting at these receptors. This was investigated by ejecting the indirectly acting cholinomimetic, physostigmine. As illustrated in Fig. 27, prior ejection of physostigmine (10-20 pmol, n=6), enhanced the responses elicited by glutamate (6-8 pmol). Not only was the monophasic pressure wave increased, but, in some cases a propulsive component occurred in the distal oesophagus 30 s to 5 min following physostigmine administration. Responses to ACh were enhanced in amplitude and duration by physostigmine applied either as a prepulse from an adjacent barrel (10-20 pmol, n=6; Fig. 27) or by intravenous injection (0.15-0.3 μmol; n=3). This enhancement was greater in terms of amplitude and duration and persisted longer than did the enhancement of the glutamate-evoked response (Fig. 27). The facilitating effect of physostigmine occurred in the absence of overt changes in oesophageal or pharyngeal baseline pressures or respiration.

6.3.4 *Intraambigual nicotinic cholinoceptor blockade and fictive oesophageal peristalsis*

Facilitation of the glutamate-evoked response by ACh and, more to the point, by physostigmine suggests that local ACh release modifies the excitability of AMB_c neurons via a nicotinic cholinoceptor-mediated mechanism. It seemed appropriate to examine if nicotinic cholinoceptor antagonists ejected to the AMB_c exerted any effect on oesophageal peristalsis evoked by muscarine from the NTS. As shown in Fig. 28, topical application of muscarine 50-100 pmol to the dorsal surface of the NTS induced fictive secondary peristalsis. Ejection of the nicotinic receptor blockers, DßE (n=2, 80-100 pmol) or

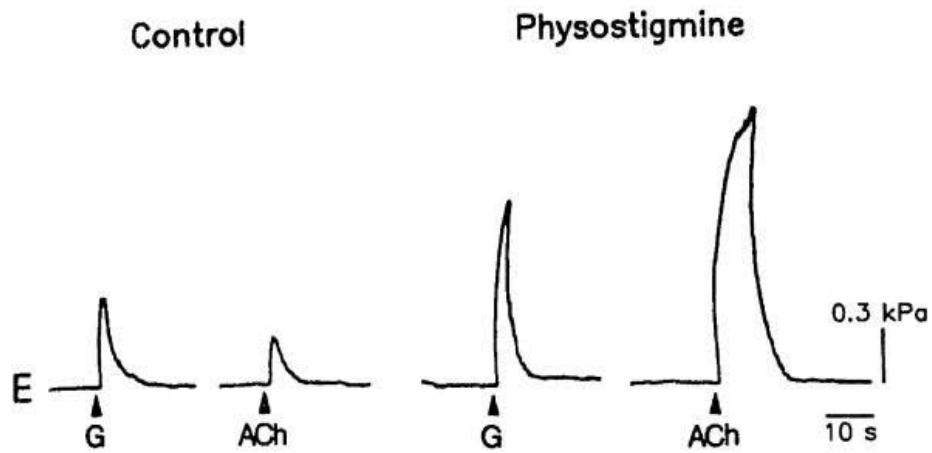


Figure 27. Enhancement by physostigmine of distal oesophageal response elicited by glutamate and ACh. **Left:** Control responses to glutamate (8 pmol) and ACh (25 pmol). **Right:** Enhanced response to glutamate at 2 min and ACh at 20 min after application of physostigmine ejected from same pipette in AMB_c.

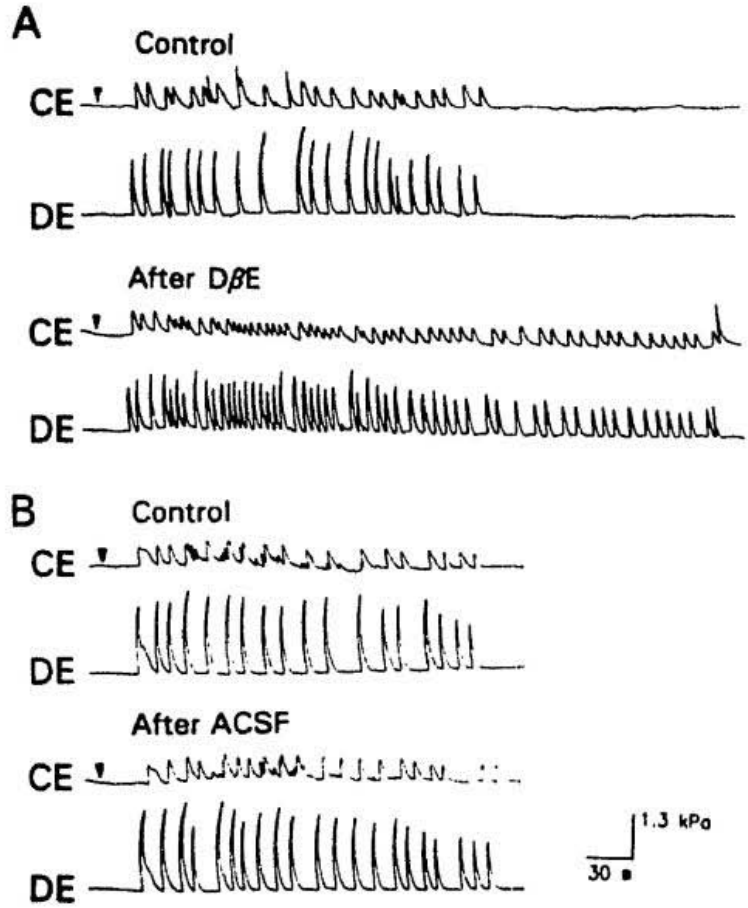


Figure 28. Effects of AMB_c nicotinic cholinoceptor blockade on fictive oesophageal peristalsis. **A:** Muscarine (0.1 nmol; downward triangles) applied to the extraventricular surface of the NTS elicits oesophageal peristalsis (control). Injection of DßE (2 nmol) into the ipsilateral AMB_c prolongs the duration of MUSC-evoked oesophageal peristalsis with increased frequency and decreased amplitude of the oesophageal pressure waves (after DßE). **B:** Injection of the same volume of artificial cerebral spinal fluid (ACSF) at the same site in the AMB_c did not alter MUSC-evoked response significantly.

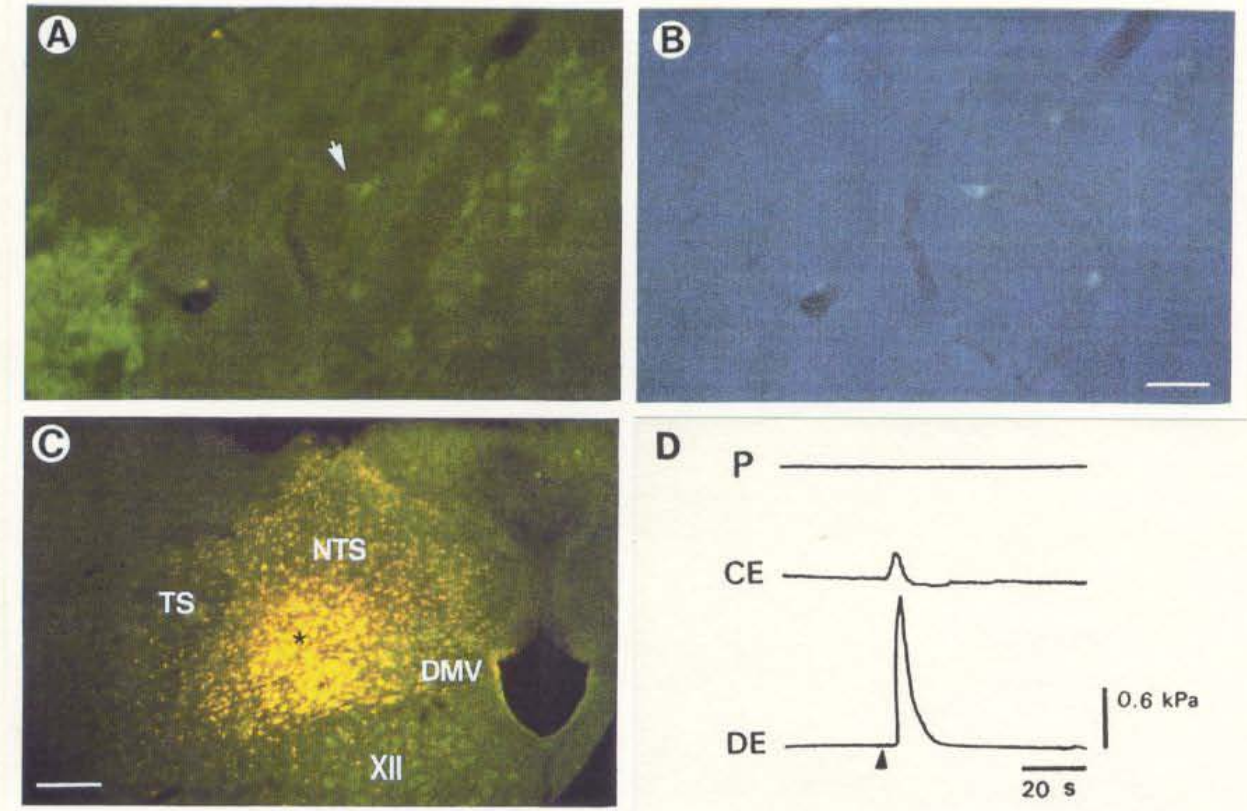
pancuronium (n=5, 80-100 pmol) at the glutamate responsive site in the ipsilateral AMB_c via an adjacent barrel of the pipette did not result in any change in basal oesophageal pressure, but altered the muscarine-evoked response markedly. Thus, within 10 min following the injection, the duration of oesophageal peristalsis evoked from the ipsilateral NTS was prolonged with decreased amplitude and increased frequency of oesophageal waves (Fig. 28A). Similar results were obtained in all animals tested (n=7). The effects were reversible within 30 to 60 min. Fictive oesophageal peristalsis evoked from the contralateral NTS was not affected. Injections of an equal volume of vehicle (ACSF) at the same site (n=4) had no effect either on resting oesophageal pressure or on muscarinic-evoked peristalsis (Fig. 28B).

6.3.5 *Retrograde tracing and ChAT immunocytochemistry*

The above results prompted further investigations into the propriobulbar cholinergic projection to the AMB_c by means of combining retrograde tracing and ChAT immunocytochemistry. Brainstem cholinergic input to the NTS_c was also investigated in light of the suggested requirement of such an input for the generation of oesophageal peristalsis (section 3).

In the medulla oblongata, ChAT-immunoreactive (ChAT-IR) neurons, in addition to their presence in all cranial motor nuclei including the AMB_c, were found throughout the parvicellular reticular formation. The largest population of ChAT-IR neurons outside of the motor nuclei (Fig. 29A) comprised a newly described group of cholinergic neurons (164,181) coextensive with the zona intermedialis reticularis parvicellularis (ZIRP). ChAT-IR neurons in this region first appeared 200 to 300 μm medial and just rostral to

Figure 29. ChAT-immunoreactive neurons in the ZIRP project to NTS. **A:** Photomicrograph under ultraviolet illumination of a transverse section showing a group of ChAT-IR neurons at the level of the rostral AMB_e (lower left corner), coextensive with the zona intermedialis reticularis parvicellularis (ZIRP) as visualised by FITC fluorescence. **B:** Same section under a filter for visualizing whitish fluorogold fluorescence showing that one of the ChAT-IR neurons in the ZIRP region (indicated by the arrow in A) is retrogradely labelled by fluorogold deposited at the locus in the NTS_e shown in C. **C:** Photomicrograph of a transverse section through the NTS_e showing the site of fluorogold injection, which yields the retrogradely labelled ChAT-IR neuron in A and B. The center of the NTS_e was identified under darkfield illumination and indicated by the '*'. **D:** Oesophagomotor response evoked by glutamate (8 pmol) delivered from the other barrel of the pipette at the site depicted in C. NTS, nucleus of the solitary tract; TS, solitary tract; DMV, dorsal motor nucleus of vagus and XII, twelfth nerve nucleus. Calibration bars represent 50 μm in A and B, 150 μm in C.


A
B
C
NTS
TS
DMV
XII
D
P
CE
DE
0.6 kPa
20 s

the AMB_c and then continued further medial up to 400 μm and rostral 650 μm, respectively. These neurons appeared ovoid or oblong in shape and about 10 μm in diameter in transverse sections (Fig. 29A).

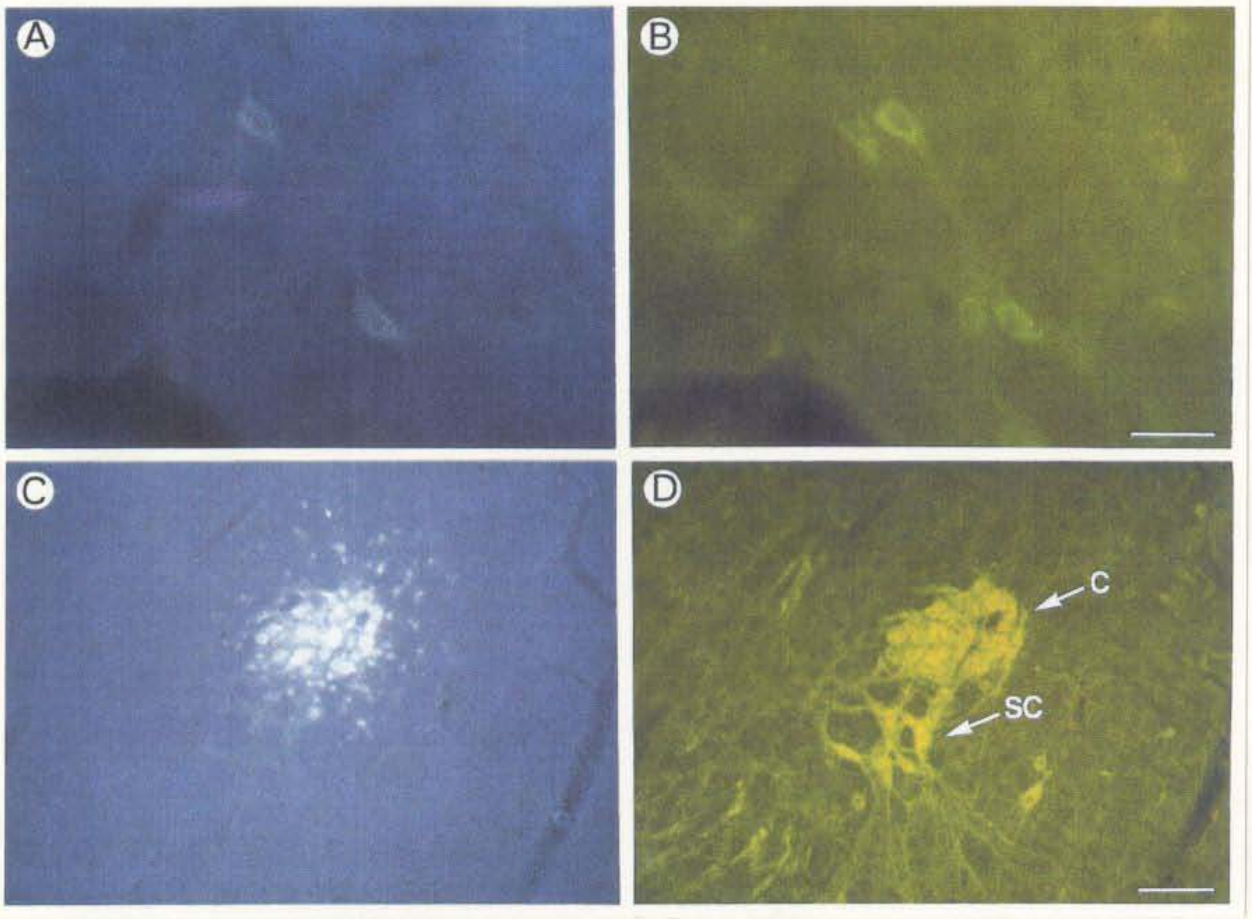
Following injections of fluorogold at functionally-defined oesophageal loci in either the NTS_c (Fig. 29 C,D) or the AMB_c (Fig. 30 C to E), retrogradely labelled neurons were found in the ZIRP region on both sides of the medulla oblongata (Fig.29 A,B and Fig. 30 A,B). Contralaterally, fluorescence of the labelled neurons appeared slightly more intense. The majority of the retrogradely labelled cells were also immunoreactive for ChAT; however, they represented only a small fraction of the total population of ZIRP ChAT neurons. For instance, in one case in which ejection of the tracer was made in the AMB_c (Fig. 30), cell counts from 16 brainstem sections, extending from the level of the AMB_c to the rostral medulla, indicated that 70% (23/32) of the fluorogold labelled neurons were double-labelled with FITC, i.e. were ChAT-IR neurons. These double-labelled neurons represented 14% (23/186) of the total population of the ZIRP ChAT-IR neurons. No obvious difference in shape or size was observed between double-labelled ZIRP neurons resulting from injections of tracer into the NTS_c and the AMB_c.

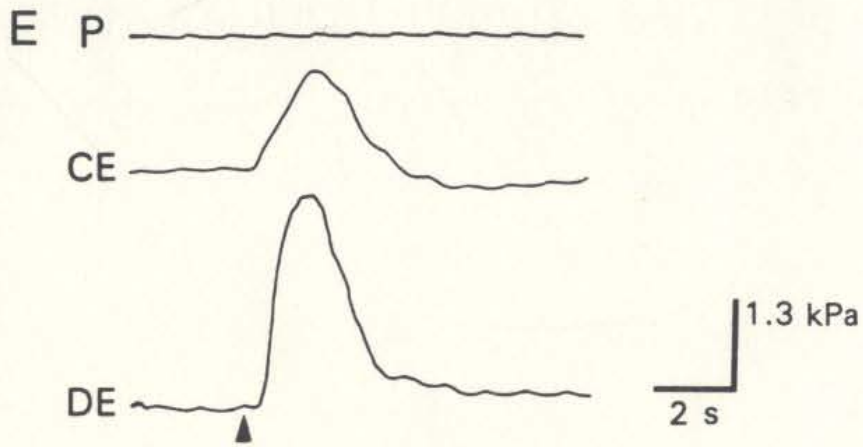
Although scattered retrogradely labelled cell bodies were also found in other regions of the medulla reticular formation, double-labelled cells were not observed outside of the ZIRP region.

6.3.6 *AMB_c neurons and cholinoceptor agonists in vitro*

To determine whether the oesophageal motor response to ACh was due to a direct

Figure 30. ZIRP neurons project to the AMB_c. Retrogradely labelled perikarya as visualised by fluorogold fluorescence (A), which are ChAT-immunoreactive as visualised by the FITC fluorescence (B), are observed in the ipsilateral ZIRP after the tracer is deposited in the AMB_c (C). Note that only neurons within the ambigual compact formation (c) but not the subjacent perikarya of the semicompact formation (sc) are labelled as seen in panel D, showing ChAT-immunoreactivity in the same section. **E:** Oesophagomotor response to a pulse of glutamate (8 pmol) evoked from the fluorogold injection site shown in E and F, which yields retrogradely labelled ChAT-IR neurons shown in A and B. Calibration bars represent 20 μm in A and B, 100 μm in E and F.

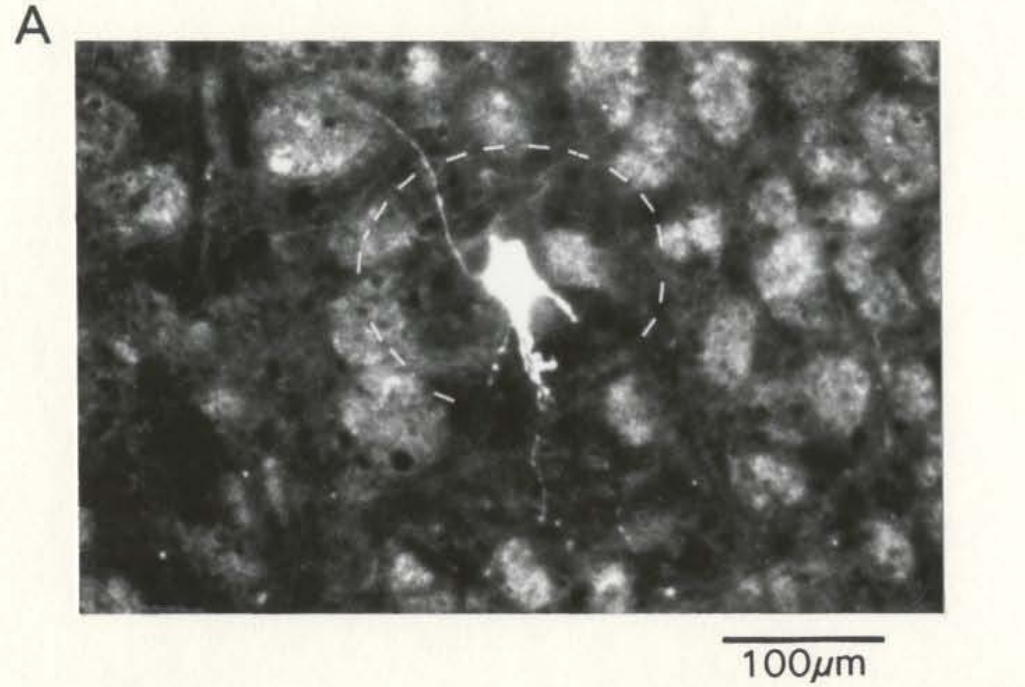

A
B
C
D
C
SC


E P
CE
DE
1.3 kPa
2 s

or indirect action on motoneurons, intracellular recordings were made from the AMB_c in brainstem transverse slices. In brainstem slices the AMB_c was readily visualized by its translucence and position with respect to the facial nucleus. All dye-filled neurons were confirmed histologically to lie within the AMB_c. A dye-filled neuron is shown in Fig. 31A. Only neurons with resting membrane potentials greater than -50 mV (-66.3 $\pm$ 2.8 mV; n=33) and capable of generating Na^+ spikes in response to intracellular current injection were included in the analysis. The apparent membrane input resistance of these cells was 42.3 $\pm$ 3.3 MΩ (n=12).

Pneumophoretic application of ACh (3-5 pmol) elicited a rapid depolarizing response in 31 out of 33 AMB_c neurons (Fig. 31B). In the remaining two cells, ACh induced a slow DßE-insensitive depolarization in one and a small hyperpolarization in the other. The fast depolarization in response to ACh was dose-dependent and, when of sufficient amplitude, was accompanied by spiking. Firing rates reached 60 spikes per s at the highest dosage tested (Fig. 31B, upper panel). Bath application of physostigmine (10^{-6} M; n=3) for 10-20 min enhanced the response to ACh. The enhancement reached its maximum within 30 min and lasted more than 2 hours after washing. The addition of tetrodotoxin (TTX, 10^{-6} M) to the perfusion medium abolished the spiking induced by ACh whilst the depolarizing response persisted at a slightly increased amplitude and duration (Fig. 31B, lower panel), presumably reflecting loss of an inhibitory input. Mn^{2+} (5 mM; n=4) applied by bath perfusion also failed to alter the response to ACh (not shown). A pulse of DßE (0.5 - 2 pmol, 10 - 20 s prior to the ACh pulse) completely and reversibly blocked the ACh-induced response. This blockade lasted from

Figure 31. AMB_e neuron response to ACh *in vitro* **A:** A lucifer yellow-injected AMB_e neuron in a transverse section visualized under UV darkfield illumination. Dashed line encircles compact formation (medial is toward left, ventral towards bottom) **B:** Depolarizing response to ACh of AMB_e motoneuron recorded in a brainstem transverse slice. Upper panel: 5 pmol pulse of ACh gives rise to depolarization and spiking (spike size attenuated by recorder frequency response) which are blocked by prior application of DßE (1 pmol). Recovery after 2 min. Lower panel: same neuron in the presence of TTX 10^{-6} M. Note that the depolarization is actually increased in amplitude and duration. DßE antagonism toward ACh persists.

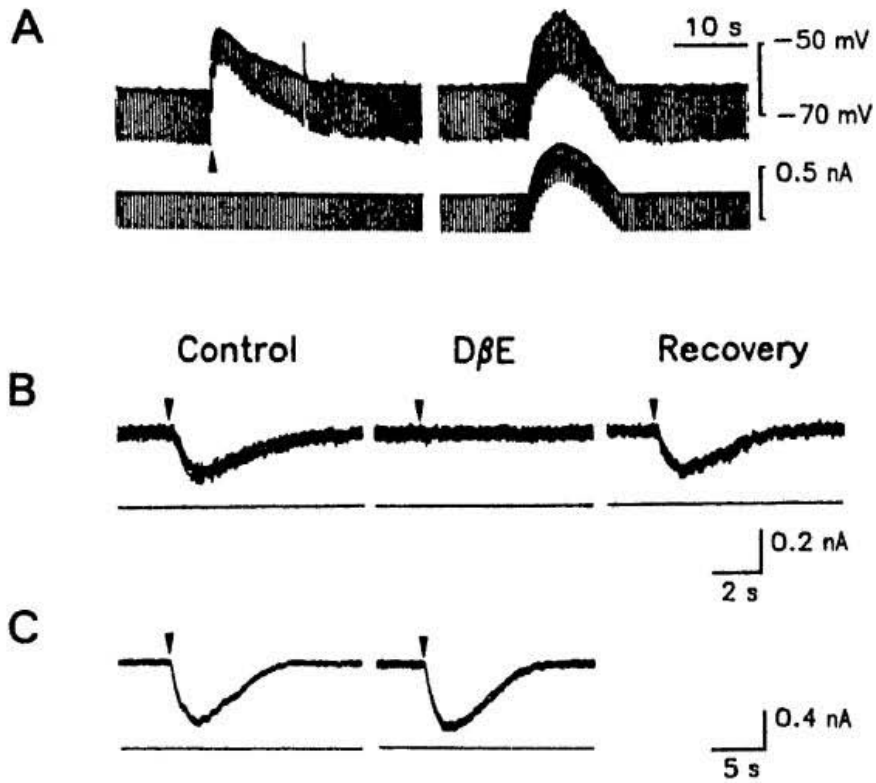

A
100μm
B
Control
DβE
Recovery
-73 mV
-76 mV
20 mV
2 s

5 to 10 min and occurred both in the absence (n=8) and in the presence (n=4; Fig. 31B, lower panel) of TTX. Besides blocking ACh responses, DßE at higher doses occasionally elicited a small transient and inconstant depolarization on its own. Ejection of an equal volume of ACSF neither elicited a response nor blocked the ACh-evoked response.

Limited testing of antagonists by bath application revealed that both hexamethonium (200-500 μM; n=6) and D-tubocurarine (10-20 μM; n=3) had effects of their own; the former producing a sustained depolarization (up to 25 mV) and the latter a moderate hyperpolarization. The sizeable depolarization induced by hexamethonium obviated evaluation of ACh-evoked depolarization in the presence of this agent; however, at the lower concentration tested, clear antagonism was evident with the ACh test pulses utilized (3-5 pmol). D-tubocurarine reduced the response to ACh by 65-80%.

As shown in Fig. 32A, application of ACh increased the apparent membrane conductance as revealed by applying hyperpolarizing current pulses through the recording electrode (40% increase; n=2). The increase in membrane conductance had a time course coincident with the depolarization. Membrane depolarization induced by current injection did not mimic the change in conductance observed with ACh application. Under voltage clamp, pulses of ACh or glutamate elicited an inward current (n=3). DßE selectively blocked the current evoked by ACh without depressing that evoked by glutamate (Fig. 32B and 32C).

Figure 32. ACh-induced conductance increase and inward current in two AMB_e motoneurons *in vitro*. **A:** Upper and lower traces show membrane potential and current injection (300 ms pulses), respectively. **Left:** Pulse of ACh (5 pmol) produces depolarization associated with increase in conductance, indicated by decreased voltage deflection in response to hyperpolarizing current pulses; **Right:** Depolarizing the neurone by current injection does not mimic the membrane conductance change observed with ACh. **B** and **C:** Voltage clamp recording from another AMB_e motoneuron showing the inward current evoked by ACh. Upper and lower traces are current and holding potential (-72 mV), respectively. Control pulse of ACh (5 pmol) evokes an inward current (left) which is inhibited by a pulse of DßE (0.5 pmol) 7 s prior to the ACh pulse (middle). ACh-evoked response recovers 2 min after DßE. **C.** glutamate (10 pmol) evokes an inward current in the same neurone (left) not blocked by DßE (right).

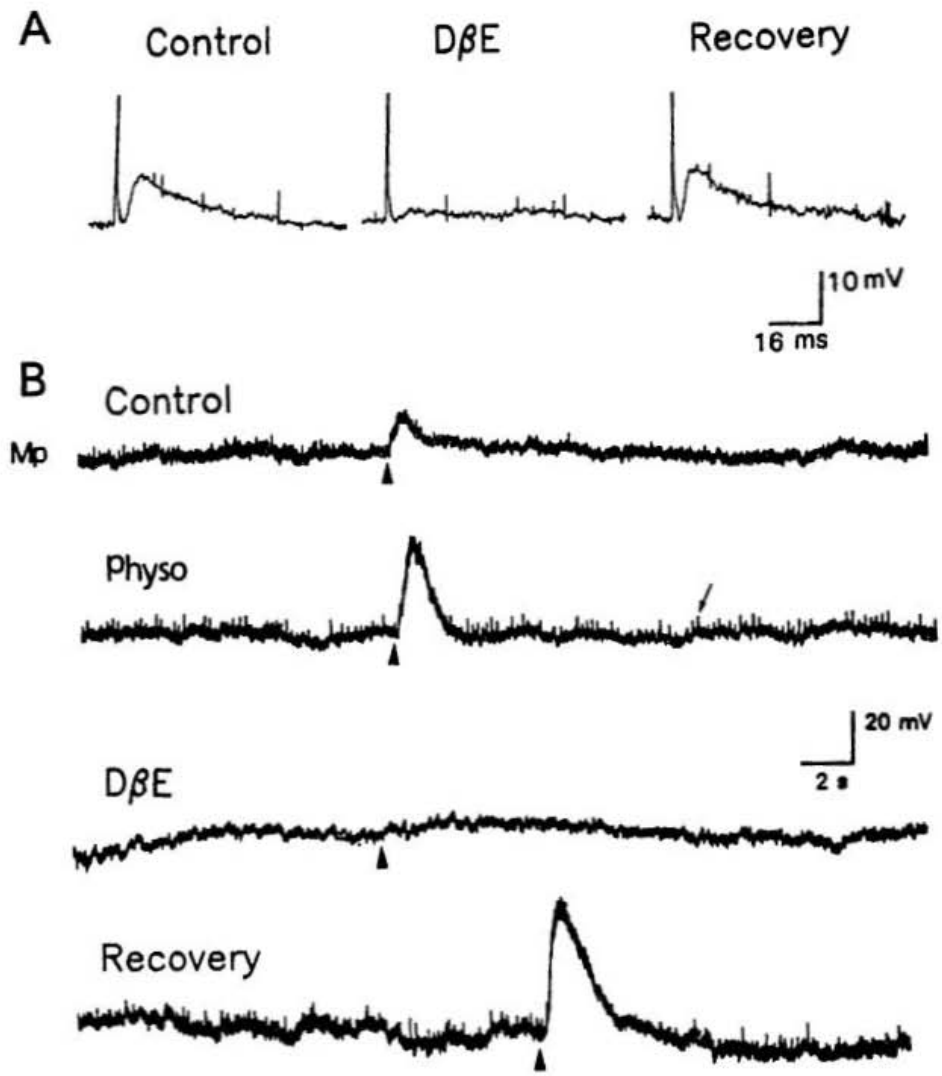

A
10 s
−50 mV
−70 mV
0.5 nA
B
Control
DβE
Recovery
0.2 nA
2 s
C
0.4 nA
5 s

6.3.7 *Nicotinic cholinoceptor-mediated EPSP*

Ambigual EPSPs in response to electrical stimulation of ZIRP region were next studied in slices. AMB_e EPSPs were only recorded in one out of three slices from three animals. As shown in Figure 33A, the amplitude of the EPSP was markedly depressed by bath application of DßE (100 μM). This inhibition was reversible; full recovery of the EPSP occurred within 10 min after wash-out of the antagonist.

Recordings from AMB_e neurons occasionally revealed spontaneous miniature EPSPs, whose amplitude and frequency markedly increased in the presence of physostigmine (10 μM) in the perfusate (Fig. 33B). Bath application of DßE (100 μM; Fig. 33B) inhibited both miniature EPSPs and the depolarizing response to pulses of ACh (3 pmol).

Figure 33. Nicotinic cholinoceptor antagonist-sensitive ambigual EPSPs. **A:** EPSP elicited in AMB_e neuron by single shock stimulation in the ZIRP region of the rostral medulla oblongata in a transverse brainstem slice preparation. Traces are averaged from 5 consecutive sweeps. DßE, 100 μM, applied by bath perfusion reversibly depresses the EPSP, which fully recovers after 20 min washout of the antagonist. **B:** DßE-sensitive spontaneous miniature EPSPs. Traces from a different neuron show resting membrane potential (Mp) and depolarizing responses evoked by pulses of ACh (10 pmol, triangles) pressure-ejected from a micropipette. Spontaneous miniature EPSP, one of which is indicated by the arrow, and ACh-induced depolarization (control) are enhanced by physostigmine (Physo, 10 μM) applied by bath perfusion and blocked by DßE (100 μM), also bath-applied.


A
Control
DβE
Recovery
10 mV
16 ms
B
Control
Mp
Physo
20 mV
2 s
DβE
Recovery

6.4 Discussion:

The present findings demonstrate that oesophagomotor neurons of the nucleus ambiguus are endowed with cholinoceptors which: (i) meet pharmacological criteria of neuronal nicotinic cholinoceptors; (ii) mediate a fast, agonist dose-dependent membrane depolarization; (iii) are subject to activation by a local source of ACh, presumably the cholinergic neurons in the ZIRP which also project to the NTS_c, and (iv) may modulate oesophageal motor output.

6.4.1 *Pharmacological properties of AMB_c nicotinic cholinoceptors*

The *in vivo* blockade of ACh responses by DßE, a neuronal nicotinic cholinoceptor antagonist (37,194), but not by D-tubocurarine or hexamethonium, suggests that the nicotinic cholinoceptors in AMB_c pharmacologically resemble those present in prefrontal cortex (194) and on Renshaw cells (36). Thus, the cholinoceptors in question might differ not only from those in peripheral tissues, but also from those described in other CNS structures, viz. the locus coeruleus (53), the medial habenula (132), the dorsal motor nucleus of the vagus (86), and spinal motoneurons (97). Neuronal nicotinic cholinoceptors in the mammalian CNS are thought to be of several subtypes (121,149,157), as yet unclassified.

The *in vitro* antagonist data are preliminary at this point and not entirely in agreement with the *in vivo* observations. It should be noted that at the high concentrations employed, the antagonists had actions of their own. For a complete pharmacological characterization of this receptor, clearly more work is required including a wider selection of antagonists, an analysis of dose-response data and tests for

competitive antagonism. The latter is necessary in view of the known channel blocking actions of hexamethonium and D-tubocurarine.

In situ hybridization techniques have revealed the existence of a gene family that encodes four different receptor subunits (α_2, α_3, α_4 and $ß_2$) of the nicotinic cholinoceptor (197). To form a functional receptor, a $ß_2$ subunit has to combine with one of the α subunits. When expressed in Xenopus ooxytes, receptors containing different α subunits differ pharmacologically (16,198), suggesting the possible presence of three subtypes of nicotinic cholinoceptors (121). Based on the *in vitro* data, the nicotinic cholinoceptors on AMB neurons reported here resemble those observed in the dorsal motor nucleus of the vagus (86) and the locus coeruleus (53), both of which contain the same subunit mRNAs as the AMB (197). The apparent discrepancy between the present *in vitro* and *in vivo* data remains to be resolved.

6.4.2 *Electrophysiological properties of AMB_e nicotinic receptors*

In keeping with other observations on neuronal nicotinic receptors in both the peripheral (110) and central (52,86,132,206) nervous system, the ACh-induced depolarization of AMB_e neurons is associated with an increased membrane conductance. Furthermore, during voltage clamp, ACh induced an inward current. Taken together, these results suggest that the ACh-evoked excitation in AMB_e motoneurons is mediated by an increased membrane conductance to cations. This mechanism may account for facilitation of glutamate responses by ACh observed *in vivo*. By reducing the membrane potential, ACh would move the cell closer to its firing threshold (52) and thus enhance glutamate excitability. However, other mechanisms such as allosteric modulation or

'unblocking' of the NMDA receptor-activated channel remain for further investigation.

It is significant that ACh-responses were evident when indirect excitatory (or disinhibitory) mechanisms requiring synaptic transmission via Na^+-dependent action potentials or Ca^{2+}-dependent excitation-secretion coupling had been eliminated by exposure to TTX or manganese. Under these conditions responses to ACh can only be generated by activation of postsynaptic nicotinic cholinoceptors on perikarya or dendrites of AMB_c neurons.

6.4.3 *Physiological role of AMB_c nicotinic cholinoceptors*

The effects of intraambigual injection of nicotinic cholinoceptor antagonists suggest that activation of this receptor plays at least two linked roles in shaping oesophageal output at the AMB_c level, increasing amplitude and decreasing frequency of oesophageal peristaltic contractions. The present experiments do not explain the mechanism through which modulation of oesophageal peristalsis by activation of nicotinic receptors is accomplished.

Increased amplitude might reflect increased synchronous excitation of individual oesophageal motoneurons in the AMB_c as a result of nicotinic receptors being activated. The dendrites of these neurons are reported to form extensive dendritic bundles (10) which may synchronize AMB_c activity via cholinergic synapses formed by AMB_c axon collaterals and efferent dendrites.

The NTS_c may generate rhythmic activity at a higher frequency than that normally expressed at the level of the AMB_c. Thus, the increased frequency of oesophageal peristalsis observed following blockade of AMB_c nicotinic cholinoceptors suggests the

role of these receptors, at least in part, is to filter this input. The underlying mechanisms remain to be determined.

Whatever the exact mechanism, the potential modifiability of oesophageal motor output by activation of nicotinic cholinoceptors suggests that AMB_c neurons do not behave merely as passively driven output elements. ACh, acting on AMB_c nicotinic cholinoceptors, might function to modulate the moment to moment oesophagomotor output at the AMB_c level.

6.4.4 *Origins of the cholinergic input to the AMB_c*

Facilitation of the glutamate evoked response by inhibiting ACh enzymatic hydrolysis and modification of muscarine-evoked oesophageal peristalsis by intra-ambigual nicotinic cholinoceptor antagonism supports the contention that local ACh release, tonically or phasically, modifies oesophageal motor output at the AMB_c level. This notion is further reinforced by the presence of DßE sensitive spontaneous miniature EPSPs. The question thus arises as to the source of this cholinergic input. AMB_c neurons receive dense afferent inputs from NTS_c premotoneurons (6,28). However, since nicotinic cholinoceptor antagonists failed to block either ambigual EPSPs evoked by stimulation of the NTS_c afferent fibers (section 4.3) or oesophageal peristalsis evoked from the NTS, it is unlikely that NTS_c neurons utilize ACh as transmitter. Moreover, ChAT immunostaining (present work) fails to reveal the presence of the cholinergic marker enzyme in NTS_c cell bodies. Instead, the present observations suggest that the cholinergic input to the AMB_c arises, at least in part, from the ZIRP region, because: (i) cholinergic neurons in the ZIRP were retrogradely labelled by depositing tracer at

functionally defined esophagomotor loci in the AMB_c and (ii) DßE-sensitive AMB_c EPSPs were evoked by stimulation of the ZIRP region in a slice preparation.

In the present study, fluorogold was delivered at a site where ejection of glutamate yielded a short latency oesophageal response. In the case illustrated in Fig. 30, the injection site is clearly identified as lying within the AMB_c. Its adjacent subdivision, the semicompact formation of the nucleus, is not invaded by the injected tracer. Thus, the method used for the functional identification of AMB_c oesophageal loci afforded considerable precision.

The presence of DßE-sensitive AMB_c EPSPs, in response to electrical stimulation of the ZIRP region, is particularly significant, although the number of observations were limited. ACh, in fact, is the first substance to be established pharmacologically as a neurotransmitter in the CNS (75). However, to date, the spinal motoneuron synapse on Renshaw cells remains the only clear example of a nicotinic cholinergic synapse in the mammalian CNS. The DßE-sensitivity of EPSPs observed in the present work tentatively implicates ZIRP neurons as the source of cholinergic input to the AMB_c.

Like other motoneurons, AMB_c neurons innervating striated muscles of the oesophagus utilize ACh as a transmitter. Thus, it is not unreasonable to suppose that nicotinic cholinoceptors on AMB_c neurons receive input from neighbouring motoneurons via axon collaterals or efferent dendrites. Similar intra-ambigual cholinergic connections have been proposed for feline laryngeal motoneurons (62). Evidence for such collateral connections in the rat remains to be determined.

6.4.5 *Projections of ZIRP cholinergic neurons to NTS_c*

Besides their projection to the AMB_c, cholinergic ZIRP neurons project to the NTS. However, it will be noted that, as illustrated in Fig. 29, the tracer injection exceeded the limits of the NTS_c, neurons in the lateral part of the dorsal motor nucleus of the vagus (DMV) being also labelled. Since dendrites of DMV cells extend dorsally into the NTS and immediate vicinity of the NTS_c (159,171), labelling of DMV cell bodies could have resulted from tracer being taken up by their dendrites. Therefore, results from the present work must be interpreted with caution. If the labelled ZIRP neurons terminate in the NTS_c, this projection might be responsible for muscarinic cholinoceptor-mediated coupling of buccopharyngeal and oesophageal stages of swallowing (Chapters 2 and 3). These ZIRP neurons may be required for programming primary oesophageal peristalsis. Thus, ZIRP neurons projecting to the NTS_c could be activated by input originating from the buccopharyngeal NPG. In support of this hypothesis, recent anterograde tracing suggests that the ZIRP region may receive inputs from the NTS_i, the location of buccopharyngeal premotoneurons (68).

Apart from the ZIRP, the NTS_c may also receive cholinergic inputs from the oesophageal primary afferents. Thus, ChAT-IR neurons are present in the vagal afferent system (154,185). Moreover, oesophageal afferents, after surgical anastomosis with the accessory nerve, reinnervate the denervated muscle originally supplied by the accessory nerve, suggesting that ACh is the transmitter (55). Given that secondary peristalsis is initiated by distention of the oesophagus (41,134,140,161), peripheral cholinergic input may well be involved in initiating and programming this type peristalsis. Thus, it may

be surmised that different sources of cholinergic input to the NTS_c may represent the major difference between the central mechanisms involved in organizing primary and secondary oesophageal peristalsis.

In conclusion, the present findings provide clear evidence for the existence of functional postsynaptic nicotinic cholinoceptors on oesophageal motoneurons of the nucleus ambiguus. ACh, acting at these receptors, modifies oesophageal motor output at the AMB_c level. These observations are consistent with the idea that AMB_c motoneuronal output represents an integrated response to solitarial and propriobulber inputs.

Chapter Seven

Interactions between SST, ACh and glutamate at the AMB_c

7.1 Introduction:

As described above, AMB_c neurons receive a cholinergic input, which presumably originates in the ZIRP and impinges on nicotinic postsynaptic cholinoceptors (Chapter 6). In addition, they receive two more prominent noncholinergic inputs from the NTS_c, utilizing an EAA-like mediator and SST (Chapters 4 and 5). The interaction of these agents at the level of the AMB_c is likely of importance in shaping oesophageal output.

In light of: (i) the postulated link between SST and the NMDA component of the ambigual EPSP (Chapter 5) and (ii) the antagonistic interactions between SST and ACh at pre- (63,64) and postsynaptic (88,145,200) levels, SST might differentially modulate cholinergic and noncholinergic excitatory inputs to AMB_c neurons.

The objective of the experiments reported below was to investigate this hypothesis with particular emphasis on SST-ACh interactions at AMB_c nicotinic cholinoceptors.

7.2 Methods:

Methods used both *in vivo* and *in vitro* experiments were similar to those described in previous sections.

7.3 Results:

7.3.1 *SST and the AMB_c responses to ACh in vitro*

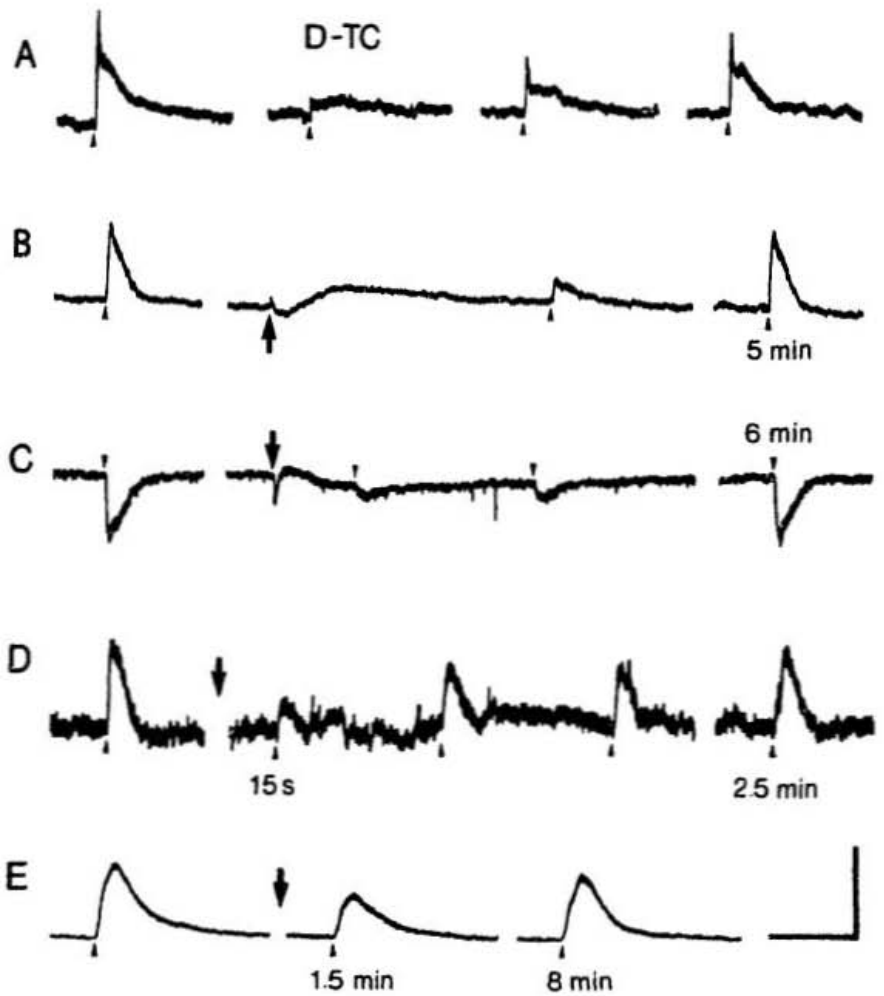
Data were obtained from 21 AMB_c neurons which had resting membrane potentials of -50 to -86 mV (-67±9 mV) and were capable of generating Na^+ dependent spikes. As described in the previous section, pneumophoretic applications of ACh elicited a rapid depolarizing response in all neurons tested which was reversibly inhibited by 10 μM D-tubocurarine, a nicotinic cholinoceptor antagonist, (n=4; Fig. 34A). Ejection of SST (0.3-0.5 pmol) evoked simple, complex or no change in the resting membrane potential (Fig. 34B, middle; see also section 5.3). However, SST applied prior to the ACh pulse reversibly inhibited the ACh-evoked depolarization, regardless of the effect of SST on membrane potential (Fig. 34B). The inhibition peaked within 15-30 s, reducing the amplitude of membrane depolarization induced by ACh to 43.1±19.2% of the control (n=10). Complete recovery was obtained within 3-10 min. In addition to the decrease in amplitude, SST slowed the rising phase of ACh-evoked depolarization.

Under voltage clamp, ACh evoked an inward current which was also inhibited by a prepulse of SST. The time course was similar to that revealed under current clamp (Fig. 34C). Furthermore, the inhibitory effect of SST on ACh-evoked responses persisted in the presence of Mn^{2+} (5 mM; n=2; Fig. 34D) and TTX (1 μM; n=2; Fig. 34E).

7.3.2 *SST and AMB_c excitability in vitro*

In contrast to the response induced by ACh, the glutamate-evoked depolarization was, to some extent, enhanced (40.2±21.4%) by prepulses of SST (0.3-0.5 pmol) in 6

Figure 34. Inhibition by SST of nicotinic cholinoceptor-mediated excitation in ambigual motoneurons. Panels A-C are intracellular records from the same neuron with a resting membrane potential of -61 mV. Panels D and E are from two other neurons with resting membrane potentials of -67 and -57 mV, respectively. Control responses are shown on the left. Time intervals refer to time after SST application. Small arrowheads indicate ACh (5-8 pmol) ejections, the bold arrows that of SST (0.3-0.5 pmol). **A:** ACh evoked depolarization is inhibited by 10 μM D-tubocurarine (D-TC) bath-applied for 6 min. The response progressively recovers at 8 and 12 min after wash-out. **B:** ACh-depolarization is inhibited following a pulse of SST and recovers after 5 min. Note triphasic complex response to SST. **C:** Current record, during voltage clamp corresponding to sequence shown in **B** demonstrates attenuation of ACh-evoked inward current by SST at a holding potential of -61 mV. The antagonism of SST towards ACh was evident in Mn^{2+} 5 mM **(D)** and TTX 1 μM **(E)** applied by perfusion. Calibration: horizontal bar 15 s; vertical bar 15 mV in A,B,D and E, 0.6 nA in C.


A
D-TC
B
5 min
C
6 min
D
15 s
2.5 min
E
1.5 min
8 min

out of 10 neurons (Fig. 35A). This is in agreement with the effects of SST on the AMB_e EPSP described in Chapter five. In the remaining 4 cells, SST did not alter the amplitude or shape of the depolarization induced by glutamate. SST enhanced the excitability of these neurons as evidenced by increased spiking during current injection through the recording microelectrode (0.6 nA, 150 ms pulses). Within 15-60 s after SST ejection, the number of spikes generated by 5 successive pulses increased from 10 to 15 (each single pulse from 2 to 3, Fig. 35B). Furthermore, the latency of the first spike was reduced from 26.2 ± 7.7 to 16.4 ± 1.9 ms.

7.3.3 *Interactions between SST, ACh and glutamate in vivo*

A similar interaction between SST, ACh, and glutamate was observed *in vivo*. As described in the previous section, pressure ejection of ACh (20-40 pmol) in the AMB_e evoked a short-latency, single nonpropulsive or propulsive oesophageal pressure wave with little evidence of desensitization when repeated at 2-3 min intervals (Fig. 36). Prepulses of SST (0.1-0.3 pmol), which produced feeble, inconsistent oesophageal pressure waves on their own, reversibly inhibited the ACh response (Fig. 36A). Complete recovery was obtained within 20 min of the SST ejection. The SST-mediated inhibition could be surmounted by doubling or tripling the duration of pressure pulse used to eject ACh (Fig. 36B). In contrast, following application of SST (0.1-0.3 pmol; Fig. 37A), the glutamate-evoked oesophageal response was enhanced. Enhanced responsiveness to glutamate coincided with the SST-ACh antagonism (Fig. 37).

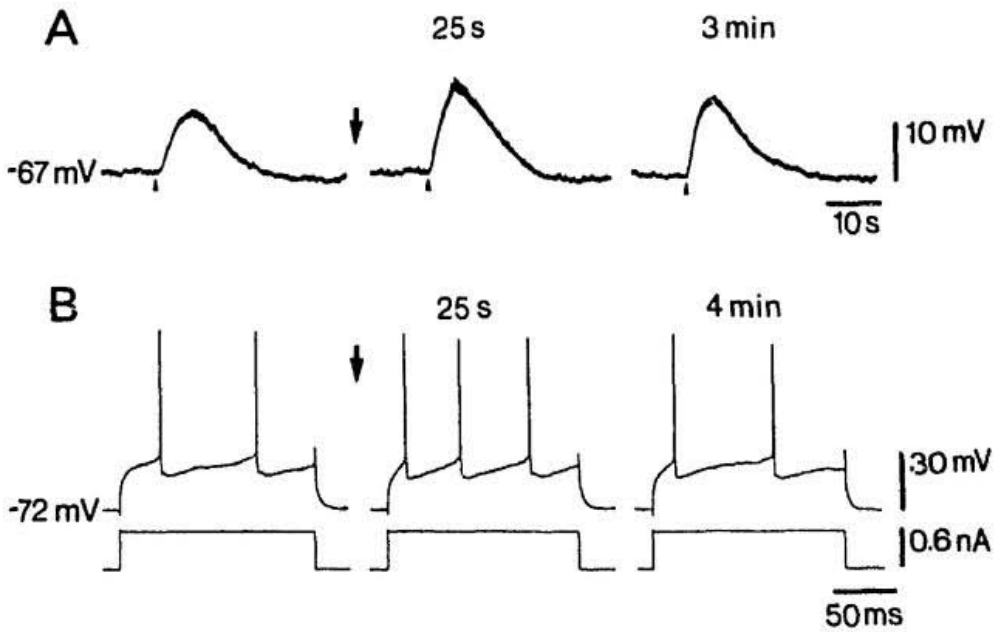


Figure 35. Increased responsiveness to glutamate and depolarizing current injection induced by SST. Control responses with resting membrane potentials are shown on left; middle and right records were taken after SST application at times indicated. **A:** glutamate pressure-ejections (6 pmol; small arrowheads) evoke depolarization which is enhanced following SST pulse (0.5 pmol; bold arrow). **B:** A depolarizing current pulse (lower trace) elicits 2 spikes during control but 3 spikes following SST pulse. Note decreased latency to first spike following SST. In the same cell a slight enhancement of the glutamate response was observed (not illustrated).

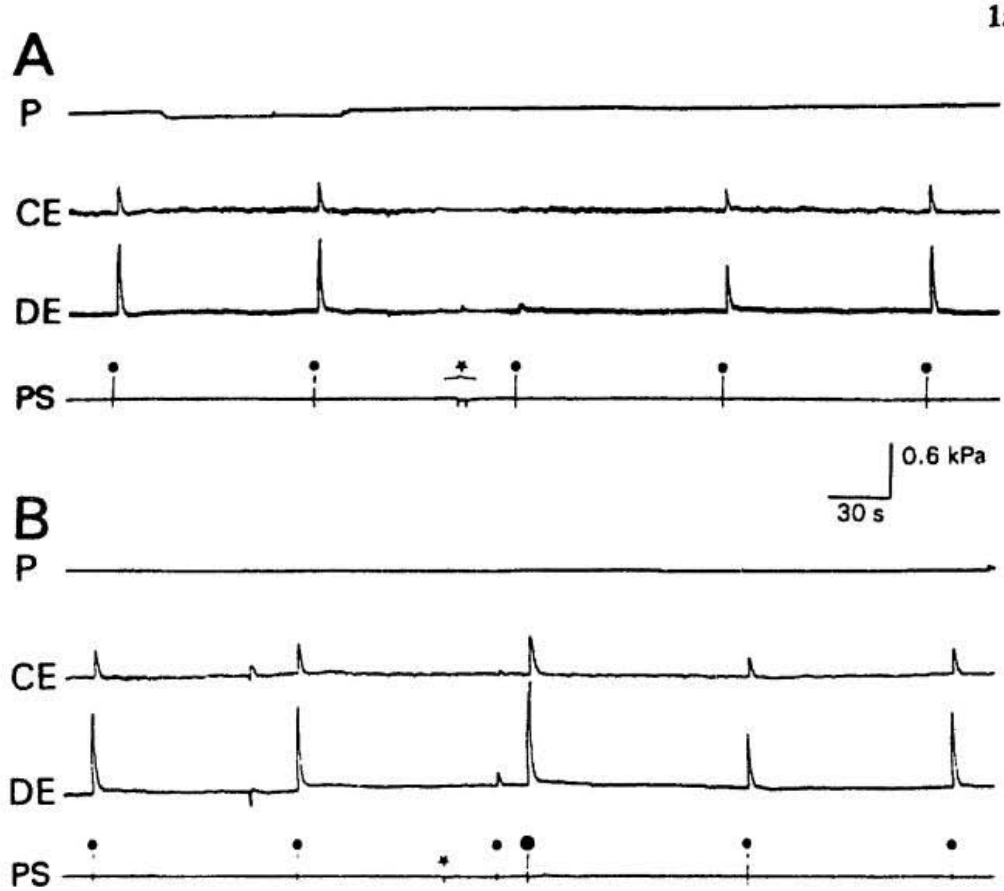


Figure 36. SST inhibits ACh-evoked activation of AMB_c oesophagomotor neurons. **A:** Continuous record showing monophasic non-propulsive oesophageal contraction induced by pressure-ejection of ACh (20 pmol, small dots) into the AMB_c at intervals of 90 s. Pulses of SST (1 pmol, star) delivered from adjacent barrel of the pipette reversibly inhibit the ACh-induced responses without causing any change in oesophageal basal pressure. In this and the following two figures, the bottom trace indicates marking pulses from the Picospritzer (PS). **B:** SST-ACh antagonism is surmountable. SST (0.5 pmol) inhibits ACh (20 pmol)-evoked oesophagomotor response, but this inhibition is surmounted by increasing pulse of ACh to 40 pmol (larger dot).

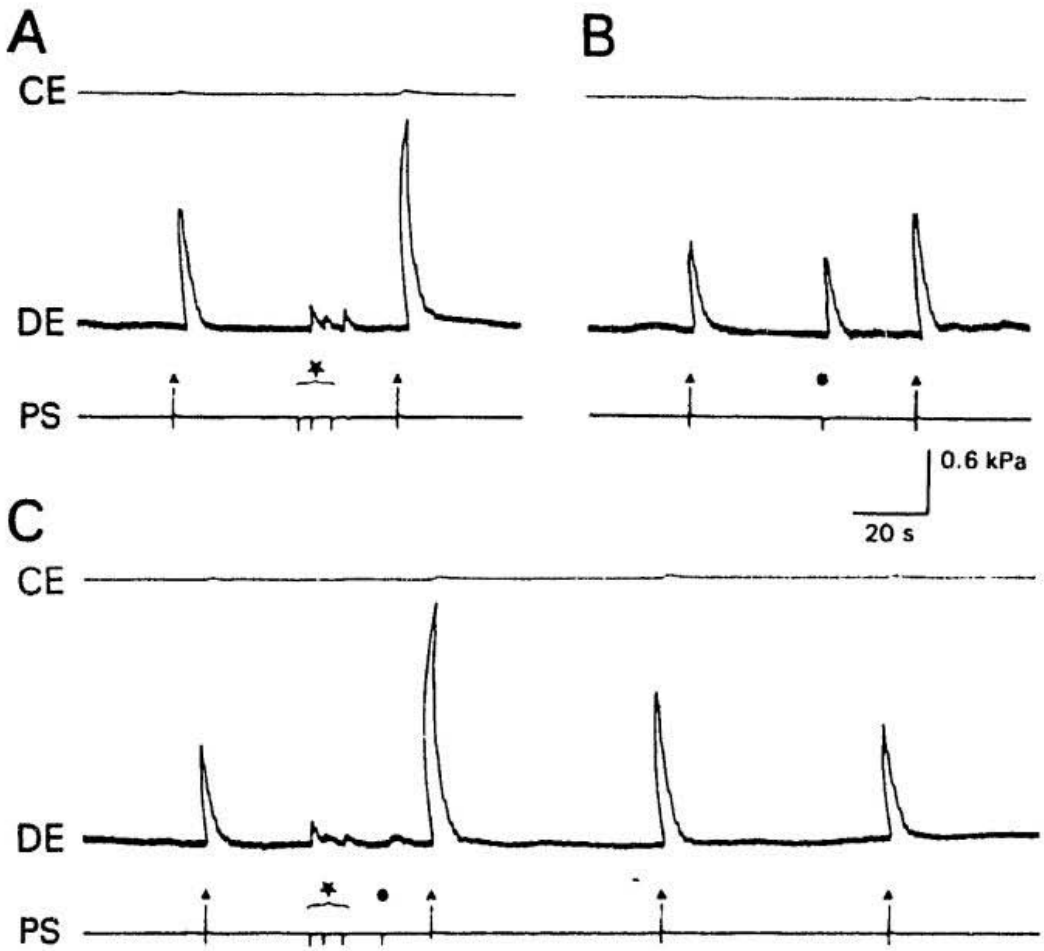


Figure 37. SST inhibition of ACh-induced oesophagomotor response coincides with enhancement of response to glutamate. Glutamate (8 pmol, triangle) pressure-ejected into the AMB_c evokes oesophagomotor responses similar to those induced by ACh (A to C). The glutamate response is enhanced by both SST (A; 1.5 pmol, star) and ACh (B; 20 pmol, dot). C: SST augments glutamate response while inhibiting that induced by ACh.

7.4. Discussion:

The results show that SST is capable of differentially modulating excitatory inputs to AMB_c neurons, i.e. SST reduces excitation of AMB_c neurons evoked by ACh acting at nicotinic cholinoceptors, while enhancing that produced by glutamate.

7.4.1 *SST antagonizes ACh at AMB_c nicotinic cholinoceptors*

Persistence of the SST-ACh antagonism in the presence of TTX or Mn^{2+} implies a direct action at the level of the postsynaptic membrane. Since this antagonism coincides with SST-glutamate synergism, SST might exert its inhibitory effect by interacting with ACh at nicotinic receptors, rather than by reducing overall excitability. The interaction between SST and ACh entails selective inhibition of an inward current, a situation analogous to the recently described SST-ACh antagonism on chromaffin cells (84). It is of interest to note that in CNS neurons under muscarinic cholinoceptor control, SST increases the M-current, a K^+ current which is inhibited by activation of muscarinic cholinoceptors (88,145,200). The common result in both these studies and the present investigation is decreased responsiveness to cholinergic input. In view of the failure of SST to inhibit glutamate-evoked AMBc excitation, the question arises as to whether this peptide serves as an endogenous inhibitory modulator of neuronal nicotinic cholinoceptor-mediated cation channels, analogous to its proposed function as endogenous 'agonist' at the M-current channel (145,200).

The precise mechanism by which SST achieves this effect remains to be elucidated. There are at least two possibilities. First, SST may interact with ACh

directly at the nicotinic receptor. The dissimilarity between the polypeptide structure of SST and known antagonists of ACh at nicotinic receptors renders it unlikely that ACh and SST simply compete for the ACh binding site. The nicotinic receptor is however known to contain more than one binding site (201). SST may exerts its modulatory effect via binding to one of the allosteric sites. Two allosteric sites appear relevant. One site is where local anaesthetics (147) achieve open channel block. The other site binds substance P (SP), which is thought to enhance the rate of desensitization of the nicotinic cholinoceptor (201). The present findings do not permit identification of the site at which SST acts. However, the inability of ACh to excite neurons following SST ejection suggests that SST does not function as a use-dependent channel blocker. Interaction at an allosteric site remains an alternative and should be investigated further.

Second, it has recently become apparent that not only voltage-activated channels, but also ligand-gated channels, such as EAA (23,66,199) and nicotinic (50,81,82,137) receptor operated channels, are modulated by second messengers. Thus, SST may act via a second messenger to modulate the nicotinic channel analogous to the proposed mechanism underlying the SP-ACh interaction (174). Several second messengers reportedly modulate the nicotinic cholinoceptor-gated channel (81,137). Among them, cAMP- (82) and a protein kinase C-dependent phosphorylation of nicotinic cholinoceptors (50) hold particular interest. Both pathways have been shown to inhibit nicotinic cholinoceptor-gated currents by increasing the rate of desensitization via phosphorylation of the receptor.

7.4.2 *SST enhances AMB_c responsiveness to glutamate*

An intriguing aspect of the inhibitory effect of SST on the nicotinic cholinoceptor-mediated response is its coincidence with enhancement of glutamate responsiveness. The synergistic interaction between SST and glutamate is in accord with the concept that SST plays an important role in NTS_c-AMB_c synaptic transmission (Chapter 5). The requirement of SST for expression of the NMDA-mediated component of the EPSP prompts the assumption that this peptide may selectively interact with NMDA receptors. However, an involvement of non-NMDA receptors can not be excluded since facilitation of the NMDA component could be secondary to an enhancement of the non-NMDA component, providing of course, that SST enhances the response of non-NMDA receptors.

The present study does not provide definitive evidence with regard to mechanisms underlying the SST-glutamate interaction. In addition to an allosteric interaction at the NMDA receptor cation channel complex, SST may enhance glutamate responsiveness by activating a second messenger. Second messenger-mediated enhancements of EAA receptor-gated currents have recently been suggested. Specifically, the NMDA current may be subject to enhancement mediated by activation of protein kinase C (23), whereas non-NMDA currents are enhanced via a cAMP-dependent mechanism (66,199). It is particularly interesting that, as noted above, the nicotinic cholinoceptor-gated current is subject to a protein kinase C- and a cAMP-mediated inhibition (50,82,137). The question arises as to whether SST exerts its differential action in modulating nicotinic cholinoceptors and EAA receptors via activation of a common intracellular second

messenger. Although plausible, at present this is no more than a conjecture.

7.4.3 *Differential modulation of excitatory inputs to the AMB$_c$ by SST*

General thought on postsynaptic modulation is dominated by the idea that modulators alter neuronal activity by modifying characteristics of voltage-gated channels (116) and consequently, alter membrane conductance of soma or dendrites. Such modulation would be relatively non-selective for incoming synaptic events on nearby regions of the membrane. In contrast, the present findings reveal that SST exerts a differential action in modulating nicotinic cholinoceptor- and EAA-mediated excitatory inputs. This suggests a different model of modulating synaptic inputs by mediator released from terminals. In this model, a modulator need not necessarily change the excitability of the cell to all synaptic inputs, but may inhibit synaptic action by one chemical mediator while leaving unaffected or even enhancing that of another. This kind of postsynaptic modulation provides a potentially important mechanism for selectively controlling the efficacy of synaptic transmission and may have general applicability. It is particularly significant for modulation of a neuronal network generating rhythmic motor activity. In one sense, such a network must be modulated or modified to provide response flexibility in the face of internal and environmental demands. In another, the network must ensure that parameters can be modulated without disrupting the network's ability to operate. In the system studied, by differentially modulating cholinergic and noncholinergic excitatory inputs to AMB$_c$ neurons, SST presumably plays a pivotal role in fine-tuning the final oesophagomotor output at the motoneuronal level, thereby ensuring the network generates the most appropriate output for the situation.

7.4.4 *AMB_c neurons shape oesophageal motor output*

The present study has demonstrated that AMB_c oesophageal motoneurons possess complex transmitter-regulated membrane conductances. These, as well as intrinsic electrical membrane properties such as postinhibitory rebound (39), endow these neurons with the ability to respond differentially and nonlinearly to synaptic inputs, thereby permitting dynamic shaping of oesophageal rhythmic output at this "final common pathway". It is clear now from results of the present study and others (109) that the concept of vertebrate motoneurons as purely passively driven followers must be revised. Instead, they should be considered as active participants involved in shaping and timing motor behaviour. Perhaps it is not unreasonable to conclude that they constitute one of intrinsic components of the NPG in generating basic motor rhythm.

Chapter Eight

SUMMARY AND SYNTHESIS

The above investigations have furnished new insights into the central organization of oesophageal peristalsis in the rat. The general objective of the analysis undertaken was to define the NTS_c-AMB_c projection as the common premotor substrate responsible for generating oesophageal peristaltic activity initiated within the CNS. Specific issues studied encompass: (i) neurotransmitter mechanisms which operate at the level of oesophageal premotoneurons to activate or inhibit the NTS_c-AMB_c central oesophagomotor pathway; (ii) the laterality of the NTS_c-AMB_c projection; (iii) the identity of the transmitters mediating synaptic transmission of this pathway and (iv) neuromodulation of the NTS_c-AMB_c synaptic transmission at the AMB_c motoneuronal level.

The results of this research are summarized as follows:

1. Solitarial GABAergic neurons acting at $GABA_A$ receptors serve multiple functions in central deglutitive control.
2. Solitarial GABA neurons exert tonic inhibition on both the buccopharyngeal and the oesophageal stage of swallowing by counteracting excitatory inputs impinging via NMDA and muscarinic receptors on pharyngeal (NTS_i) and oesophageal (NTS_c) premotoneurons, respectively.
3. Inhibition of solitarial GABA neurons underlies the postulated central mechanism

by which pharyngo-esophageal coupling is effected; conversely, activation of these neurons is in part responsible for deglutitive inhibition of the oesophagus.

4. $GABA_A$ receptor blockade in NTS_c leads to fictive secondary peristalsis which depends on endogenous muscarinic cholinoceptor activity.

5. NTS_c neurons are oesophageal premotoneurons. Activation of muscarinic cholinoceptors associated with NTS_c neurons projecting to the AMB_c constitutes the penultimate step in the generation of oesophageal peristalsis.

6. Unilateral muscarinic cholinoceptor-mediated activation of NTS_c neurons is functionally distributed to ipsilateral AMB_c motoneurons and is capable by itself of supporting oesophageal peristalsis. The apparent independence of each NPG in the organization of oesophageal peristalsis suggests that extrinsic inputs from outside the brainstem are required for coordination of the two NPGs so as to produce bilateral synchronized rhythmic activity in oesophageal motoneurons during oesophageal peristalsis.

7. NTS_c neurons projecting to the AMB_c utilize a glutamate-like substance as primary transmitter. The latter acts through both NMDA and non-NMDA receptor subtypes to excite AMB_c motoneurons.

8. Activation of the NMDA subtype contributes to the rising phase of the ambigual EPSP and is necessary for spike generation, thereby playing a critical role in fast information transfer in this central oesophagomotor pathway. These findings add substantially to the growing body of evidence for activation of NMDA receptors under physiological conditions.

9. By permitting expression of the NMDA-mediated component of the ambigual EPSP, SST functions as a co-transmitter in the central oesophagomotor pathway. This observation provides evidence for peptide participation in an EPSP in the mammalian CNS.

10. Glycine, via acting at a strychnine-resistant and non-NMDA site, modulates co-activation of this peptidergic and EAAergic input at AMB_c neurons. The physiological relevance of the glycine action remains to be elucidated.

11. Oesophageal motoneurons in the AMB_c are endowed with functional cholinoceptors, which have pharmacological and electrophysiological characteristics of neuronal nicotinic cholinoceptors. These receptors appear to be activated by a propriobulbar cholinergic input originating from the ZIRP region and are involved in fine-tuning oesophagomotor output. The exact mechanism by which activation of the AMB_c nicotinic cholinoceptors alters both amplitude and frequency of oesophageal peristalsis, initiated from the NTS_c, requires further investigation.

12. SST postsynaptically reduces nicotinic cholinoceptor-mediated excitation whereas it enhances glutamate-evoked excitation of AMB_c neurons. By differentially modulating cholinergic and noncholinergic excitatory inputs to AMB_c neurons, SST presumably plays an important role in modifying the final oesophagomotor output at the motoneuronal level, thereby enabling the network to generate the most appropriate output.

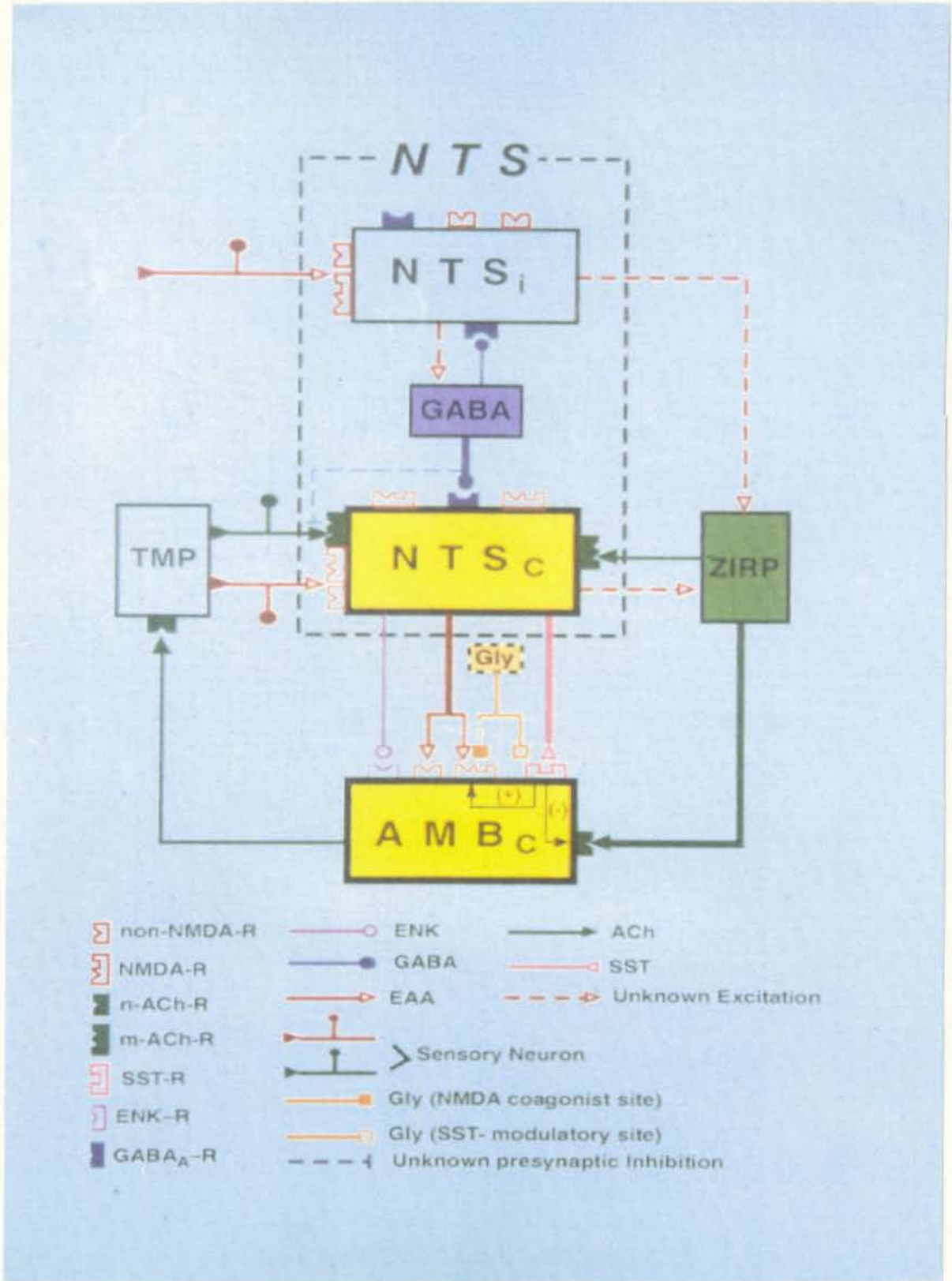
Operation of the brainstem oesophageal NPG:

Based on information provided from the present investigation and others, it is proposed that in the rat, there exists separate unilateral NPGs organizing oesophageal peristalsis. Figure 38 depicts the proposed NPG circuit.

The NTS_e contains principal components of the NPG, i.e. premotoneurons involved in initiating and programming oesophageal peristalsis. These premotoneurons receive: (i) two cholinergic inputs impinging on muscarinic cholinoceptors, which arise from the ZIRP region and oesophageal afferents respectively and (ii) an intrinsic GABAergic input acting at $GABA_A$ receptors which originates from interneurons within the NTS. Activation of muscarinic cholinoceptor is required for production of oscillatory activity in NTS_e neurons, whereas activation of $GABA_A$ receptor-mediated inhibition prevents these neurons from oscillating. Functional antagonism between these cholinergic and GABAergic inputs may occur at a pre- or postsynaptic level. Thus, oesophageal peristalsis, whether primary or secondary, is an "emergent" consequence of interactions between the $GABA_A$ receptor-mediated inhibition and muscarinic cholinoceptor-mediated excitation. The mode of operation of EAA-mediated excitatory inputs from peripheral and central sources remains to be established.

Central and peripheral neuromodulatory inputs to the NTS_e play important instructive roles in determining the moment-to-moment output of NTS_e elements by altering these excitatory and inhibitory synaptic interactions and intrinsic cellular properties of NTS_e neurons. For instance, in the case of primary peristalsis, patterned activity generated from the buccopharyngeal premotoneurons in the NTS_i is sufficient to

Figure 38. Schematic diagram of the proposed brainstem NPG for oesophageal peristalsis in the rat. For clarity, neuronal modulatory inputs other than EAA-, GABA- and cholinergic to NTS_i and NTS_e have been omitted. Note that thicker lines represent connections supported by compelling evidence. The NTS_e and AMB_e are emphasized since the present work focused on these nuclei. Abbreviations: AMB_e, compact formation of the nucleus ambiguus; NTS, nucleus tractus solitarii; NTS_e, subnucleus centralis of NTS; NTS_i, subnucleus intermedialis of NTS; TMP, tunica muscularis propria of the oesophagus; ZIRP, zona intermedialis reticularis parvicellularis; ACh, acetylcholine; m-ACh-R, muscarinic cholinoceptor; n-ACh-R, nicotinic cholinoceptor; EAA, excitatory amino acid; NMDA-R, N-methyl-D-aspartate receptor; non-NMDA-R, non-NMDA receptor; GABA, gamma-aminobutyric acid; $GABA_A$-R, GABA-A receptor; GLY, glycine; ENK, enkephalin; ENK-R, ENK receptor; SST, somatostatin; SST-R, SST receptor.


NTS
NTS$_i$
GABA
TMP
NTS$_C$
ZIRP
Gly
AMB$_C$
(+)
(-)
non-NMDA-R
NMDA-R
n-ACh-R
m-ACh-R
SST-R
ENK-R
GABA$_A$-R
ENK
GABA
EAA
Sensory Neuron
Gly (NMDA coagonist site)
Gly (SST- modulatory site)
Unknown presynaptic Inhibition
ACh
SST
Unknown Excitation

activate both cholinergic neurons in ZIRP and solitarial GABA neurons projecting to NTS_e, although activation of the latter presumably requires a higher oscillation rate of the NTS_i. Thus, when swallowing is elicited at a low rate, the cholinergic pathway is predominantly activated, enabling oesophageal NTS_e neurons to oscillate at a 1:1 coupling ratio with appropriate phase delay. At a high rate, the GABAergic pathway is activated, resulting in deglutitive inhibition of the oesophagus.

In the case of secondary peristalsis, afferent input initiates oscillation of NTS_e neurons via muscarinic cholinoceptor excitation or inhibition of $GABA_A$ receptor activity, thereby giving rise to oesophageal movements independent of buccopharyngeal activity. Lack of phasic modulatory input to the NTS_e from the buccopharyngeal NPG may, at least in part, contribute to the greater pattern variability of oesophageal movement observed during secondary as opposed to primary peristalsis.

Rhythmic motor commands programmed by premotoneurons are monosynaptically conveyed to ipsilateral AMB_e motoneurons by an array of chemical mediators. An EAA-like substance acting through both NMDA and non-NMDA receptors has a dominate role in mediating this NTS_e-AMB_e synaptic transmission. Activation of the NMDA receptor is required for functional information transfer in this central oesophagomtor pathway. By its permissive action, SST functions as a co-transmitter or synergistic mediator of EAA transmission. The former implies colocalization in the same terminal, the latter release from a separate afferent terminal. In addition, enkephalin is also present in this pathway; its actions on AMB_e neurons, however, remains unknown.

At least two other, non-solitarial, inputs to the AMB_e regulate NTS_e-AMB_e

transmission. Glycine acting at a strychnine-resistant and non-NMDA receptor-complex site modulates the action of SST at the postsynaptic level, thereby inhibiting EAA-mediated synaptic transmission. The origin of the glycine input remains to be investigated. ACh originating from cholinergic neurons of the ZIRP acts at AMB_c postsynaptic nicotinic cholinoceptors to modify oesophageal motor output during oesophageal peristalsis. The exact mechanisms by which activation of these motoneuron nicotinic cholinoceptors shapes the final oesophageal motor output and its physiological significance remain to be established.

Multiple receptor-mediated membrane conductances confer on AMB_c motoneurons substantial flexibility with respect to their response to synaptic inputs. Therefore, the AMB_c motoneuron, combined with the diversity of neurochemical inputs impinging on it, forms an important component of the NPG, actively shaping and timing the oesophageal peristalsis.

Future directions:

The present investigation has raised several important issues which should be addressed in future studies. They are outlined below:

A. The NTS_c contains elements intrinsic to the oesophageal NPG. Their proposed functions and multiple messenger systems suggest that neurons in this nucleus are not homogenous. Combined electrophysiological, anatomical and immunohistochemical studies will be required to categorize subpopulations of NTS_c neurons with respect to their neuromessenger specificity, responses to afferent stimuli and efferent discharge

patterns. It will be difficult to fully appreciate the organizational role of NTS_c neurons in the generation of oesophageal peristalsis without specific knowledge on how particular categories of neurons within the nucleus function.

B. It can be assumed that at least one subpopulation of NTS_c neurons has pacemaker or conditional pacemaker properties which provide basic rhythms for a variety of patterned oesophageal movements. Extensive electrophysiological studies are needed to elucidate intrinsic membrane properties which contribute to the rhythmic burst production in these neurons. Such studies would ultimately facilitate our understanding of the manner in which such pacemakers are turned on and off by activation of muscarinic cholinoceptors and $GABA_A$ receptors, respectively. There is also a need for further efforts to investigate interactions between muscarinic cholinoceptor-mediated excitatory and GABA-mediated inhibitory processes at both pre- and postsynaptic levels.

C. Combination of ChAT-immunohistochemistry with two retrograde tracers deposited in the NTS_c and AMB_c, respectively, would be useful in determining if cholinergic inputs to these two nuclei originate from the same or different populations of ChAT-IR neurons in the ZIRP region. This is an issue whose resolution can be expected to produce important clues to our understanding of the physiological role of these ZIRP cholinergic elements in the central control of oesophageal motility.

D. Understanding the active role of AMB_c motoneurons in generating oesophageal peristalsis will ultimately require further information concerning the intrinsic membrane properties of AMB_c neurons and the diversity of neuromediators to which these neurons are exposed. Combined whole-cell recording and neuropharmacological

studies would yield important insights into the mechanisms by which oesophageal motor output is further fine-tuned at this final common pathway.

E. Although the NPG operate without sensory input, both ongoing primary and secondary peristalsis is strongly affected by such input. How sensory feedback interacts with the NPG, as in other rhythmic motor systems, has not yet been established. Specific questions with respect to sensory inputs raised by the present work are: (i) does the sensory signal function as a physiological trigger for the oesophageal NPG through inhibiting solitarial GABA neurons which tonically suppress NTS_c neurons? (ii) do oesophageal afferents utilize ACh as transmitter and thereby initiate secondary peristalsis? (iii) is sensory input organized to coordinate activity of the two unilateral NPGs during ongoing peristalsis? As these questions are answered, the role of sensory input would be clarified with respect to how such activity is integrated with the NPG to form a complete pattern generator circuit. Thus, specifying the role of sensory inputs in the generation and maintenance of oesophageal peristalsis is clearly an important direction for future research.

As advances are made on all of these fronts, a more coherent picture will emerge of the central organization of oesophageal peristalsis.

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