

PREMOTONEURONAL ORGANISATION OF  
SWALLOWING IN THE RAT

CENTRE FOR NEWFOUNDLAND STUDIES

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**PREMOTONEURONAL ORGANISATION OF  
SWALLOWING IN THE RAT**

**By**

**© Mir Ali Hashim, M. Sc.**

**A thesis submitted to the School of Graduate Studies  
in partial fulfilment of the requirements for  
the degree of Doctor of Philosophy**

**Faculty of Medicine  
Memorial University of Newfoundland**

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*To my grandmother (Ammi Jaan)*

*and*

*My parents (Abbi and Baji)*

## ABSTRACT

The overall aim of this research was to examine the role of the neuronal solitary network in the neural organization of swallowing. The specific objectives were: 1) to map deglutitive premotor loci within the nucleus tractus solitarii (NTS), 2) to examine their pharmacological characteristics and 3) trace their intrabulbar connections. Experiments were done on anaesthetised rats utilising techniques which included micropneumophoresis, neuronal tracing and immunocytochemistry. Deglutitive loci were mapped by local applications of glutamate; the pharmacology of glutamate receptor subtypes involved in the various components of swallowing were examined; and both anterograde and retrograde tracing techniques were employed.

The results indicate that, within the NTS, pharyngeal loci extend from the level of the obex to 900  $\mu\text{m}$  rostrally overlapping the subnuclei ventralis and intermediolateralis; both cervical and distal oesophageal loci are coextensive with the subnucleus centralis of the NTS.

Based on a comparison of excitatory amino acid agonist potency and the effects of *N*-methyl-D-aspartate (NMDA)-selective and non-selective antagonists on glutamate-evoked responses, kainate (KA) receptors were found linked to pharyngeal sites whereas NMDA receptors were preferentially associated with the subnucleus centralis. Under physiological conditions, the response to NMDA

receptor-activation in the subnucleus centralis appears to be dependent in part on an intact cholinergic input directed at muscarinic cholinceptors. In contrast, the presumptive KA receptor-mediated responses at solitary deglutitive loci appear independent of an excitatory serotonergic input insofar as the involvement of excitatory 5-HT<sub>2</sub> and/or 5-HT<sub>1c</sub> receptors is concerned. The 5-HT input to the pharyngeal territory of the solitary complex originates, at least in part, from cells in the raphe obscurus, magnus and pontis nuclei.

Anterograde fibre tracing by the use of *Phaseolus vulgaris* leucoagglutinin revealed dense projections from the deglutitive loci to the pharyngo- and oesophagomotor portions of the nucleus ambiguus. In addition, solitary efferents displayed a distinct pattern of projection to other motoneuronal pools controlling the muscles of deglutition viz., the hypoglossal, the facial and its accessory nucleus, the motor trigeminal and its accessory nucleus and the dorsal vagal motor nucleus. Thus, the pharmacological data together with the neuroanatomical evidence of direct neural projections from deglutitive loci in the solitary complex to the motor nuclei involved in swallowing lend strong credence to the hypothesized role of solitary interneurons as generators of the deglutitive motor pattern.

**KEY WORDS:** Swallowing Deglutition Nucleus tractus solitarii Glutamate  
NMDA receptors Acetylcholine Serotonin Nucleus  
ambiguus Premotor neurons

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## TABLE OF CONTENTS

ABSTRACT .....	ii
ACKNOWLEDGEMENTS .....	iv
TABLE OF CONTENTS .....	vii
LIST OF FIGURES .....	xii
LIST OF TABLES .....	xvii
LIST OF ABBREVIATIONS .....	xviii
INTRODUCTION .....	1
I. Swallowing - an overview .....	1
II. Neural control of swallowing .....	5
1. The efferent limb .....	5
2. The afferent limb .....	11
3. The organisational stage .....	13
III. Pharmacology of swallowing .....	18
IV. Objectives .....	20
MATERIALS AND METHODS .....	23
I. Experimental procedures .....	23
II. Mapping .....	29



III. Pharmacology . . . . .	30
1. Micropneumophoretic application of drugs . . . . .	30
2. Topical application of drugs . . . . .	31
3. Electrical stimulation . . . . .	31
IV. Neuronal Tracing . . . . .	32
V. Immunocytochemistry . . . . .	36
1. Peroxidase-antiperoxidase method for PHAL . . . . .	36
2. Immunofluorescence technique for 5-HT . . . . .	38
VI. Chemicals and drug solutions . . . . .	40
RESULTS . . . . .	42
I. Characterisation of the solitary deglutitive loci . . . . .	42
1. Characteristics of evoked responses . . . . .	42
A. Pharyngeal responses . . . . .	42
B. Oesophageal responses . . . . .	45
C. Complete swallow responses . . . . .	49
2. Responsive loci . . . . .	49
3. Non-responsive loci . . . . .	62
II. Pharmacologic observations at solitary deglutitive loci . . . . .	63
1. Effects of excitatory amino acid receptor agents . . . . .	63
A. Excitatory amino acid receptor antagonists . . . . .	63
i) Pharyngeal responses . . . . .	63
ii) Oesophageal responses . . . . .	63
iii) Complete swallow responses . . . . .	68
a) CS responses evoked by glutamate microinjections into the NTS . . . . .	68
b) CS responses evoked by systemic quipazine . . . . .	68
c) Pharyngo-oesophageal responses evoked by electrical stimulation of the superior laryngeal nerve . . . . .	72
B. Excitatory amino acid receptor agonists . . . . .	76
i) Pharyngeal and CS responses . . . . .	76
ii) Oesophageal responses . . . . .	80
iii) Glutamate <i>versus</i> ACh (muscarine) . . . . .	80

2. Effects of 5-HT receptor agents . . . . .	91
A. Quipazine-induced swallowing . . . . .	91
B. 5-HT <i>versus</i> glutamate . . . . .	96
i) Elicitation of pharyngeal responses by 5-HT . . . . .	96
ii) Effect of 5-HT on glutamate-evoked pharyngeal responses . . . . .	101
iii) Effect of chemical microstimulation of the medullary raphe nuclei . . . . .	108
iv) Oesophageal responses . . . . .	108
v) CS responses . . . . .	111
III. Neural connections of solitary deglutitive loci . . . . .	112
1. Retrograde tracing combined with 5-HT immunocytochemistry . . . . .	112
A. Injection sites . . . . .	112
B. Retrogradely labeled perikarya . . . . .	115
C. 5-HT immunoreactivity . . . . .	115
D. Retrogradely-labeled 5-HT-IR perikarya . . . . .	118
2. Anterograde Tracing . . . . .	121
A. Injection sites . . . . .	121
B. Efferents from pharyngeal loci . . . . .	126
i) The nucleus ambiguus . . . . .	126
ii) The trigeminal motor nucleus . . . . .	131
iii) The facial nuclear complex . . . . .	132
iv) The hypoglossal nucleus . . . . .	132
v) The dorsal vagal motor nucleus . . . . .	137
C. Efferents from oesophageal loci . . . . .	140
i) The nucleus ambiguus . . . . .	140
ii) The dorsal vagal motor nucleus . . . . .	143
iii) Other motor nuclei . . . . .	143
D. Efferents from CS loci . . . . .	146
E. Solitary projections to other pontomedullary regions . . . . .	146
i) The NTS . . . . .	153
ii) Nuclei of the spinal trigeminal tract, the paratrigeminal islands, the principal sensory trigeminal nucleus and the parabrachial complex . . . . .	153
iii) "Lacrimal" neurons . . . . .	163
iv) The inferior olivary complex . . . . .	163

F. Unlabeled pontomedullary regions .....	163
G. Controls .....	166

## DISCUSSION ..... 169

I. Topography and functional characteristics of solitary deglutitive loci .....	169
1. Response types .....	169
2. Topography of the solitary deglutitive loci .....	171
II. Pharmacologic characterisation of deglutitive response types ...	178
1. Excitatory amino acids .....	178
2. Serotonergic mechanisms .....	181
3. Interaction of Glutamatergic and Cholinergic mechanisms ..	188
III. Neural connections of the NTS .....	192
1. Methodological considerations .....	192
A. Retrograde tracing and 5-HT immunocytochemistry experiments .....	192
B. Anterograde tracing experiments .....	193
2. Serotonergic afferents to solitary deglutitive loci .....	196
3. Efferents of the NTS .....	201
A. Solitary deglutitive efferents to motoneuronal pools .....	201
i) The nucleus ambiguus .....	201
ii) The trigeminal motor nucleus .....	204
iii) The facial nuclear complex .....	206
iv) The hypoglossal nucleus .....	207
v) The dorsal motor nucleus of the vagus ....	211
B. Solitary efferents to other pontomedullary structures .....	213
i) The solitary complex .....	213
ii) Nuclei of the spinal trigeminal tract, the principal trigeminal sensory nucleus, the paratrigeminal islands and the parabrachial complex .....	215
iii) "Lacrimal neurons" .....	217

C. Unlabeled pontomedullary regions . . . . .	218
i) The medullary reticular formation . . . . .	218
ii) The pontine reticular formation . . . . .	219
D. Proposed circuit model . . . . .	220
 SUMMARY AND CONCLUSIONS . . . . .	 224
Future studies . . . . .	227
 LITERATURE CITED . . . . .	 229

## LIST OF FIGURES

Figure 1	Pharyngeal response evoked by glutamate . . . . .	43
Figure 2	Oesophageal responses evoked by glutamate and ACh . . . .	46
Figure 3	Rhythmic oesophageal responses resembling secondary peristalsis evoked by muscarine . . . . .	48
Figure 4	Complete swallow elicited by pressure ejection of glutamate .	50
Figure 5	A fluorochrome-marked pharyngeal locus in the rat nucleus tractus solitarii . . . . .	52
Figure 6	A fluorochrome-marked cervical oesophageal site in the rat nucleus tractus solitarii . . . . .	54
Figure 7	Fluorochrome-marked CS and distal oesophageal loci in the rat nucleus tractus solitarii . . . . .	56
Figure 8	Fluorochrome-marked deglutitive and non-deglutitive sites . .	58
Figure 9	Map of deglutitive loci . . . . .	60
Figure 10	Effects of excitatory amino acid receptor antagonists at a pharyngeal NTS site . . . . .	65
Figure 11	Effect of NMDA receptor blockade at an oesophageal site .	66
Figure 12	Effect of NMDA receptor blockade at a CS site . . . . .	69
Figure 13	Effect of MK-801 on quipazine-induced automatic swallowing . . . . .	70

Figure 14	Blockade by DGG of quipazine-induced swallowing . . . . .	73
Figure 15	Selective blockade by MK-801 of oesophageal components of reflex swallowing . . . . .	75
Figure 16	A: Potency comparison of excitatory amino acid agonists at a pharyngeal site . . . . .	77
	B: Extent of pharyngo-oesophageal progression of a CS response evoked by pressure ejection of the three EAA agonists . . . . .	77
Figure 17	Potency comparison of excitatory amino acid agonists at an oesophageal site . . . . .	82
Figure 18	Enhancement of a glutamate-evoked oesophageal response by ACh . . . . .	83
Figure 19	Enhancement of a glutamate-evoked oesophageal response by muscarine . . . . .	84
Figure 20	Inhibition of a glutamate-evoked oesophageal response by methscopolamine . . . . .	87
Figure 21	Effect of muscarine receptor blockade on a propulsive oesophageal response evoked by glutamate . . . . .	89
Figure 22	Spontaneous oesophageal contractions evoked by intravenous physostigmine . . . . .	92

Figure 23	Blockade of quipazine-induced automatic swallowing by ketanserin . . . . .	93
Figure 24	Inhibition of quipazine-induced swallowing by 8-OH-DPAT . .	94
Figure 25	Elicitation of pharyngeal responses by 5-HT . . . . .	97
Figure 26	Selective tachyphylaxis of 5-HT-evoked responses . . . . .	99
Figure 27	Enhancement of 5-HT- and glutamate-evoked pharyngeal responses by systemic quipazine . . . . .	102
Figure 28	Facilitation of a glutamate-evoked pharyngeal response by 5-HT . . . . .	104
Figure 29	Selective blockade by ketanserin of facilitation by 5-HT of a glutamate-evoked pharyngeal response . . . . .	105
Figure 30	Effect of 5-HT on oesophageal and CS responses . . . . .	109
Figure 31	Fluorogold injection site . . . . .	113
Figure 32	Retrogradely-labeled 5-HT-immunoreactive and non-immunoreactive perikarya in the raphe obscurus nucleus . .	116
Figure 33	Retrogradely-labeled 5-HT-immunoreactive perikarya in the raphe magnus nucleus . . . . .	119
Figure 34	Glutamate-responsive pharyngeal and oesophageal sites of application of PHAL in the rat solitary complex . . . . .	122
Figure 35	A glutamate responsive CS site of application of PHAL in the rat solitary complex . . . . .	124

Figure 36	Photomicrographs showing lectin-filled neurons . . . . .	127
Figure 37	Projections to nucleus ambiguus from a solitary pharyngeal site . . . . .	129
Figure 38	Projections to the facial nucleus from a solitary pharyngeal site . . . . .	133
Figure 39	Projections to the hypoglossal nucleus from a solitary pharyngeal site . . . . .	135
Figure 40	Projections to the dorsal motor nucleus of the vagus from a solitary pharyngeal site . . . . .	138
Figure 41	Projections to the nucleus ambiguus from a solitary oesophageal site . . . . .	141
Figure 42	Projections to the dorsal motor nucleus of the vagus from a solitary oesophageal site . . . . .	144
Figure 43	Projections to the nucleus ambiguus from a solitary CS site . . . . .	147
Figure 44	Projections to the nucleus ambiguus from a solitary CS site . . . . .	149
Figure 45	Projections to the accessory trigeminal motor nucleus from a solitary CS site . . . . .	151
Figure 46	Projections to the subnucleus centralis from a solitary pharyngeal site . . . . .	154



Figure 47	Projections to the rostralateral NTS from a solitarily pharyngeal site . . . . .	156
Figure 48	Projections to the contralateral NTS ('mirror-image') from a solitarily oesophageal site . . . . .	158
Figure 49	Projections to the parabrachial complex from a solitarily oesophageal site . . . . .	161
Figure 50	Projections to the "lacrimal" neurons from a pharyngeal solitarily site . . . . .	164
Figure 51	PHAL Control . . . . .	167
Figure 52	Summary diagram of solitarily deglutitive efferents . . . . .	221

## LIST OF TABLES

TABLE I	Muscles participating in deglutition and their sources of innervation in the rat. . . . .	8
TABLE II	Volume of <i>Phaseolus vulgaris</i> leucoagglutinin (PHAL) pressure-injected at glutamate-responsive deglutitive loci in the rat nucleus tractus solitarii. . . . .	34
TABLE III	Volume of fluorogold or latex microspheres injected at glutamate-responsive pharyngeal sites in the rat nucleus tractus solitarii. . . . .	35
TABLE IV	Characteristics of deglutitive responses evoked by glutamate micropneumophoresed into the rat nucleus tractus solitarii. .	44
TABLE V	Effective dose-range (picomoles) of antagonists at deglutitive loci in the rat nucleus tractus solitarii. . . . .	64
TABLE VI	Equieffective molar doses (ED50) of EAA agonists* at deglutitive loci in the solitarius complex of the rat. . . . .	79
TABLE VII	Incidence of pharyngo-oesophageal progression observed at deglutitive loci in rat nucleus tractus solitarii. . . . .	81

## LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine (serotonin)
8-OH-DPAT	8-hydroxy-2-(di-n-propylamino)tetralin
ACh	acetylcholine
Amb	nucleus ambiguus
Amb <sub>c</sub>	compact formation of Amb
Amb <sub>sc</sub>	semicompact formation of Amb
Amb <sub>l</sub>	loose formation of Amb
AP	area postrema
APV	D,L-2-amino-5-phosphonovaleric acid
AP7	D,L-2-amino-7-phosphonoheptanoic acid
BRL 43694	(endo)-N-[9-methyl-9-azabicyclo(3,3,1)non-3-yl]-1-methyl- indazole-3carboxamide
CC	central canal
CE	cervical oesophagus
CS	complete swallow
CSF	cerebrospinal fluid
d	dorsal
DAB	3,3'-diaminobenzidine tetrachloride
DE	distal oesophagus

DGG	Gamma-D-glutamylglycine
DMX	dorsal motor nucleus of the vagus
EAA	excitatory amino acid
FTTC	fluorescein isothiocyanate
GF	gracile fascicle
ICS 205-930	(3- $\alpha$ -troponyl)-1H-indole-3-carboxylic acid ester
IR	immunoreactive/-immunoreactivity
IV	fourth ventricle
KA	kainate
MK-801	(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine
MSCP	scopolamine methylbromide
NMDA	N-methyl-D-aspartate
nSpV	nucleus of spinal trigeminal tract
NTS	nucleus tractus solitarii
NTS <sub>cen</sub>	subnucleus centralis of NTS
NTS <sub>int</sub>	subnucleus intermedius of NTS
NTS <sub>v</sub>	subnucleus ventralis of NTS
P	pharynx
PAP	peroxidase-antiperoxidase complex
PB	parabrachial complex

PBS	phosphate-buffered saline
PHAL	<i>Phaseolus vulgaris</i> leucoagglutinin
PTI	paratrigeminal islands
Py	Pyramidal tract
QA	quisqualate
R (r)	respiratory rate
r	rostral
SCP	scopolamine hydrobromide
SLN	superior laryngeal nerve
SO	superior olivary complex
SpV	spinal trigeminal tract
TFMPP	1-(3-trifluoromethylphenyl) piperazine
ts	solitary tract
V <sub>m</sub>	trigeminal motor nucleus
V <sub>m</sub> <sup>ca</sup>	ventromedial division of V <sub>m</sub>
VII <sub>m</sub>	facial nucleus
VII <sub>m</sub> <sup>ca</sup>	accessory facial nucleus
VII <sub>n</sub>	facial nerve
XII <sub>m</sub>	hypoglossal nucleus

## CHAPTER ONE

### INTRODUCTION

#### **I. Swallowing - an overview**

According to the 'biogenetic law' (Baer, 1828; Haeckel 1868), ontogeny recapitulates phylogeny. If the biogenetic law were to hold for motor development as well as for morphogenesis, it would predict motor functions during ontogeny to emerge the earlier the more ancient their origin. The early appearance of swallowing during foetal development (Humphrey, 1964; Mistretta and Bradley, 1975) suggests the primordial nature of this motor synergy.

Swallowing has been the subject of scientific enquiry since antiquity. One of the first authoritative accounts is that of Galen (165-179 A. D.) which, although partly based upon animal experimentation, remained entirely speculative and dogmatic, being guided by deductive argumentation. Accurate descriptions of the act of swallowing date back to the 1600's (Harvey, 1628, cited from Doty, 1968). More than two-and-a-half centuries elapsed before it was proposed that this complex act must be under the control of a "center of deglutition" (Wassilieff, 1888 and Marckwald, 1889, cited in Doty, 1968; Meltzer, 1899). The search for this elusive swallowing centre is still continuing.

Comprehensive reviews on deglutition have been compiled in previous years (Ingelfinger, 1958; Doty, 1968; Diamant and El-Sharkawy, 1977; Hockman *et al.*, 1979; Miller, 1982, 1986, 1987; Roman, 1982, 1986; Hiimae and Crompton, 1985; Carpenter, 1986; Kennedy and Kent, 1988). Therefore, the present dissertation will not attempt a detailed survey of the literature but concentrate on the major current concepts of the neural control of swallowing.

Swallowing may functionally be divided into three stages, the preparatory (linguopalatal), the pharyngeal and the oesophageal (Magendie, 1813, cited from Miller, 1982).

In the linguopalatal stage, food is rendered suitable for swallowing by intraoral manipulation and mastication. Although this stage of swallowing is predominantly under voluntary control, current evidence suggests that particular patterns of oral stimuli facilitate repetitive jaw and tongue movements (e.g. Willigan *et al.*, 1986).

The pharyngeal stage is initiated as the bolus makes contact with specific receptive zones of the oropharynx (Miller and Sherrington, 1916). Two principal movements are then performed by the pharynx: an elevation of the whole pharyngeal tube followed by a descending peristaltic wave (Negus, 1943; 1948; Shelton *et al.*, 1960). The latter consists of sequential contractions of the pharyngeal constrictors, which propel the bolus through the pharynx.

Simultaneously, the larynx moves up and is pulled forward under the root of the tongue.

The pharyngeal stage of swallowing is characterised by a complex pattern of muscle contractions that proceeds in an all-or-none sequence. The muscles that discharge at the onset of the pharyngeal stage (the "leading complex"; Doty and Bosma, 1956) include: superior constrictor, palatopharyngeus, palatoglossus, posterior intrinsic tongue muscles, styloglossus, stylohyoid, geniohyoid and mylohyoid. The activity of the digastric, genioglossus, geniohyoid and the mylohyoid forms a tensed floor for the oral cavity and the tongue base. Against this tensed floor, the posterior tongue contracts and is forced backward against the food. This posterior movement of the tongue appears to be caused in part by powerful activity of the hyoglossal musculature (Hiimae and Crompton, 1985).

The final stage of the pharyngeal phase is the propulsion of the bolus from the pharynx into the oesophagus. The pharyngoesophageal junction, consisting of the cricopharyngeal muscle of the inferior pharyngeal constrictor and the muscles positioning the cricoid cartilage into which the inferior pharyngeal constrictor inserts, normally remains closed passively by elasticity of surrounding tissues, according to the analysis by Doty (1968). Whether or not the cricopharyngeal muscle is also tonically active remains somewhat controversial. However, it is generally agreed that the cricopharyngeal muscle must relax to allow the bolus to enter the oesophagus (see Miller, 1982 for review).



Entry of the bolus into the oesophagus marks the initiation of the oesophageal stage of swallowing. Meltzer (1899; 1907) divided oesophageal peristalsis into primary and secondary types. The oesophageal components of swallowing, consisting of propulsive oesophageal contractions, are termed 'primary' (or deglutitive) peristalsis. The oesophagus also displays peristalsis independently of swallowing in response to a local stimulus such as balloon distension. Such peristaltic contractions of the oesophagus are termed 'secondary' peristalsis. Yet another type of peristalsis is seen which is termed 'tertiary' (Cannon, 1907; Jurica, 1926, cited from Roman, 1982) or 'autonomous' (Roman, 1982). This is the term applied to peristaltic contractions displayed by the smooth muscle oesophagus in the absence of extrinsic innervation.

When swallows are elicited in quick succession, the oesophageal component remains inhibited until the last swallow following which primary peristalsis resumes (Meltzer, 1899). This phenomenon has been termed 'deglutitive inhibition' (Helleman and Vantrappen, 1967; Helleman *et al.*, 1974).

It is generally agreed that, at rest, the lower oesophageal (gastroesophageal) sphincter remains closed due to a tonic contraction of the sphincteric muscles (see Ingelfinger, 1958; Helleman and Vantrappen, 1974). Relaxation of this sphincteric region occurs shortly after initiation of the pharyngeal stage (see Doty, 1968 for a review of the literature). The latency of this relaxation is relatively short so that it invariably occurs well before the oesophageal peristaltic wave approaches

the area. Thus, it appears that the gastroesophageal junction is an entity separable from the other stages of swallowing insofar as the organisation of deglutition is concerned. This statement is further supported by the observation that deglutitive inhibition does not extend to the gastroesophageal junction: the latter remains open during repeated swallowing and until the oesophageal peristaltic wave of the last swallow passes through the opening (Kronecker and Meltzer, 1881, cited from Doty, 1968).

## **II. Neural control of swallowing**

With the recognition of swallowing as a reflex synergy (Bidder, 1865, Blumberg, 1865, Waller and Prevost, 1870, and Wassilieff, 1888, cited from Doty, 1968; Miller and Sherrington, 1916), three separate levels of the swallowing pathway have traditionally been discerned:

1. The efferent limb.
2. The afferent limb.
3. The organisational stage.

### **1. The efferent limb**

The efferent limb of the deglutitive neural pathway represents the coordinated output of cranial nerves V, VII, IX, X and XII. Motoneurons

controlling the oropharyngeal and oesophageal muscles are distributed in the brainstem in several pools beginning from the level of the trigeminal motor nucleus and extending at least as far caudally as the first cervical segment of the spinal cord. Within this distribution, two major groupings can be distinguished, the dorsally located hypoglossal nucleus and the ventrally located column of special visceral motor nuclei innervating the branchiomeric and striated oesophageal musculature. Contrary to the analysis of Doty (1968), specific grouping of motoneurons active in deglutition is apparent, at least within some of the branchiomotor nuclei (e.g. the principal column of the nucleus ambiguus; the accessory nuclei of the facial and trigeminal motor nuclei; see Table I). The locations of motoneurons innervating the major muscles participating in swallowing in the rat are listed in Table I.

The source of the extrinsic innervation of the oesophageal smooth muscle has been a subject of debate. Physiological evidence has attested to the presence of an extrinsic source of nerve supply to the smooth muscle oesophagus (Janssens *et al.*, 1976; Janssens, 1978; Tieffenbach and Roman, 1972). Pharmacological evidence (Bartlett, 1968; Kamikawa and Shimo, 1979) indicates the innervation to be of the general visceral efferent type and, therefore, points to the dorsal motor nucleus of the vagus (DMX) as the source. This is supported by histological and tracing studies (Marinesco and Parhon, 1907; Coil and Norgren, 1979; Niel *et al.*, 1980; Hudson and Cummings, 1985; Vyas *et al.*, 1987).

TABLE I

Muscles participating in deglutition  
and their sources of innervation in the rat.

Muscle	Location of motoneurons	Peripheral nerve branch	Reference
Pharyngeal constrictors	Semicompact division of nucleus ambiguus (Amb <sub>sc</sub> )	Pharyngeal branch of Xth nerve	Bieger and Hopkins, 1987.
Intrinsic laryngeal muscles	Loose formation of nucleus ambiguus (Amb <sub>l</sub> )	Recurrent laryngeal	*
Cricothyroid muscle	Overlaps rostral portion of Amb <sub>sc</sub>	Superior laryngeal	*
Stylopharyngeus muscle	Tip of nucleus ambiguus	IXth nerve	*
Mylohyoid, anterior belly of digastric	Ventromedial division of trigeminal motor nucleus (V <sub>m</sub> <sup>tr</sup> )	Posterior trunk of mandibular branch of Vth nerve	Sasamoto, 1979; Székely and Matesz, 1982
Stylohyoid, posterior belly of digastric	Accessory facial nucleus (VII <sub>af</sub> <sup>n</sup> )	Stylohyoid and digastric rami of VIIth nerve.	Székely and Matesz, 1982; Shohara and Sakai, 1983.

TABLE I - continued

Muscle	Location of motoneurons	Peripheral nerve branch	Reference
Styloglossus, hyoglossus	Dorsal subdivision of hypoglossal nucleus (XII <sub>m</sub> )	Styloglossal and hyoglossal rami of XIIth nerve	Krammer <i>et al.</i> , 1979.
Genioglossus	ventromedial subdivision of XII <sub>m</sub>	Genioglossal ramus of XIIth nerve	"
Geniohyoid	ventrolateral subdivision of XII <sub>m</sub>	Geniohyoidal ramus of XIIth nerve	"; Kitamura <i>et al.</i> , 1983
Thyrohyoid	Spinal portion of XII <sub>p</sub> and ventral horn of C1	First cervical spinal nerves	Kitamura <i>et al.</i> , 1983.
Oesophageal striated muscle	compact division of nucleus ambiguus (Amb <sub>c</sub> )	Cervical vagus nerve	Bieger and Hopkins, 1987.
Oesophageal smooth muscle	Dorsal vagal motor nucleus (?)	Xth nerve	Vyas <i>et al.</i> , 1987.

In addition to this parasympathetic nerve supply, histofluorescence microscopic demonstration of a well developed adrenergic ground plexus in the muscularis mucosae of the cat or rhesus monkey (Baumgarten and Lange, 1969) suggests the existence of an extrinsic sympathetic innervation of the smooth muscle oesophagus. However, the exact source of this innervation remains obscure.

The origin of the central nervous control of the lower oesophageal sphincter is even more uncertain due to the paucity of experimental data. Ongoing investigations (Vyas *et al.*, 1987; in preparation) demonstrate that, in the rat, the spinal portion of the dorsal vagal motor nucleus contains neurons retrogradely labeled following injection of tracer into the gastroesophageal junction, thereby indicating this motor nucleus to be the source of the smooth muscle component of the rat lower oesophageal sphincter. It should be pointed out that smooth muscle in the lower sphincteric region of the oesophagus interdigitates with striated muscle fibers in the rat (Marsh and Bieger, 1987) and the guinea pig (Thomas and Trounce, 1960).

Intrinsic innervation of the oesophagus derives from the plexuses of Meissner and Auerbach which are well developed in the smooth muscle of the oesophagus (Kuntz, 1947, cited from Roman, 1982). Intramural neurons of the myenteric plexus of Auerbach were generally believed to be relay neurons interposed between the vagal efferent fibers and the smooth muscle. However, the presence of these intramural neurons also in the striated muscle portion of the

oesophagus refute such a simple explanation of their role. On the other hand, the inherent ability of the oesophageal smooth muscle to display autonomous peristalsis as well as the results from a plethora of studies on this preparation (e.g. Bieger and Triggle, 1985; see Roman, 1982 for literature) indicate the profound influence the intramural neurons must exert on oesophageal motility.

Several electrophysiological recording studies have been performed on the activity of the motoneurons of the hypoglossal, ambiguus and trigeminal motor nuclei during swallowing (Sumi, 1964; 1969; Car and Jean, 1971; Jean, 1972a; 1978; 1984; Amri *et al.*, 1984; Kessler and Jean, 1985a; Car and Amri, 1987; Amri and Car, 1988; Tomomune and Takata, 1988). The data indicate that the motoneurons display a burst of spiking activity during swallowing evoked by stimulation of the superior laryngeal nerve (SLN) but with synaptic delays (5-8 ms) that suggest the pathway to be polysynaptic. In addition, data suggest that the synaptic inputs to the motoneurons are not purely excitatory; for example, in hypoglossal motoneurons, a complex combination of inhibition and excitation is often observed during swallowing (e.g. Tomomune and Takata, 1988). Following a stimulus pattern delivered via the SLN, the motoneurons demonstrate two stages of depolarisation (Sumi, 1969): the first of these is probably due to the polysynaptic input while the second is accompanied by greater depolarisation, displaying a spiking discharge for 200-400 ms. The latter probably represents the synaptic drive of the central pattern generator for swallowing.

## 2. The afferent limb

Various receptive regions in the oropharynx can elicit deglutition upon appropriate stimulation (Miller and Sherrington, 1916). Originally discovered by Bidder (1865) and Blumberg (1865), the nerve that elicits swallowing most readily upon electrical stimulation in many species including cat, dog, monkey, sheep and rat is the superior laryngeal nerve branch of the vagus (Doty, 1951; Doty and Bosma, 1958; Miller, 1972; Jean, 1972a; Bieger *et al.*, 1977; Weerasuriya *et al.*, 1980). Other nerve routes are also effective in giving rise to reflex swallowing. The IX nerve, when electrically stimulated, evokes swallowing (Reid, 1837, cited from Miller and Sherrington, 1916) though less readily than the SLN in most species (Doty, 1951; Priima, 1958, cited from Miller, 1982). In the dog, bilateral sectioning of the IX nerve impairs deglutition (Ogura *et al.*, 1964) but does not affect swallowing in human (Ballantine *et al.*, 1954), indicating the variation between species. In the cat, Weerasuriya *et al.* (1980) showed that simultaneous stimulation of the IX and the SLN results in spatial summation *i.e.* an increased response (number of swallows) as compared to stimulation of a single nerve alone. In the macaque, the afferent pathway carried by the Vth nerve has been reported to be the primary one for the initiation of swallowing (Kahn, 1903, cited from Doty, 1968).

Electrical stimulation of the lingualis-chorda nerve has been reported to inhibit swallowing (Miller and Sherrington, 1916).



Since other synergies (vomiting, coughing, vocalisation) can also be evoked by electrical stimulation of the SLN, it is believed that the sensory code eliciting swallowing comprises a particular stimulus pattern. This is supported by the observation that electrical stimulation of the SLN releases separate synergies at different frequencies with a swallowing optimum of 30-50 Hz in various species (Doty, 1951; Miller, 1972; Weerasuriya *et al.*, 1980) and 20-40 Hz in the rat (Bieger *et al.*, 1977). Exactly what comprises the diverse sensory codes and how they are deciphered by the central programs organizing the different synergies remains unclear.

Peripheral sensory feedback is important not only for triggering the central program for swallowing, but also for continually modifying it depending upon the size and consistency of the bolus during its transit in the course of a swallow or a series of swallows (e.g Hrychshyn and Basmajian, 1972). Unlike the opossum and the rhesus monkey (Janssens *et al.*, 1976; Janssens, 1978), the dog and baboon (Longhi and Jordan, 1971; Janssens *et al.*, 1973; Roman and Tieffenbach, 1972; Diamant and El-Sharkawy, 1977 for review) depend on sensory input from the cervical oesophagus for the aboral progression of the peristaltic wave. Peripheral sensory feedback is especially important in the elicitation of the pharyngeal stage of neonatal deglutition (Sumi, 1967; 1975).

Sensory deglutitive information is relayed to the brainstem mainly via the IXth and the Xth cranial nerves in most species. The nucleus tractus solitarii

(NTS) is a major recipient of afferent information from the upper alimentary tract and thoracoabdominal viscera. The afferent termination fields in the NTS from the pharynx and the oesophagus have been very well characterized in the rat (Bieger and Hopkins, 1986; Altschuler *et al.*, 1989). These authors showed that the palatopharyngeal afferent representation overlaps the intermediate and interstitial subnuclei of the NTS while the oesophageal afferentation is coextensive with the central subnucleus of the NTS.

In the rat, afferents carried by the SLN terminate within the interstitial dorsal intermediate, and rostral half of the central subnucleus. In the lamb, SLN afferentation is seen in the interstitial, medial, ventral and ventrolateral subnuclei (Sweazey and Bradley, 1986) and conceivably includes the intermedialis. In the cat, the SLN was found to project to the dorsolateral, intermediate and interstitial subnuclei (Lucier *et al.* 1986). In the rabbit, SLN afferentation overlaps "central and lateral" portions of the NTS (Hanamori and Smith, 1989), conceivably including the subnuclei intermedialis, ventralis, interstitialis and centralis.

### 3. The organisational stage

The concept of a functional swallowing centre was advanced a century ago (Wassilieff, 1888; Marckwald, 1889; Meltzer, 1899) and has been described in neurophysiological terms by Doty (Doty, 1951, 1968; Doty *et al.*, 1967). A centre or pattern generator may be defined as "a group of neurons whose coordinated

action produces a stereotyped response" (Miller, 1982). In other words, the inherent organisation of this neuronal circuit produces the patterned response by directing appropriate excitation-inhibition to the motoneurons. The principle of central pattern generation is either well established or strongly indicated for a number of behaviours throughout the animal kingdom (For reviews, see Delcomyn, 1980; Grillner, 1985; Bässler, 1986; Harris-Warrick and Flamm, 1986). Such behaviours include swimmeret beating in crayfish (Wiersma and Ikeda, 1964); locomotion in lamprey (Grillner *et al.*, 1987; Buchanan *et al.*, 1989); locust flight (Wilson, 1961); breathing in cat (Wang *et al.*, 1957); and masticatory movements in guinea pig (Nozaki *et al.*, 1986), to name but a few.

By definition, the operation of a pattern generator should not depend upon peripheral afferent input. During reflex swallowing in adult cats, the basic sequence of motoneuronal activation remains essentially unchanged following either physical or anaesthetic-induced sensory deprivation (Doty and Bosma, 1956; Miller, 1972a). Indeed, the very existence of a 'deglutitory automatism' (Bieger, 1981; see below) attests to the existence of a central pattern generator for swallowing.

At the turn of this century, the split-brainstem experiments of Ishihara (1906) gave rise to some concepts regarding the "swallowing centre". Following midline splits in the medulla of dogs and rabbits, Ishihara (1906) demonstrated that swallowing could be elicited in such preparations only on the side ipsilateral to the SLN stimulation. From these experiments emerged the concept that the

swallowing centre consists of two independent 'half-centres' one on each side of the brainstem, each of which is capable of supporting "unilateral swallowing", a phenomenon that was subsequently confirmed in cat and monkey (Doty *et al.*, 1967). The lesion experiments of Doty *et al.* (1967) suggested, in addition, that, throughout the sequence of swallowing, each 'half-centre' excites and inhibits various components of the other via cross-connections that run 'at levels posterior to the obex and at the level of the trapezoid body'.

Various structures have been imputed to subserve the important function of coordinating the firing of the different motoneurons so as to generate the spatiotemporal pattern that characterizes the act of swallowing. Prominent among such postulated "swallowing centres" are the medullary reticular formation (Doty *et al.*, 1967; Manchanda and Aneja, 1972; Sumi, 1974); the pontine reticular formation (Holstege *et al.*, 1983); and the nucleus tractus solitarii and adjacent reticular formation (Jean, 1972a, 1972b, 1984; Kessler and Jean, 1985a).

On the basis of lesion experiments in cat, dog and macaque, Doty *et al.* (1967) proposed that the swallowing centre resides within the reticular substance extending from the rostral pole of the inferior olive to the posterior pole of the facial motor nucleus. This area corresponds to the nucleus gigantocellularis of the reticular formation, or, according to modern neuroanatomists, spans the rostral ventrolateral medulla, which is believed to contain a 'tonic vasomotor centre' (Ross *et al.*, 1984; Ross *et al.*, 1985).

By employing anterograde autoradiographic tracing in the cat, Holstege *et al.* (1983) found that in the caudal pontine tegmentum, an area is located dorsal to the superior olivary complex that sends projections contralaterally to the ventromedial trigeminal, dorsal group of ambiguus (conceivably, the Amb<sub>sc</sub>) and ventral hypoglossal motor nuclei. Based on these findings, it was proposed that the latter pontine area may correspond to the swallowing centre.

Electrophysiological, neuroanatomical and pharmacological evidence accumulated thus far has supported the status of the NTS as an important neural substrate involved in the organisation of deglutition for a number of reasons. First, it was demonstrated in sheep and rat that, with swallowing elicited by electrical stimulation of the SLN, some NTS units ('early' neurons) fired during or even prior to the pharyngeal stage while others ('late' and 'very late' neurons) discharged during the oesophageal stage (Jean, 1972a; Kessler and Jean, 1985a). The activity of these 'swallowing neurons' remained unchanged after curarisation of the animal. 'Early' neurons were located either around the solitary tract, i. e. in the NTS and the adjacent reticular formation (the 'dorsal group') or in the lateral reticular formation above the nucleus ambiguus (the 'ventral group'). The authors suggested that the neurons of the dorsal group are involved in initiating and organising the sequence of swallowing while the ventral group of neurons are 'switching' neurons that receive their input from the dorsal group and distribute this input to the different deglutitive motoneurons.

Second, there is strong evidence supporting the view that the central pattern generator for swallowing can be broken down into at least two components governing the pharyngeal and oesophageal stages (Jean, 1972b): in sheep, a unilateral lesion placed between the solitary tract and the DMX suppressed the oesophageal stage of swallowing elicited by electrical stimulation of the SLN ipsilateral to the lesion. The integrity of the oesophageal motor apparatus was ascertained by the oesophageal contractions elicited upon stimulation of the nucleus ambiguus on the lesioned side. As well, stimulation of the contralateral SLN produced normal swallowing. These results suggested that the oesophageal pattern generator includes the NTS (Jean, 1972b).

Third, the chemical microstimulation and retrograde tracing experiments of Bieger (1984) provide a precise neuroanatomical localisation of the oesophageal premotor area within the solitary complex of the rat. The oesophageal subregion *i. e.* the subnucleus centralis of the NTS, not only projects directly to the rostral oesophagomotor portion of the nucleus ambiguus (Bieger, 1984; Ross *et al.*, 1985; Cunningham and Sawchenko, 1989), but also receives the oesophageal primary afferent input (Bieger and Hopkins, 1986; Altschuler *et al.*, 1989).

Finally, it was demonstrated that 'automatic' swallowing could be elicited by application of 5-HT or a 5-HT mimetic to the fourth ventricle (Bieger, 1981); conceivably, the amine was acting upon underlying structures, including the solitary complex. Indeed, direct chemical microstimulation, with glutamate and ACh or

muscarine, of structures within the confines of the NTS gave rise to either complete swallows or their constituent stages, depending upon the site of stimulation (Bieger, 1984). The latter investigation also furnished pharmacological evidence confirming the identity of the oesophageal pattern generator.

Studies on the neuropharmacology of deglutition were instrumental in giving rise to the concept of a 'deglutitory automatism' in the rat. In view of the scant attention these data have received (e.g. Miller, 1982, 1986, 1987; Jean, 1984; Roman, 1986), a brief look at these pharmacologic findings and their implications seems appropriate.

### III. Pharmacology of swallowing

In 1972, Bieger *et al.*, described a periodic response consisting of spontaneous twitching of branchiomeric musculature in the floor of the mouth of the urethane-anaesthetised rat. This phenomenon was termed oral myoclonic twitch activity. Central dopaminergic and serotonergic systems were implicated in the elicitation and maintenance of this response. The paradigm was successfully utilised as a model system for the evaluation of the central effects of monoamines (Bieger, 1974; Menon *et al.*, 1976a, 1976b, 1986; Clineschmidt *et al.*, 1977; Clineschmidt and McGuffin, 1978; Tseng, 1978, 1979; Tseng *et al.*, 1978; Clineschmidt, 1979). This oral myoclonic twitch activity was subsequently identified

as the buccopharyngeal component of swallowing (Bieger *et al.*, 1977). Therefore, the neuropharmacological data from the studies listed above apply directly to swallowing.

An important concept that arises from these investigations and which was made explicit by Bieger (1981) is that of 'automatic' swallowing or 'deglutitory automatism' in contradistinction to reflex swallowing. Automatic swallowing is a truly centrally generated synergy, being independent of peripheral sensory influences insofar as its genesis and maintenance is concerned (Bieger, 1981).

The latter investigation demonstrated that the neural generator for serotonergically-driven automatic swallowing is contained in the medulla, possibly within structures in the environs of the fourth ventricle. However, Kessler and Jean (1985b; 1986a; 1986b) have denied any deglutitive excitant action of the monoamines, including 5-HT, and ascribed only inhibitory effects of these amines on deglutition at the level of the NTS.

Further demonstration of a deglutitory automatism emerged from the work of Bieger (1984). Activation of muscarinic cholinceptors within the subnucleus centralis of the NTS evoked isolated oesophageal contractions which resembled secondary peristalsis (Bieger, 1984). In addition, oesophageal components of both automatic and reflex swallowing were suppressed following administration of antimuscarinic agents. This finding, together with the retrograde tracing of efferents of the subnucleus centralis, provide strong evidence to support the



contention that the latter subnucleus of the NTS contains premotor elements forming part of the internuncial network organising oesophageal peristalsis.

The study by Bieger (1984) directs particular attention to three pharmacologic substances: i) glutamate, because it is capable of giving rise to either a complete swallowing sequence or isolated parts thereof, depending upon the site of application within the NTS ii) 5-HT, because it appears to show selectivity for eliciting the pharyngeal stage and iii) ACh or muscarine, because of their ability to selectively evoke the oesophageal stage of swallowing when applied to the NTS.

The foregoing synopsis draws attention to the many pieces of evidence implicating the NTS as an important substrate supporting deglutition. However, it is also evident that analysis of data so far is not unequivocal regarding the exact location/s of the central pattern generator for swallowing.

#### IV. Objectives

This research was aimed at examining certain pharmacological properties of, and tracing the neural connections of deglutitive substrates within the solitary complex of the rat in order to understand the role of the NTS in the neural organization of swallowing. The anaesthetized rat was chosen as the model since previous investigations had established the validity of this paradigm (Bieger *et al*,

1972; Bieger, 1974; 1981; 1984; Bieger *et al.*, 1977) and a wealth of neuroanatomical information is available on this species (e.g. Kalia and Sullivan, 1982; Bieger, 1984; Shapiro and Miselis, 1985; Bieger and Hopkins, 1986; 1987; Cunningham and Sawchenko, 1989; Altschuler *et al.*, 1989).

The first important step was to delineate the deglutitive region of the NTS. The method of chemical microstimulation by pneumophoresis was employed in these investigations because it had been successfully used in a previous study (Bieger, 1984). However, further refinement was needed to allow microapplication of multiple substances with a high degree of accuracy and precision. Two substances, glutamate and acetylcholine (ACh) or muscarine, were chosen for mapping the deglutitive sites of the NTS owing to their reported role as deglutitive excitants (Bieger, 1984).

The second phase of the investigations involved identification of excitatory amino acid receptor mechanisms mediating the deglutitive actions of glutamate within the solitary deglutitive loci: glutamate had been demonstrated as a selective deglutitive excitant, capable of triggering a complete swallow or isolated parts thereof (Bieger, 1984). Pharmacological agents were used to characterize the receptor types mediating the deglutitive effects of glutamate.

The third objective was to analyse the non-specific deglutitive excitant action of glutamate by comparison with ACh (or muscarine) and 5-HT. Both 5-HT and ACh were known to give rise to 'fictive' or automatic swallowing (Bieger, 1981,

1984) and could be utilised as pharmacological tools owing to their specificity for either the pharyngeal (5-HT) or the oesophageal (muscarine) stage of swallowing (Bieger, 1984). In addition, a side issue that warranted clarification was the apparent disparity between the the excitatory (Bieger, 1981) and inhibitory (Kessler and Jean, 1985b) effects of 5-HT on deglutition. To locate the central source of the serotonergic input to the solitary complex, retrograde tracing was employed in conjunction with immunocytochemical localisation of 5-HT.

The fourth objective was to trace the projections issuing from the solitary deglutitive loci. Previous findings had suggested that the central subnucleus of the NTS is interposed between the oesophageal afferent and efferent limbs (Bieger, 1984; Bieger and Hopkins, 1986; Cunningham and Sawchenko, 1989; Altschuler *et al.*, 1989) implicating this subnucleus as the organiser of oesophageal motility patterns. If the analogy is extended to the remainder of the solitary deglutitive network, the NTS could be hypothesised as the key substrate involved in the neural organization of deglutition. Thus, the tracing of solitary efferents was expected not only to test this hypothesis but also to reveal the pontomedullary structures involved in swallowing. For this purpose, the anterogradely transported plant lectin, *Phaseolus vulgaris* leucoagglutinin (PHAL; Gerfen and Sawchenko, 1984), was the tracer of choice. The approach was to make discrete deposits of the tracer at identified deglutitive loci by the technique of micropneumophoresis and to examine its presence in terminals originating from the solitary deglutitive sites.

## CHAPTER TWO

### MATERIALS AND METHODS

#### **I. Experimental procedures**

Experiments were done on male Sprague-Dawley rats weighing 250-450 g. A surgical plane of anaesthesia was induced either by: i) an intraperitoneal (i.p.) injection of 20% urethane (1.2 g/kg) or ii) an initial dose of i.p. sodium pentobarbital (45 mg/kg) followed by intravenous (i.v.) urethane administered in boluses of 150-175 mg/kg every 30 min.

The animal was tracheostomized and mounted in a stereotaxic frame following cannulation of the right external jugular vein for intravenous infusion of drugs. The tooth bar was 5 mm below the intra-aural line, therefore, the head was in maximum ventroflexion permitted by the apparatus.

Changes in intraluminal pressure within the pharynx and the oesophagus were recorded using miniature balloon-tipped catheters. PE-90 polyethylene tubing (Becton-Dickenson) was used to construct the balloon-tipped catheters. Using the flame of a spirit lamp, one end of the tubing was sealed and melted while air was gently blown through the other end to produce a small balloon 5-8 mm in diameter and 1-1.5 cm long. Three such balloon-tipped catheters were filled with water and each connected to a Statham pressure transducer. The balloon

catheters were thickly coated with 2% xylocaine jelly (Astra Pharma Inc.) and inserted into the laryngopharynx, and the cervical and the supradiaphragmatic oesophagus, respectively. Oesophageal balloons had a diameter of about 5 mm while the pharyngeal balloon was larger (diameter=8 mm). The approximate length of the balloon catheters to be inserted was first marked for each as measured in a number of animals. After allowing the animal to swallow it, the distal oesophageal balloon was inserted into the oesophagus until a slight obstruction could be felt, indicating the point at which the diaphragm encircles the oesophagus. The cervical oesophageal balloon was secured in place as soon as the animal swallowed it, ensuring a position just below the pharyngo-oesophageal junction. The pharyngeal balloon was placed far back into the oral cavity, between the pharyngeal palate and the dorsal aspect of the posterior tongue.

A catheter, connected externally to a pressure transducer, was inserted into the tracheal cannula. Corresponding intraluminal pressure signals registered by the four Statham pressure transducers were amplified and displayed on a pen-recorder (Grass polygraph). Correct placement of the balloons was ascertained by evoking reflex swallowing by intra-tracheal tactile stimulation. Respiratory rate was derived from airway pressure fluctuation and monitored by means of a tachograph. Rectal temperature was maintained between 36.5° and 37.5° C. by means of a heat lamp.

In order to expose the caudal floor of the fourth ventricle and the surrounding structures on the dorsal medulla oblongata, a suboccipital craniotomy was performed. Briefly, a midline incision on the head was made, about 2.5 cm

long, running from the level of the animal's ears down to its neck. The underlying musculature was retracted and the posterior aspect of the occipital bone was removed. Under visual control through a Wild M650 stereomicroscope, a pair of fine hooks were used for lifting the dura mater and the arachnoid membrane while cutting them with a pair of microscissors. The cerebrospinal fluid was continuously drained using small wicks made of 'Kimwipes' tissue paper.

For the microstimulation of deglutitive neurons in the NTS, 2-, 3- or 4-barreled glass micropipettes (tip O.D. 2-5  $\mu\text{m}$ ) were used. The micropipettes were pulled from glass capillary tubing (O.D.=1 mm; I.D.=0.7 mm; Glass Company of America) on an electrode puller (PUL-1, W-P Instruments, Inc., Conn.) to a shank length of 2.5-3.5 cm. The long shanks provided a certain degree of flexibility that could accommodate the movements of the animal due to breathing and blood pressure pulsations. Drug solutions, mixed with different fluorescent dyes for marking ejection sites (see below), were back-filled into each barrel of the micropipettes using 1 ml syringes fitted with 5 cm long, 30 gauge stainless steel needles (Becton-Dickenson). Pressure connections to the micropipettes were made as per the technique described by Neuman (1986). Briefly, stainless steel cannulae made from 26 gauge, 3/8 or 5/8 inch needles (Becton-Dickenson) were bent in the middle at an angle of about 45° using a pair of nose-pliers. The cannulae were inserted into each barrel of the micropipette and cemented either with dental acrylic (L. D. Caulk Company, Delaware) or with 'krazy' glue gel.

Drug solutions were ejected from the micropipettes by means of a Picospritzer II micropneumophoresis pump (General Valve Corporation) using a nitrogen pressure source.

Pipettes were individually calibrated before insertion into the brain tissue and after withdrawal according to the technique described by McCaman, McKenna and Ono (1977). The diameter of the ejected droplet was measured with the aid of an ocular micrometer (Bausch and Lomb, U.S.A.). The volume ejected with each pressure pulse (400-500 kPa; 50-300 ms) was kept in the range of 20-100 pl (droplet diameter=34-57  $\mu\text{m}$ ). This corresponded to an injection site diameter of 50-100  $\mu\text{m}$  (see below).

The micropipettes were fixed to an electrode-holder assembly tilted at an angle of 28° in the antero-posterior plane and controlled by a Narishige micromanipulator. Stereotaxic placement of the micropipettes with reference to anatomical landmarks on the dorsal medullary surface was achieved under visual control through the stereomicroscope. The zero reference point was identified as a small ridge marking the rostral border of the area postrema. This corresponded to a point about 160  $\mu\text{m}$  rostral to the obex as identified histologically (see below).

Swallowing responses were characterised as: pharyngeal, if a contraction in the pharynx was not followed by an oesophageal peristaltic wave; complete swallow (CS), if a pharyngeal contraction was followed by phasic cervical and distal oesophageal propulsive contractions; and cervical oesophageal or distal oesophageal,

if a contractile response was elicited primarily in either the cervical or distal oesophagus. Pharyngeal responses were considered deglutitive in nature if, in terms of amplitude, rate of pressure rise and duration, they were indistinguishable from pharyngeal contractions forming part of a CS.

At the end of the experiment, the brain was fixed *in situ* by perfusing the animal either transcardially or retrogradely through the abdominal aorta with 50 ml of 1% magnesium chloride in saline followed by 500 ml of phosphate-buffered (0.1 M) mixture of paraformaldehyde (4%) and glutaraldehyde (0.5%). The brain was removed and kept in the fixative overnight. The brainstem was blocked for sectioning in the transverse plane and the left side was marked by placing a small knife-cut in the pyramidal tract. 40  $\mu$ m thick serial sections of the medulla oblongata were cut on a vibratome and collected on gel-coated slides. The slides were dried and coverslipped using paraffin oil. Injection sites were visualized under ultraviolet darkfield trans-illumination, with the aid of a Zeiss microscope equipped with excitation-suppression filters suitable for the detection of the fluorescent dyes. Photomicrographs of injection sites were taken on 400 ASA colour or black-and-white film with a Winder 35 mm camera attached to the fluorescence microscope. Drawings of the sections were made using a Zeiss camera lucida phototube.

The diameter of the fluorochrome-marked injection sites was measured with the aid of an ocular micrometer and verified by measuring the same in camera lucida drawings or photomicrographs. With fluorochrome concentrations of



0.03-0.1% and ejection parameters as outlined above, dye-marked ejection sites were nearly spherical, their diameters ranging between 50 and 100  $\mu\text{m}$ . A crude verification of these measures was further provided by counting the number of sections containing the fluorochrome. No correction was made for shrinkage of the tissue.

The neuroanatomical identification of stimulation sites in the NTS was made with reference to recent accounts of the subnuclear subdivisions of this region (Kalia and Sullivan, 1982) and studies of the viscerotopic afferent representation of the upper alimentary tract in the NTS (Bieger and Hopkins, 1986; Altschuler *et al.*, 1989). Histologically, the obex was identified as the point where the dorsal wall of the central canal merges with the area postrema. The neuroanatomical collection of Dr. D. Bieger was utilised for determining: i) the rostrocaudal extent of the subnucleus centralis of the NTS from a series of camera lucida drawings of serial transverse sections through the solitary complex in which the NTS<sub>cen</sub> had been retrogradely labeled by an injection of a fluorescent tracer into the oesophagomotor compact division of the nucleus ambiguus (from Bieger, 1984) ii) the location of the accessory facial subnucleus in serial sagittal sections of a case in which horseradish-peroxidase had been retrogradely transported from an injection made into the posterior belly of the digastric muscle.

## II. Mapping

For mapping the deglutitive loci within the NTS, the region was systematically explored with 4-10 pmol of sodium S-glutamate and acetylcholine (ACh) and 2-5 pmol of D,L-muscarine ejected from multibarreled glass micropipettes in volumes not exceeding 50 pl per pulse. All observations were repeated in a minimum of five separate animals and drug-ejections were made on both halves of the medulla in a given preparation. Bis-benzimide, nuclear yellow or fluorogold (0.03-0.1%), either in a separate barrel or mixed with one or more drug solutions contained in the micropipette, were used as markers to permit ultraviolet fluorescence microscopical identification of the ejection sites. Mixing the dye with the drugs did not appreciably affect the evoked responses as compared to those elicited by the drug alone at a given locus. In addition, ejection of the dye alone at a concentration of 0.03-0.1% did not affect subsequent responses evoked by the drug from the same site. Relevant criteria for the analysis of responsive loci included latency of response, pharmacological specificity, and deglutitive phase relationship.

### III. Pharmacology

#### 1. Micropneumophoretic application of drugs

Pressure pulses of glutamate were used for locating the deglutitive loci in the NTS. Glutamate pulses were typically 4-20 pmol, unless stated otherwise, and were applied in volumes of 20-100 pl. Glutamate was employed as a standard test substance for eliciting deglutitive responses since i) it can evoke different types of swallowing responses, depending upon the site of stimulation and ii) owing to the relatively rapid termination of its action in brain tissue, it is less excitotoxic than the other excitatory amino acids (Olney, 1978; Mayer and Westbrook, 1987). A computer-driven timer circuit was employed in order to enable the glutamate test-pulses to be delivered at a fixed rate. Five to eight control responses spaced at 1-2 min intervals were obtained to ensure reproducibility before drug interactions were examined. Control ejections were also done with vehicle (see below). Barring accidental displacement of the pipette, chemical microstimulation of the sites described in this study yielded responses that remained stable and reproducible even after repetitive ejections (30-120 times over a period of 30 minutes to 3 hours) of glutamate or ACh. In trials where the interaction between glutamate and 5-HT was studied, paired drug ejections were repeated up to 15 times.

Relative potency of excitatory amino acid (EAA) agonists at a given site was evaluated by determining the threshold dose of each compound required to elicit

a response using four different concentrations, 0.1, 1, 10 and 100 mM, keeping the delivery volume constant (100  $\mu$ l) and applying equimolar doses of the three agonists at each site. To ensure reproducibility at a given locus i) responses to glutamate were tested between the applications of different agonists and ii) each agonist set was tested at least twice (up to 5 times) and data were rejected if the results were inconsistent. This procedure, therefore, yielded a reproducibility of 100% at any given locus. In order to arrive at an index of relative agonist potency, the effective dose of each agonist capable of eliciting a response in 50% of the total number of test sites was determined from a plot of log molar dose vs. total percentage of responsive sites (see Table V).

## 2. Topical application of drugs

For the application of drugs to the ependymal surface of the solitary complex, 5 or 10  $\mu$ l Hamilton syringes were used. A small cannula tip made from PE-20 tubing was attached to the tip of the needle. Volumes applied were typically 1  $\mu$ l and did not exceed 5  $\mu$ l.

## 3. Electrical stimulation

In a few experiments, the effects of systemic administration of drugs on reflex swallowing elicited by electrical stimulation of the superior laryngeal nerve (SLN) was studied. In these cases, the SLN was approached from the ventral side,

dissected free of the surrounding tissue and placed on bipolar platinum electrodes. The electrical stimuli were delivered in trains (10 s duration) of pulses at 5-10 Hz, 2-10 V and of 0.1-1 ms duration generated by a Grass S88 or a Neurolog (Medical Systems Corp.) constant current (40  $\mu$ A) stimulator.

#### IV. Neuronal Tracing

Surgery was done under aseptic conditions on sodium pentobarbital-anaesthetised male Sprague-Dawley rats (N=45). All surgical instruments were sterilized before use by autoclaving. The areas to be incised were shaved and cleaned with iodine solution. Following cannulation of the external jugular vein, the animal was fixed in a stereotaxic frame. Care was taken to ensure that the tympanic membranes were not damaged by the ear bars. Endotracheal intubation was performed using a slightly modified version of the technique described by Procter and Fernando (1973). Briefly, a fibre-optic light source (Nikon Inc.) was used to illuminate the oral cavity in order to visualize the vocal cords. A blunt-ended guide wire, its other end carrying a tracheal cannula, was inserted between the vocal cords, the tracheal cannula was slipped through and the guide wire withdrawn.

Intraluminal pressure changes within the pharynx and cervical and distal oesophagus were measured as described above. Balloon-catheters were constructed

from thinner (PE-20) tubing. Respiration was recorded from the endotracheal tube. Rectal temperature was maintained between 36.5° and 37.5° C. by means of a heat lamp. A surgical level of anaesthesia was maintained throughout the experiment by i.v. boluses (150-175 mg/kg) of 10% urethane in saline containing 10 mM glucose.

A suboccipital craniotomy followed by incision of the two outer meninges exposed the dorsal medullary surface. In order to locate deglutitive sites in the solitary complex, the lateral ala cinerea was probed with glutamate 10-20 pmol pulses, pneumophoresed from a three-barreled glass micropipette (tip o.d. 8-15  $\mu$ m). Sites selected for labeling had response latencies of <1 s. At such sites, either a 2.5% solution of *Phaseolus vulgaris* leucoagglutinin (PHAL) or a solution of a fluorescent tracer (10-20% solution of fluorogold or an undiluted suspension of rhodamine-filled latex microspheres) was ejected from an adjacent barrel of the micropipette in pulses of 0.2-0.8 nl to a total of 0.2-20 nl (Tables II and III). Five min later, the pipette was gradually withdrawn and its calibrations verified. The chipped portion of the occipital bone was covered with a small piece of Gelfoam sponge (Upjohn), the wound was sutured in layers with 5-0 surgical silk sutures (Ethicon) and the incision was closed with wound clips. The balloon catheters were removed and the animal taken out of the stereotaxic frame. The external jugular was ligated following removal of the cannula and the wound was sutured. Finally, the endotracheal tube was removed, the wounds were sprinkled with sulphanilamide powder (Allen and Hanburys, Toronto) and the animal returned

TABLE II

Volume of *Phaseolus vulgaris* leucoagglutinin (PHAL) pressure-injected at glutamate-responsive deglutitive loci in the rat nucleus tractus solitarius.

Site	Case #	Volume (nl)
Pharyngeal (N=6)	19	2.0
	20	2.0
	21	1.0
	35	5.0
	37	5.5
	38	8.0
Cervical	8	1.8
Oesophageal (N=5)	11	20.0
	14	4.0
	18	3.0
	28	2.0
Distal Oesophageal (N=11)	1	2.1
	2	4.5
	3	5.0
	4	1.75
	5	1.25
	6	2.1
	7	1.05
	9	1.5
	10	10.0
	12	20.0
	17	1.0
Complete Swallow (N=7)	13	8.0
	15	2.0
	16	3.5
	24	3.0
	31	3.0
	32	3.0
	33	1.0
Nucleus of the spinal trigeminal tract (control)	27	5.0

TABLE III

Volume of fluorogold or latex microspheres injected at glutamate-responsive pharyngeal sites in the rat nucleus tractus solitarii.

Case #	Volume (nl)
22	1.5
23	0.75
25	3.0
26	0.3
29	2.0
30	2.5
34	2.5
36	5.0
39	4.5
40	5.0
41	0.5
42	2.0
43	3.0
44	0.2
45*	5.0

\* A suspension of latex microspheres was injected in this case.



to its cage. Two or three ml of dextrose-saline solution i.p. was administered in some cases to replenish the loss of fluid and electrolytes. All animals recovered completely within 24-48 h.

After a survival period of 6-10 days, brainstem sections (30-40  $\mu\text{m}$ ) of the fixative-perfused brain were subjected to immunocytochemistry. Serial sagittal sections (40  $\mu\text{m}$ ) of PHAL-labeled brains were processed by the peroxidase-antiperoxidase method (Sternberger, 1979) using diaminobenzidine as the chromogen. Horizontal sections (30 $\mu\text{m}$ ) of fluorogold-labeled brains were processed for the detection of 5-HT by fluorescence immunocytochemistry.

## V. Immunocytochemistry

### 1. Peroxidase-antiperoxidase method for PHAL

Animals were anaesthetized with an overdose of i.p. urethane and perfused through the heart with 500 ml of heparinized (1000 units/litre) saline followed by 500-700 ml of 4% paraformaldehyde in 0.1 M phosphate buffer at a pressure of 120-140 mm Hg. The brain was removed and post-fixed for 24-36 h in the fixative. The brainstem was split in the middle to yield right and left halves which were glued to the sectioning blocks on their medial sides. 40  $\mu\text{m}$  thick sagittal sections were cut on a vibratome and collected serially in 0.1 M phosphate-buffered saline

(PBS), pH 7.2. Multi-well tissue culture plates (Flow Laboratories, Virginia) were used for collection and processing of the sections.

All antibodies were diluted in 10% normal rabbit serum (ICN Immunobiologicals, Illinois) in PBS containing 0.4% Triton X-100. The sections were rinsed in PBS for 2 h at room temperature and incubated in 10% normal rabbit serum for 1 h with gentle shaking. They were then incubated with the primary antibody for 48 h at 4° C. The primary antibody (obtained from Vector Laboratories) was raised in goat against PHAL and used in a dilution of 1:2000.

Forty-eight h later, the sections were washed in PBS 3-4 times and transferred to the linking antibody for 4 h at room temperature with gentle shaking. The linking antibody was a rabbit-anti-goat IgG (obtained from Sigma) used in a dilution of 1:200. Following several washes in PBS, as above, the sections were transferred to a peroxidase-antiperoxidase antibody raised in goat (PAP; Sigma) at room temperature at a dilution of 1:400.

After incubating in PAP for 4 h, the sections were rinsed in PBS several times and transferred to freshly prepared 3,3'-diaminobenzidine tetrachloride (DAB) reaction mixture. The reaction mixture was prepared in PBS and consisted of 0.05% DAB, and, for the liberation of hydrogen peroxide, 0.1% glucose and 176 units of glucose oxidase per 100 ml of the reaction mixture. Within 5-8 min, following appearance of the brown DAB reaction product, the reaction was halted by transferring the sections to PBS. The sections were mounted on gelatinized

slides, dried in air for 1 h, dehydrated through a graded series of ethyl alcohol, cleared in xylene, and coverslipped using the permanent mounting medium 'Eukitt' (O. Kindler, W. Germany). Sections were observed and photographed on a Zeiss Photomicroscope II under both darkfield and brightfield illumination and drawings were made using a camera lucida phototube.

Unless indicated otherwise, the projections from the NTS described in the results section are all ipsilateral.

## 2. Immunofluorescence technique for 5-HT

Rats injected with fluorescent tracers were perfused as described above and their brains removed. The brainstem was sectioned ( $30\text{ }\mu\text{m}$ ) in the horizontal plane and the sections were mounted on gelatinized slides. The slides were balanced horizontally using a spirit-level in a humid chamber at room temperature and covered with a few drops of 10% normal goat serum (ICN Immunobiologicals). Normal goat serum was diluted to 10% in PBS containing 0.4% Triton X-100. After 1 h, the normal goat serum was drained and the edges of the slides wiped clean. The slides were rebalanced in the humid chamber and covered with the primary antibody. The primary antibody was raised against 5-HT in rabbit (obtained from Immunonuclear) and diluted 1:2000 in 10% normal goat serum.

Twenty-four h later, the antibody was drained and the sections washed in PBS sprayed from a wash bottle followed by rinsing in a bath of PBS for 30-45

min. After wiping the edges dry and balancing in the humid chamber, the slides were covered with the secondary antibody. The secondary antibody was a goat-anti-rabbit immunoglobulin conjugated to fluorescein isothiocyanate (FITC; obtained from Sigma) and diluted 1:40 with 10% normal goat serum. After incubation for 2 h, the slides were rinsed with PBS and coverslipped using either PBS:glycerine=1:3 (fluorogold) or methyl salicylate (latex microspheres) as mountant. Some of the fluorogold cases were mounted in a glycerol-based medium containing 0.1% *p*-phenylenediamine to reduce fading of the immunofluorescence (Johnson and Nogueira Araujo, 1981). The sections were examined through a Zeiss epi-illuminated ultraviolet microscope fitted with appropriate filters for visualizing white fluorogold (G 365, FT 395, LP 420), green FITC (450-490, FT 510, LP 520) and red rhodamine (BP 546, FT 580, LP 590) fluorescence. Darkfield illumination was used to identify various subnuclear divisions and boundaries. Photographs were taken on 400 ASA colour film (Kodak) with a Winder 35 mm camera fitted to the microscope. The slides were stored in slide-books at 4° C if mounted with glycerine or methyl salicylate and at -20° C if mounted with the *p*-phenylenediamine medium.

## VI. Chemicals and drug solutions

Drugs, reagents and enzymes used and their sources were as follows: acetylcholine (ACh) chloride, DL-2-amino-5-phosphonovaleric acid (APV), DL-2-amino-7-phosphonoheptanoic acid (APT), bis-benzimide, calcium chloride, 3,3'-diaminobenzidine tetrachloride, gamma-D-glutamylglycine (DGG), glucose, glucose oxidase, sodium S-glutamate, kainic acid (KA), methyl salicylate, DL-muscarine hydrochloride, N-methyl D-aspartic acid (NMDA), 5-HT oxalate, paraformaldehyde, p-phenylenediamine hydrochloride, quisqualic acid (QA), sodium chloride, dibasic sodium phosphate, urethane, yohimbine hydrochloride (Sigma); sodium bicarbonate, potassium chloride (Fisher Scientific); magnesium sulphate, physostigmine salicylate (BDH); 8-OH-DPAT, TFMPP (RBI); N-methyl-DL-aspartic acid (NMA; Calbiochem); sodium pentobarbital (M.T.C. Pharmaceuticals); quipazine maleate (QPZ; Miles Laboratories); 5-HT bimaleate (Koch-Light Laboratories); 5-HT creatinine sulphate (Aldrich); methysergide bimaleate, pizotifen (gift from Sandoz); cyanopindolol, ICS 205-930 (Sandoz); metergoline (gift from Farmitalia); MK-801 (gift from Dr. Dale Corbett); ketanserin (gift from Janssen); BRL 43694 (Beecham); dimethylsulphoxide (Taab Laboratories); nuclear yellow (Hoechst); fluorogold (Fluorochrome Inc.); rhodamine-filled latex microspheres (Lumafuor Inc.); *Phaseolus vulgaris* leucoagglutinin (PHAL; Vector Laboratories, Inc.); and heparin (Organon).

For micropneumophoretic application, solutions of these compounds were made in artificial cerebrospinal fluid (CSF) with the following exceptions: 5-HT creatinine sulphate in distilled water; metergoline in distilled water containing ascorbic acid 0.25%; fluorogold in distilled water containing 0.1% dimethylsulphoxide; PHAL in 0.05 M phosphate-buffered saline, pH 7.4. Artificial CSF was composed of (in mM): sodium chloride 124; sodium bicarbonate 26; glucose 10; potassium chloride 3; calcium chloride 2; magnesium sulphate 2; potassium dihydrogen phosphate 0.4. The pH of artificial CSF was adjusted to 7.2.

## CHAPTER THREE

### RESULTS

#### I. Characterisation of the solitary deglutitive loci

##### 1. Characteristics of evoked responses

###### A. Pharyngeal responses

Pharyngeal responses evoked by pressure-ejection of glutamate into the solitary complex ranged in amplitude from 1 to 24 kPa (mean=5.8 kPa) and consisted of 1-2 pressure-waves, each lasting an average of 200 ms (Fig. 1; Table IV). The responses were elicited at latencies ranging between 0.1 s and 1 s.

ACh or muscarine, ejected at glutamate-responsive pharyngeal loci in the NTS, failed to produce any consistent effects at these sites. In three cases involving high doses (>50 pmol), ACh did produce pure pharyngeal responses presumably reflecting a nicotinic action; however, these were not investigated further.

Irrespective of the mode of elicitation, all pharyngeal responses: i) coincided with a respiratory pause and ii) occurred most frequently during expiration or at the peak of the inspiratory phase of respiration.

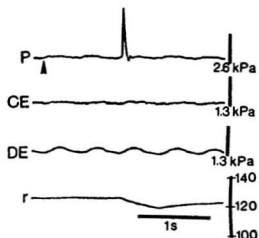


Figure 1

Pharyngeal response evoked by glutamate 8 pmol pressure ejected (arrowhead) into the subnucleus intermediolateral of the NTS (injection site shown in figure 5). In this and the subsequent figures, traces are taken from continuous records and show (from top to bottom) intraluminal pressure changes within the pharynx (P), the cervical oesophagus (CE) and the distal oesophagus (DE). The bottom trace (r) is the respiratory rate and shows the sharp dip corresponding to the interruption of breathing during the pharyngeal phase of swallowing. Respiratory rate calibration in beats per min.



TABLE IV

Characteristics of deglutitive responses evoked by glutamate micropneumophoresed into the rat nucleus tractus solitarii.

Type of Response	Latency (mean <sup>**</sup> $\pm$ S.E.)	Amplitude (mean $\pm$ S.E.)	Duration (mean $\pm$ S.E.)	Velocity* (mean $\pm$ S.E.)
Pharyngeal	0.1-1 s (0.44 $\pm$ 0.11)	1-24 kPa (5.79 $\pm$ 1.10)	0.15-0.35 s (0.22 $\pm$ 0.05)	-
Oesophageal	0.3-1 s (0.51 $\pm$ 0.11)	0.3-12 kPa (4.2 $\pm$ 0.86)	2-8 s (3.5 $\pm$ 1.05)	2.6-5 cm/s (2.8 $\pm$ 0.76)

\* Velocity of propulsive oesophageal responses as computed from the time-lag between the onset of contractions in cervical and distal oesophageal recording sites which were separated from each other by a distance of about 4 cm.

\*\* Means computed from 10 random samples of each type, each taken from a separate animal.

## B. Oesophageal responses

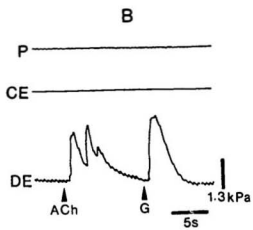
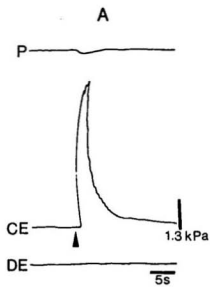
Oesophageal responses evoked by pressure-ejection of glutamate into the NTS were typically monophasic with an amplitude range of 0.3-12 kPa (mean=4.2 kPa) and a duration of 2-5 s (average=3.5 s; Table IV). The range of latency for the responses was 0.3-1 s. Depending upon the site of stimulation in the NTS, responses evoked by glutamate were observed in the cervical, distal (Fig. 2) or both regions of the oesophagus. When both cervical and distal oesophageal components were present, the response was either simultaneous or peristaltic. When the response was peristaltic, the phase-lag between the cervical and distal oesophageal components was 0.8-1.5 s. Thus, the velocity of spread of the oesophageal wave averaged 2.8 cm per s. No change in the animal's respiration was observed with the glutamate-evoked oesophageal responses.

ACh or muscarine were also effective in eliciting oesophageal responses at all glutamate-responsive oesophageal sites. In contrast to glutamate, ACh or muscarine frequently gave rise to rhythmic contractions consisting of multiple waves (Figs. 2, 3), confirming a previous investigation (Bieger, 1984). The latency range of the evoked responses for ACh was 0.3-1 s and for muscarine 1-5 s. At sites where glutamate evoked both cervical and distal oesophageal components either simultaneously or in a peristaltic fashion, ACh or muscarine invariably gave rise to propulsive, secondary peristalsis-like contractile waves (cf. Bieger, 1984).

The amplitude of the evoked responses, whether pharyngeal or oesophageal, varied directly (2-3 times the threshold response) with the dose of the agonist

## Figure 2

Oesophageal responses evoked by glutamate and ACh from the subnucleus centralis of the NTS. **A:** Monophasic cervical oesophageal response evoked by microinjection of glutamate (arrowhead) 6 pmol into the subnucleus centralis of the NTS (injection site shown in figure 6). **B:** Multiphasic contractions in the distal oesophagus evoked by ACh 5 pmol pressure injected into the subnucleus centralis of the NTS. Application of glutamate (G) 8 pmol at the same site from an adjacent barrel of the micropipette yielded the monophasic response characteristic of this substance (injection site shown in figure 7).



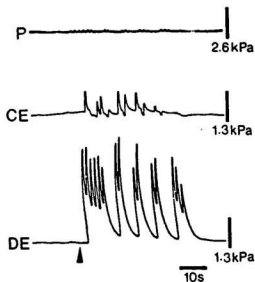


Figure 3

Rhythmic oesophageal responses resembling secondary peristalsis evoked by muscarine  
3 pmol pressure ejected (arrowhead) into the subnucleus centralis of the NTS.

applied at a given site. This variation from threshold to maximum was observed within a narrow dose range (1.5-2 times the threshold dose of ACh or glutamate) at any given site.

### C. Complete swallow responses

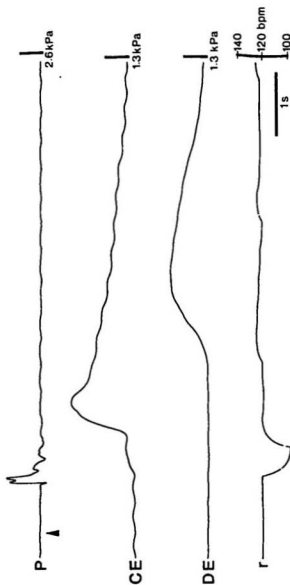
The complete swallow (CS) responses were evoked by microinjections of glutamate (Fig. 4) but not ACh or muscarine and consisted of phasic pharyngo-oesophageal contractile sequences. The latency of these responses ranged between 0.5 and 2 s. The cervical oesophageal component appeared 0.3-0.7 s (mean=0.49 s) after the pharyngeal component while the phase-lag between the onset of contractions in the cervical and distal oesophageal components varied from 0.8 s to 1.5 s (average=0.95 s). This yielded a velocity of about 3 cm per s for the spread of the oesophageal wave.

## 2. Responsive loci

Deglutitive loci mapped in this study are depicted in Figs. 5-9. The deglutitive region was found within the confines of the NTS, specifically an area overlapping its central, intermediate and ventral subnuclei and extending from 200  $\mu\text{m}$  caudal to the obex to 900  $\mu\text{m}$  rostrad. In the mediolateral axis, the loci lay between 650  $\mu\text{m}$  and 850  $\mu\text{m}$  from the midline and were found at a depth of 250-650  $\mu\text{m}$  from the dorsal surface of the medulla.

**Figure 4**

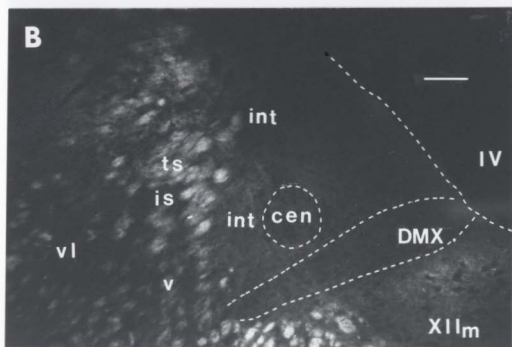
Complete swallow elicited by pressure ejection of glutamate (arrowhead) 8 pmol into the solitary complex (injection site shown in figure 7).





**Figure 5**

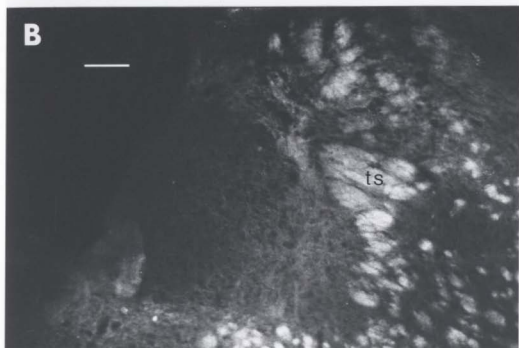
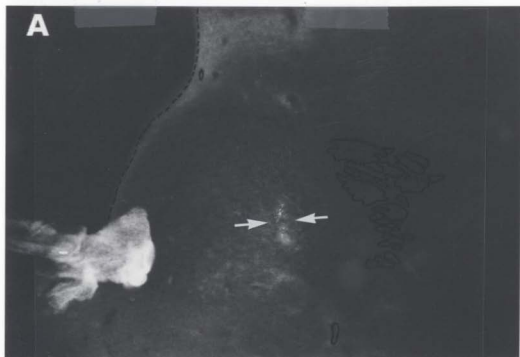
A fluorochrome-marked pharyngeal locus in the rat nucleus tractus solitarii. **A:** Darkfield photomicrograph under ultraviolet illumination of a transverse section through the solitary complex, 480  $\mu\text{m}$  rostral to obex, showing a fluorogold-labeled glutamate-responsive (response depicted in figure 1) pharyngeal site in the subnucleus intermedius. **B:** Same section under darkfield white light illumination showing anatomic detail and landmarks. Approximate locations of the subdivisions of the NTS are also indicated. Abbreviations: *cen* subnucleus centralis; *DMX* dorsal vagal motor nucleus; *int* intermediate subnucleus of NTS; *is* interstitial subnucleus of NTS; *IV* fourth ventricle; *ts* tractus solitarius; *v* ventral subnucleus of NTS; *vl* ventrolateral subnucleus of NTS; *XII<sub>9</sub>* hypoglossal nucleus. Calibration bar: 100  $\mu\text{m}$ .



### Figure 6

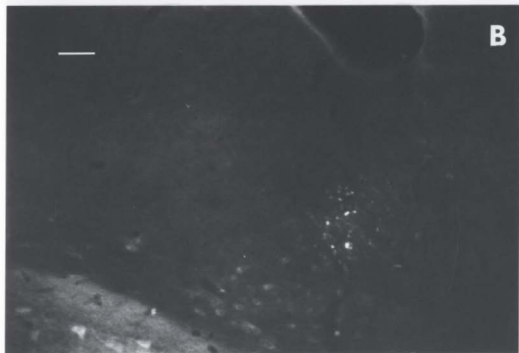
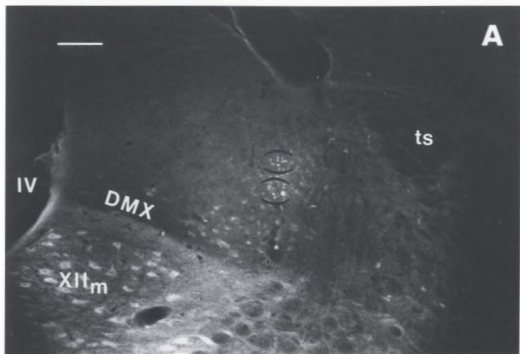
A fluorochrome-marked cervical oesophageal site in the rat nucleus tractus solitarii.

**A:** Darkfield photomicrograph under ultraviolet illumination of a transverse section through the solitary complex, 600  $\mu\text{m}$  rostral to the obex, showing a nuclear yellow-labeled ACh- and glutamate-responsive (glutamate-evoked response depicted in figure 2) cervical oesophageal site in the subnucleus centralis. **B:** The same section under darkfield white light illumination showing anatomic detail (see Figure 5 B or identification of anatomic landmarks). Calibration bar: 100  $\mu\text{m}$ .



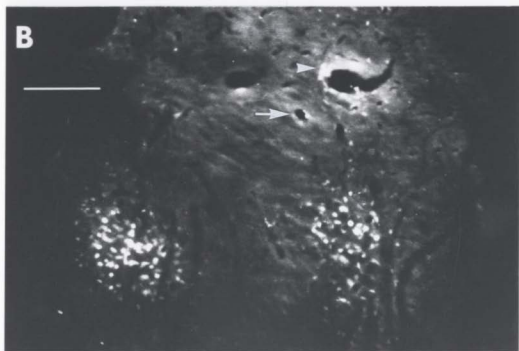
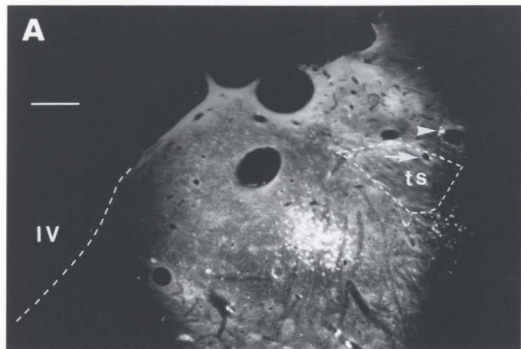
### Figure 7

Fluorochrome-marked CS and distal oesophageal loci in the rat nucleus tractus solitarii. **A:** Darkfield photomicrograph under ultraviolet illumination of a transverse section through the solitary complex, 240  $\mu\text{m}$  rostral to obex, showing two closely-situated deglutitive loci (enclosed within brackets) labeled with fluorogold. The dorsally situated locus overlaps the centro-intermediate NTS and yielded a complete swallow in response to glutamate (shown in figure 4). The ventrally located site lies within the subnucleus centralis and responded to both glutamate and ACh with mono- and multiphasic contractions, respectively, of the distal oesophagus (see figure 2). Cells in XII<sub>n</sub> exhibit autofluorescence. **B:** Same section under high power. Abbreviations: DMX dorsal vagal motor nucleus; IV fourth ventricle; ts tractus solitarius; XII<sub>n</sub> hypoglossal nucleus. Calibration bars: 100  $\mu\text{m}$  in A, 25  $\mu\text{m}$  in B.



### Figure 8

Fluorochrome-marked deglutitive and non-deglutitive sites in the rat nucleus tractus solitarius. **A:** Darkfield photomicrograph under ultraviolet illumination of a transverse section through the solitary complex 320  $\mu\text{m}$  rostral to obex showing two sites marked with bis-benzimide. The medial site is centered in the subnucleus centralis of the solitary complex and yielded a cervical oesophageal response to glutamate. The lateral site lies in the ventrolateral subnucleus of the solitary complex and failed to evoke a deglutitive response to pressure-ejection of glutamate. **B:** The two sites shown at higher magnification. Arrows in A and B indicate landmarks for orientation. Calibration bars: 100  $\mu\text{m}$

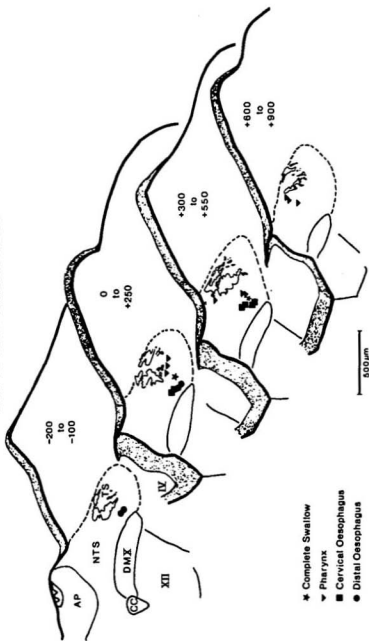




### Figure 9

Map of deglutitive loci. Transverse slabs through the solitary complex depicting CS (✱), pharyngeal (▼), cervical (■) and distal oesophageal (●) sites extending from 200  $\mu$ m caudal to obex to 900  $\mu$ m rostrally. Each symbol represents 5-8 closely overlapping loci mapped in separate experiments. Abbreviations: AP area postrema; CC central canal; NTS nucleus of the tractus solitarius; DMX dorsal motor nucleus of the vagus; IV fourth ventricle; XII hypoglossal nucleus.

# MAP OF RESPONSIVE SITES



Pharyngeal sites were found within two subnuclear divisions of the NTS viz., ventralis and intermedialis, and extended from the level of the obex to 900  $\mu\text{m}$  rostrally.

Oesophageal loci were coextensive with the subnucleus centralis. Cervical oesophageal loci extended to levels more rostral than did the distal oesophageal loci which, in turn, were clustered at more caudal levels. Cervical and distal oesophageal loci had a region of overlap extending between 50  $\mu\text{m}$  and 250  $\mu\text{m}$  rostral to obex.

Complete swallow (CS) loci were found 200-500  $\mu\text{m}$  rostral to obex and lay between the oesophageal and pharyngeal loci in the mediolateral axis *i.e.* overlapping the subnuclei centralis and intermedialis of the NTS.

### 3. Non-responsive loci

ACh or muscarine or glutamate failed to elicit swallowing responses when pressure ejected into other regions of the NTS, including its medial, commissural, dorsolateral, interstitial and ventrolateral (Fig. 8) subnuclei. Dorsal medullary regions outside the NTS also were non-responsive to agonist ejections. Regions examined included: area postrema, dorsal motor nucleus of the vagus, hypoglossal, gracile and cuneate nuclei and the paratrigeminal islands.

## II. Pharmacologic observations at solitary deglutitive loci

### 1. Effects of excitatory amino acid receptor agents

#### A. Excitatory amino acid receptor antagonists

##### i) Pharyngeal responses

At pharyngeal sites, application of the NMDA-receptor blockers, APV or AP7, in the dose range of 6-40 pmol (Table V), 1-2 min prior to the pulse of glutamate, failed to affect the evoked response (Fig. 10 B). However, a complete and reversible blockade of the response was achieved by equimolar prepulses of the non-selective excitatory amino acid receptor antagonist, DGG (Fig. 10 C,D).

None of the three excitatory amino acid antagonists caused any overt changes in basal intrapharyngeal pressure or respiratory rate.

##### ii) Oesophageal responses

Pressure pulses of APV or AP7, within 1-2 min of application, selectively inhibited the glutamate response elicited at both cervical and distal oesophageal sites, without affecting the muscarine-evoked rhythmic oesophageal contractions evoked at the same site (Fig. 11). The glutamate response gradually recovered over a period of 9-20 min. Prepulses of DGG, applied in the same dose range

TABLE V

Dose-range (picomoles) of antagonists for different levels of blockade at deglutitive loci in the rat nucleus tractus solitarii.

Agent	Type of Response	Level of Blockade*:		Nil
		>50-100%	<50%	
APV/AP7	Pharyngeal	(0)	(0)	6-40 (26)
	Cerv. Oes.	>15-35 (18)	>6-15 (21)	<5 (18)
	Dist. Oes.	>15-33 (21)	>6-15 (25)	<5 (19)
	CS**	>20-40 (13)	>8-20 (12)	<7 (9)
DGG	Pharyngeal	>18-40 (8)	>7-18 (9)	<6 (6)
	Cerv. Oes.	>16-35 (5)	>8-16 (4)	<6 (5)
	Dist. Oes.	>20-36 (5)	>8-20 (5)	<7 (7)
	CS	>20-40 (4)	>8-20 (4)	<7 (5)

\* Blockade as evidenced by reduced amplitude of pressure wave evoked by pressure ejection of L-glutamate 10-20 pmoles in nucleus tractus solitarii.

\*\* Antagonism of oesophageal components only. Number of trials are given in parentheses.

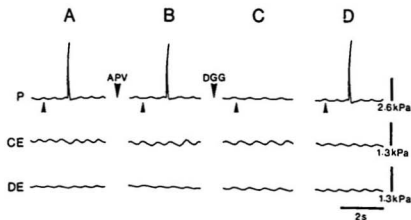


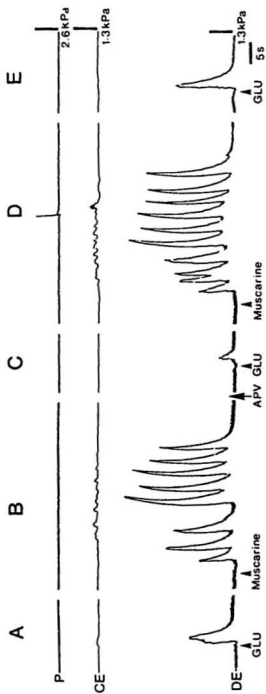
Figure 10

Effects of excitatory amino acid receptor antagonists at a pharyngeal NTS site.

**A:** Control response elicited by glutamate (small arrowhead) 14 pmol pressure ejected in the subnucleus ventralis. **B:** Insensitivity of the response to aminophosphonovaleric acid (APV) 30 pmol prepulsed 2 min prior to the glutamate pulse. **C:** Complete blockade of the response 1.5 min subsequent to pressure application of gamma-D-glutamylglycine (DGG) 28 pmol. **D:** Recovery 13 min later.

Figure 11

Effect of NMDA receptor blockade at an oesophageal site. Control responses elicited by glutamate (A) 15 pmol and muscarine (B) 6 pmol pressure ejected from adjacent barrels of the micropipette into the subnucleus centralis. C: Inhibition of the glutamate-evoked response 2 min following application of APV 20 pmol and persistence of the muscarine-evoked response (D). E: Recovery of the glutamate-evoked response 9 min later.





as APV or AP7 (Table V), also blocked these glutamate-evoked responses. Neither antagonist produced a change in intraoesophageal baseline pressure.

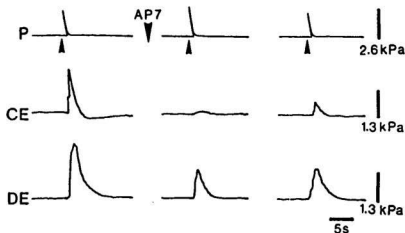
### iii) Complete swallow responses

#### a) CS responses evoked by glutamate microinjections into the NTS

At CS loci, prior application of APV or AP7 7-40 pmol (Table V) inhibited the cervical and distal oesophageal components of the swallowing sequence evoked by glutamate as evidenced by a fall in the amplitude; no corresponding effect was observed on the pharyngeal component (Fig. 12). However, recovery of the response was incomplete (25-65% of control) for up to 1 h. In contrast to APV and AP7, DGG blocked all three components of the CS response in a non-selective manner.

#### b) CS responses evoked by systemic quipazine

The effects of NMDA-receptor blockade on automatic swallowing were examined. Automatic swallowing was induced by intravenous administration of quipazine 5  $\mu\text{mol/kg}$  (cf. Bieger, 1981; see 2.A below). In about 50% of the test cases, the pharyngeal responses had accompanying oesophageal components. The frequency of the responses was 12-24/min (Fig. 13; cf. Bieger, 1981). MK-801, the non-competitive blocker of the NMDA-receptor-associated ionophore (Wong *et al.*, 1986), was given intravenously (100-300 nmol/kg). Within 20 s of administration, MK-801 selectively abolished the oesophageal components of the CS responses.

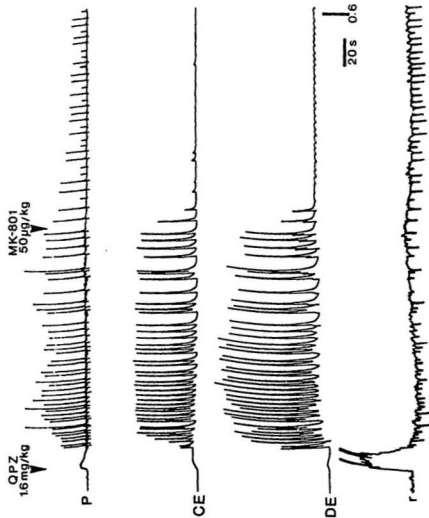


**Figure 12**

**Effect of NMDA receptor blockade at a CS site.** **Left Panel:** Control response evoked by pressure ejection of glutamate (small arrowhead) 20 pmol into the NTS. **Middle Panel:** Selective antagonism of cervical and distal oesophageal components following microinjection of APV 22 pmol at the same site 2 min prior to the glutamate pulse. **Right Panel:** Partial recovery 15 min later. Note that in this and some of the subsequent figures, pharyngeal pressure waves appear as upward spikes due to their brevity and the slow recording speed.

**Figure 13**

Effect of MK-801 on quipazine-induced automatic swallowing. Intravenous administration of quipazine (QPZ), 3  $\mu\text{mol/kg}$ , evoked automatic swallowing consisting of complete deglutitive sequences. MK-801 (148 nmol/kg of intravenous), a non-competitive antagonist at NMDA receptors, blocked the oesophageal but not the pharyngeal components. However, the amplitude of the pharyngeal component was markedly reduced.



The pharyngeal components displayed a drop in amplitude reflecting a reduction in the force of contraction without any apparent change in their frequency (Fig. 13).

Recovery of oesophageal components was either incomplete or not seen for up to 1 h.

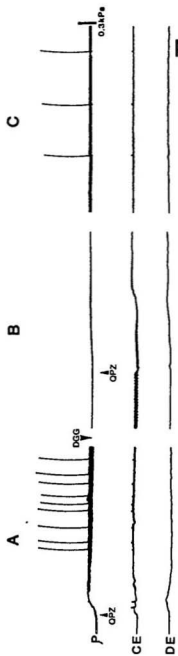
In contrast to MK-801, prior application of DGG (10 nmol) to the ependymal surface of the solitary complex completely abolished the quipazine-induced swallowing (Fig. 14) for periods up to 10 min. The swallowing activity gradually recovered over a period of 15-30 min.

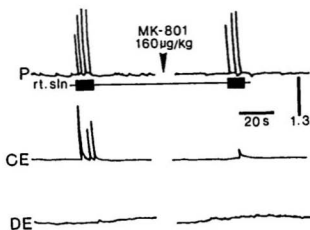
#### c) Pharyngo-oesophageal responses evoked by electrical stimulation of the superior laryngeal nerve

Reflex swallowing was evoked in 5 rats by electrical stimulation of the superior laryngeal nerve. Each train of pulses evoked 3-4 swallows consisting of pharyngo-oesophageal contractile waves which, however, seldom progressed beyond the cervical oesophagus (Fig. 15). Intravenous injections of MK-801 (300-500 nmol/kg), within 2-3 min of administration, inhibited the oesophageal component of the swallows with no corresponding effect on the pharyngeal component save for a small drop in amplitude (Fig. 15). Higher doses of MK-801 (500-600 nmol/kg) completely abolished the response, *i.e.*, both pharyngeal and oesophageal

**Figure 14**

Blockade by DGG of quipazine-induced swallowing. **A:** Control response showing automatic swallowing induced by intravenous quipazine (QPZ), 6  $\mu$ mol/kg. **B:** Absence of response to the same dose of quipazine administered 2 min after application of gamma-D-glutamylglycine (DGG) 10 nmol to the ependymal surface of the solitary complex. **C:** Appearance of automatic swallowing activity 10 min later. Calibration bar: 20 s in A and C, 10s in B.





**Figure 15**

Selective blockade by MK-801 of oesophageal components of reflex swallowing. **Left panel:** control swallowing response to electrical stimulation of the right superior laryngeal nerve (rt. sln; 6 Hz, 2.5 V, 1ms; 10 s trains). **Right panel:** Selective blockade of the oesophageal components 3 min after intravenous administration of MK-801 480 nmol/kg. Note the slight fall in amplitude of the pharyngeal component. Pressure calibration in kPa.



components, for a short period of time (2-10 min), following which the pharyngeal components reappeared but not the oesophageal components.

Recovery of the oesophageal components was not observed.

## **B. Excitatory amino acid receptor agonists**

### **i) Pharyngeal and CS responses**

At pharyngeal or CS sites, application of equimolar doses (0.01-10 pmol) of the three excitatory amino acid receptor agonists (kainate [KA], quisqualate [QA] and N-methyl-D,L-aspartate [NMA] or N-methyl-D-aspartate [NMDA]) in random sequence from adjacent barrels of the micropipette, were effective in mimicking the deglutitive action of glutamate but at potencies differing from one another by several orders of magnitude. KA displayed the highest potency followed by NMA/NMDA and QA (Fig. 16 A; Table VI). The efficacy of the agonists, as indicated by the response duration and rate of repetitive swallowing activity decreased in the order KA>NMA/NMDA>QA. No potency difference could be discerned between the racemate and the dextrorotatory enantiomer of N-methyl-aspartate. QA had the weakest action, inducing responses only at relatively higher doses (1-10 pmol).

**Figure 16**

**A:** Potency comparison of excitatory amino acid agonists at a pharyngeal site in the subnucleus ventralis of the NTS. Continuous record showing pharyngeal responses elicited by application (at arrowheads) of equal doses (0.1 pmol; 100 pl) of kainate (KA), quisqualate (QA) and N-methyl-D-aspartate (NMDA) from adjacent barrels of the micropipette. **B:** Extent of pharyngo-oesophageal progression of a CS response evoked by pressure ejection of the three EAA agonists into the NTS in equimolar doses (1 pmol). In this figure, only the first elicited response is shown at high chart speed for each of the excitatory amino acid agonists. Note the weaker oesophageal components of the KA-evoked response. Pressure calibration bars as in **A**.

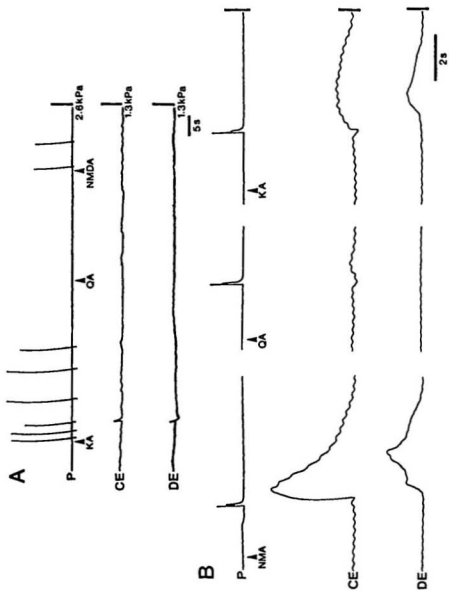


TABLE VI

Equieffective molar doses (ED50) of EAA agonists\* at deglutitive loci in the solitarius complex of the rat.

Type of site (N)	Agonist dose (picomoles)		
	NMA/NMDA	KA	QA
Pharyngeal and CS (87)	0.24	0.02	3.3
95% confidence limits	0.09-0.6	0.009-0.046	0.64-n.d.**
Oesophageal (105)	0.016	0.3	0.7
95% confidence limits	0.006-0.036	0.056-1.7	0.34-1.6

\* Agonists were micropneumophoretically applied in pulses of 100 picoliters each, in random order at concentrations of  $10^{-4}$ ,  $10^{-3}$ ,  $10^{-2}$  and  $10^{-1}$  M. The values given represent doses required to elicit a response in 50% of test sites, as determined by graphic interpolation from a plot of log molar dose vs. percentage of responding sites.

\*\* n.d.=not determined.

Abbreviations: NMDAN-methyl-D-aspartate; NMN-methyl-D,L-aspartate; KA kainate; QA quisqualate; CS complete swallow.

Whereas pharyngeal swallows or CS responses evoked by KA were either devoid of or had weak accompanying oesophageal waves, those elicited by NMA/NMDA frequently exhibited a marked oesophageal progression (Fig. 16 B; Table VII).

## ii) Oesophageal responses

As at the pharyngeal loci, the three excitatory amino acid agonists were tested in random fashion at glutamate-responsive oesophageal loci. All three were equally efficacious in eliciting oesophageal responses but NMA/NMDA proved to be the most potent (Fig. 17; Table VI).

All sites at which the three excitatory amino acid agonists were applied showed evidence of highly localized parenchymal damage in the form of interstitial oedema. This was especially evident at sites where NMA/NMDA was applied. The radius of the oedematous area was roughly half that of the injection sites (15-25  $\mu\text{m}$ ) as determined by the radius of the fluorochrome-labeled locus.

## iii) Glutamate *versus* ACh (muscarine)

Subthreshold pulses of ACh (4-10 pmol) or muscarine (0.1-1 pmol; N=8 rats), applied 5 s prior to the glutamate test pulse, enhanced the glutamate-evoked oesophageal response by a factor of 2-5 (Figs. 18, 19). The response returned to baseline levels within 1-3 min. This interaction between glutamate and ACh or muscarine was highly reproducible and could be repeated up to 30 times at a given

TABLE VII

Incidence of pharyngo-oesophageal progression observed at deglutitive loci in rat nucleus tractus solitarii.

AGONIST*	% OF TOTAL TRIALS
S-glutamate	77 (N=26)
NMA/NMDA	75 (N=28)
Quisqualate	33 (N=12)
Kainate	23 (N=21)

\* Agonists were micropneumophoretically applied in the dose range of 0.01 - 10.0 pmoles.

Abbreviations: NMA N-methyl-D,L-aspartate; NMDA N-methyl-D-aspartate.  
N = total number of sites tested (in a total of 25 animals).

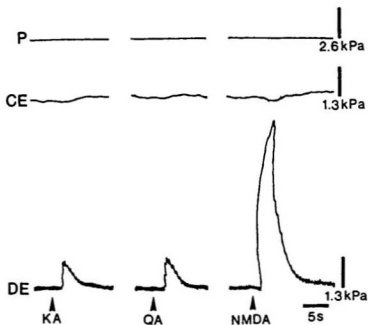


Figure 17

Potency comparison of excitatory amino acid agonists at an oesophageal site in the subnucleus centralis of the NTS. Equal doses (1 pmol; 100 pl) of the three excitatory amino acid agonists were applied (at arrowheads) at the same site from adjacent barrels of the micropipette.

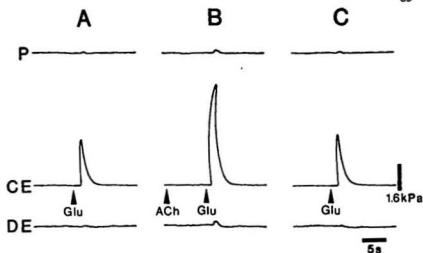


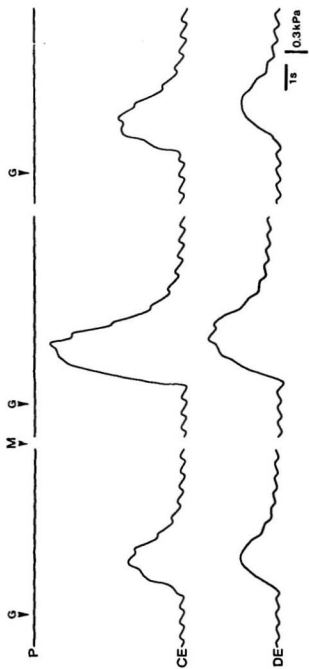
Figure 18

Enhancement of a glutamate-evoked oesophageal response by ACh. A: Control response in the cervical oesophagus to 12 pmol of glutamate (Glu) pressure ejected into the subnucleus centralis of the NTS. B: Enhancement of the glutamate-evoked response 9 s after application of a subthreshold pulse (6 pmol) of ACh at the same site. C: Recovery 2.5 min later.



**Figure 19**

Enhancement of a glutamate-evoked oesophageal response by muscarine. **A:** Control response in the distal oesophagus elicited by pressure ejection of 15 pmol of glutamate (G) into the subnucleus centralis of the NTS. **B:** Enhancement of the glutamate-evoked response 5 s after the application of a subthreshold pulse (0.4 pmol) of muscarine (M) at the same site. **C:** Recovery 2 min later.



site. Prior ejection of glutamate was without effect on ACh- or muscarine-evoked oesophageal responses.

The glutamate test pulse was gradually reduced in steps of 5-10 ms until the response could no longer be elicited. Subthreshold pulses of ACh or muscarine, applied 5 s prior to the test pulse of glutamate, caused the glutamate response to appear. This facilitation by ACh or muscarine of the glutamate-evoked oesophageal responses lasted 1-2 min.

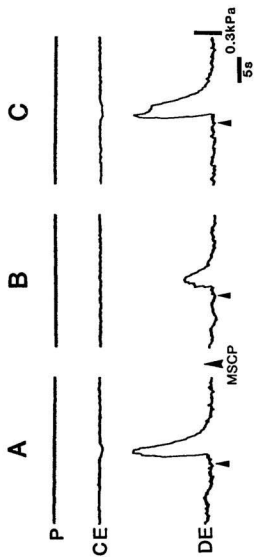
Prior ejection of the muscarinic cholinceptor antagonist, methscopolamine (MSCP; 2-5 pmol), markedly attenuated the glutamate-evoked oesophageal response (Fig. 20; cf. Bieger, 1984). The response remained inhibited for periods lasting up to 10-30 min. Increasing the dose of glutamate failed to overcome the inhibition by MSCP.

In 3 cases, at cervical oesophageal sites, glutamate evoked a propulsive oesophageal response (Fig. 21). Application of MSCP (2-5 pmol) at these sites 50 sec prior to the glutamate pulse or intravenous administration of scopolamine (3  $\mu$ mol/kg) rendered the evoked response non-peristaltic (N=1) or entirely suppressed the distal oesophageal component (N=2; Fig. 21). Recovery was not seen for up to 1 h.

Oesophageal responses evoked by ACh were consistently potentiated by intravenous administration of physostigmine 0.15-0.3  $\mu$ mol/kg; there was a weaker, inconsistent increase in glutamate-evoked responses. In about 50% of test cases,

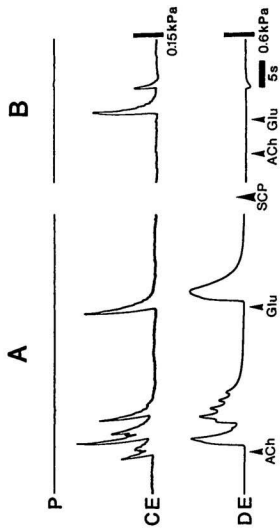
**Figure 20**

Inhibition of a glutamate-evoked oesophageal response by methscopolamine. **A:** Control response evoked by an intrasolitary 16 pmol pulse of glutamate (small arrowhead). **B:** Attenuation of the glutamate-evoked response 50 s after application of methscopolamine (MSCP) 4 pmol to the same site from an adjacent barrel of the micropipette. **C:** Recovery 16 min later.



**Figure 21**

Effect of muscarine receptor blockade on a propulsive oesophageal response evoked by glutamate. **A:** Control response to an intrasolitary pulse of glutamate (Glu) 17 pmol consisting of a propulsive oesophageal contractile wave originating in the cervical oesophagus. Note application of 18 pmol of ACh at the same site evokes secondary peristalsis-like response. **B:** Loss of the distal oesophageal component of the glutamate-evoked response as well as blockade of the ACh-evoked response 3 min after intravenous administration of scopolamine (SCP) 3  $\mu\text{mol/kg}$ . The small upward deflection in the cervical oesophageal trace is produced by "sighing" of the animal. Note corresponding relaxation of the distal oesophagus.



administration of physostigmine alone produced rhythmic oesophageal contractile activity (Fig. 22) which was indistinguishable from the muscarine-evoked responses.

High doses of ACh (>30 pmol; 150 pl) or muscarine (>10 pmol) caused an increase in the amplitude of inspiratory and expiratory waves. The latency of this effect was 5-15 s.

## 2. Effects of 5-HT receptor agents

### A. Quipazine-induced swallowing

The 5-HT mimetic, quipazine was administered intravenously (3-15  $\mu\text{mol/kg}$ ) to evoke repetitive swallowing activity (cf. Bieger, 1981) which consisted of pharyngeal swallows with or without accompanying oesophageal components. The swallows thus elicited were reversibly blocked by intravenous administration of the 5-HT<sub>2</sub> and 5-HT<sub>1c</sub> receptor antagonist, ketanserin (1-2  $\mu\text{mol/kg}$ ; Fig. 23) as well as by the non-selective 5-HT receptor antagonists, methysergide and metergoline (1-2  $\mu\text{mol/kg}$ ) and potently inhibited by the 5-HT<sub>1A</sub> receptor agonist, 8-OH-DPAT (0.5-1.5  $\mu\text{mol/kg}$ ; Fig. 24) for periods upto 2 h. Intravenous administration of a number of 5-HT receptor agents failed to produce any appreciable effects either on the quipazine-induced swallowing or on the 8-OH-DPAT inhibition of quipazine-induced swallowing. These (see Bradley *et al.*, 1986, Fozard, 1987 and Peroutka,





Figure 22

Spontaneous oesophageal contractions evoked by intravenous physostigmine. Periodic contractions in the distal oesophagus 3.5 min after administration of physostigmine ( $0.25 \mu\text{mol/kg}$ , i. v.).

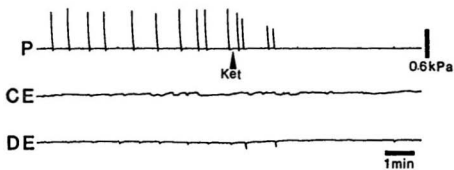
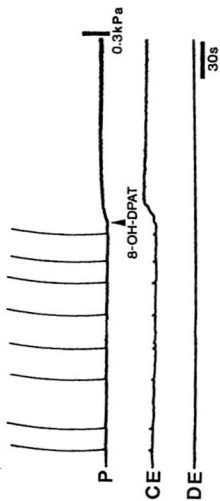


Figure 23

Blockade of quipazine-induced automatic swallowing by ketanserin. Continuous trace showing automatic swallowing induced by intravenous administration of quipazine 5  $\mu$ mol being blocked after intravenous ketanserin (Ket) 1  $\mu$ mol. Recovery (not shown) occurred 30 min later.

Figure 24

Inhibition of quipazine-induced swallowing by 8-OH-DPAT. Continuous trace showing automatic swallowing initiated by an intravenous bolus of quipazine 6  $\mu\text{mol/kg}$  being suppressed by 8-OH-DPAT 1.5  $\mu\text{mol/kg}$ , administered intravenously.



1988 for an overview of 5-HT receptor classification and pharmacology; see also Hoyer and Middlemiss, 1989) included:

- i) Cyanopindolol (5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> antagonist; 0.15-3  $\mu$ mol/kg)
- ii) TFMP (1-(3-trifluoromethylphenyl) piperazine; 5-HT<sub>1B</sub> and 5-HT<sub>1C</sub> agonist; 5-HT<sub>2</sub> antagonist; 0.5-1.5  $\mu$ mol/kg)
- iii) Pizotifen (5-HT<sub>1C</sub> and 5-HT<sub>2</sub> antagonist; 0.1 mg/kg)
- iv) Yohimbine (5-HT<sub>1D</sub> antagonist; 2.5  $\mu$ mol/kg)
- v) 5-HT<sub>3</sub> receptor antagonists, ICS 205-930 and
- vi) BRL 43694 (1.5  $\mu$ mol/kg),

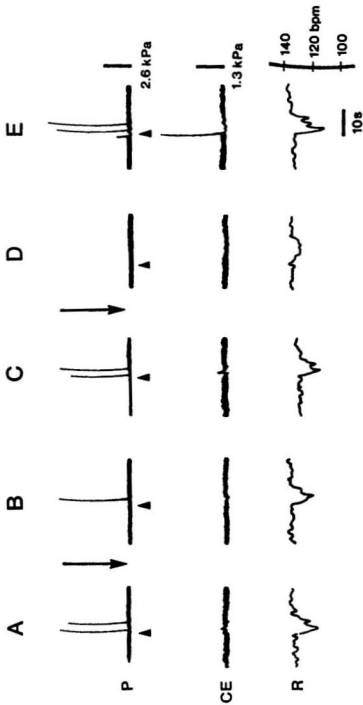
## B. 5-HT *versus* glutamate

### i) Elicitation of pharyngeal responses by 5-HT

In 6 out of 32 experiments, 5-HT 5-50 pmol pressure ejected at glutamate-responsive pharyngeal sites within the subnuclei ventralis and intermedius of the NTS produced short-latency (1-2 s) pharyngeal responses on its own, revealing an overt excitatory action (Fig. 25). The 5-HT responses, obtained with either the bimalate or creatinine sulphate salt, were qualitatively similar to those evoked by glutamate at the same site except that their latencies were 5-10 times longer (Fig. 26 A). Pressure ejections of equal volumes (60  $\mu$ l) of either vehicle, viz., artificial CSF or distilled water, failed to produce any effect. Prior ejection

Figure 25

Elicitation of pharyngeal responses by 5-HT pressure ejected into the NTS. **A:** Two pharyngeal pressure waves elicited at a latency of 2 s by a single pulse (at arrowhead) of 5-HT 24 pmol. **B:** Antagonism by ketanserin (bold arrow) 20 pmoles ejected at the same site 60 s earlier. Note also the increased latency of response, 3 s. **C:** Recovery 2.5 min later. **D:** Complete blockade of 5-HT effect after a second pulse of ketanserin 36 pmol. Note incomplete inhibition of bradypneic effect of 5-HT. **E:** Recovery 3.5 min later. Upward deflections prior to the pulse of 5-HT in top two traces correspond to spontaneous "sigh".



**Figure 26**

Selective tachyphylaxis of 5-HT-evoked responses. **A:** Two pharyngeal swallows (P) elicited by glutamate 18 pmol (arrow) and a single pharyngeal swallow by 5-HT 24 pmol (arrowhead) ejected at 1 min intervals at the same site into the NTS. **B:** Weaker response to fourth 5-HT pulse. **C:** Failure of response after ninth 5-HT pulse and persistence of glutamate effect.



A

B

C



CE



(1-2 min) of the 5-HT<sub>2</sub>- and 5-HT<sub>1C</sub>-receptor antagonist, ketanserin (25-40 pmol) reversibly blocked the 5-HT-evoked responses (see Fig. 29 B, D) for 2-5 minutes.

However, after 5-10 repetitions, over a period of 10-20 min, the responses could no longer be elicited by 5-HT, whereas glutamate, ejected from the other barrel of the micropipette, continued to produce the response (Fig. 26 C). The cumulative dose of 5-HT producing such tachyphylaxis totalled 100-300 pmol.

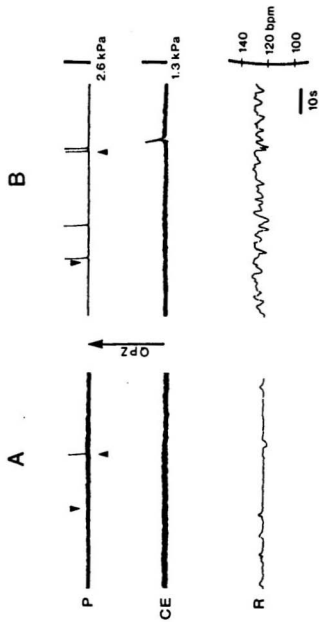
A low intravenous dose of quipazine (0.3-1  $\mu$ mol/kg) was injected (N=6) while swallowing responses were being tested to pressure-ejections of 5-HT and glutamate in the NTS. Within 3-5 minutes after quipazine administration, responses to 5-HT were markedly enhanced (Fig. 27) as indicated by: i) appearance of responses with subliminal doses or ii) an increased frequency of pharyngeal pressure waves elicited per 5-HT pulse (5-10 pmol). Glutamate-evoked responses, elicited alternately at the same site, were also enhanced by quipazine (Fig. 27 B). This facilitatory effect of quipazine was reversibly blocked by intravenous administration of methysergide, metergoline or ketanserin (1-2  $\mu$ mol/kg).

## ii) Effect of 5-HT on glutamate-evoked pharyngeal responses

At pharyngeal sites, pulses of 5-HT (1-10 pmol), applied 5-10 s before the glutamate pulse, facilitated the glutamate-evoked pharyngeal responses as evidenced by 1.5 - 2 fold increase in their amplitude and/or number as well as a decrease in their latency by a factor of 0.5-0.3 (Figs. 28 A,B and 29 A,B). The responses reverted to baseline levels 1-4 minutes after application of 5-HT (Figs. 28 C and

**Figure 27**

Enhancement of 5-HT- and glutamate-evoked pharyngeal responses by systemic quipazine. **A:** Absence of response to a subliminal pulse (▼) of 5-HT 20 pmol applied at an NTS site responsive to glutamate (▲) 16 pmol ejected from adjacent barrel. **B:** Facilitated responses 4 min after intravenous quipazine (QPZ, at arrow) 0.3  $\mu$ mol/kg to 5-HT 14 pmol and glutamate 16 pmol.



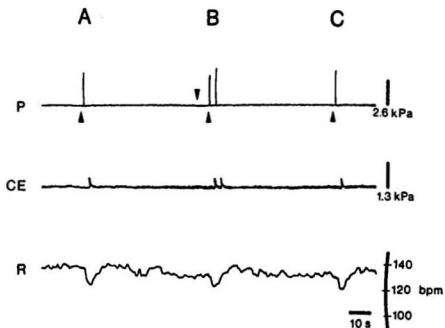
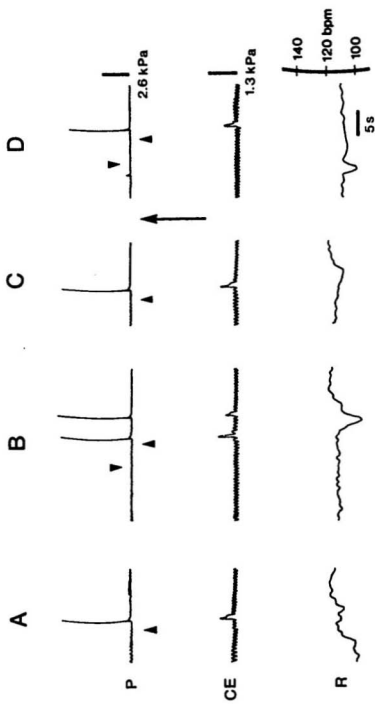


Figure 28

Facilitation of a glutamate-evoked pharyngeal response by 5-HT. A: Control swallowing response to glutamate (▲) 12 pmol ejected into the NTS. B: Facilitation of glutamate response by prepulse of 5-HT (▼) 1.5 pmol. C: Recovery 1 min later.

**Figure 29**

Selective blockade by ketanserin of facilitation by 5-HT of a glutamate-evoked pharyngeal response. **A:** Control response to intrasolitary pulse of glutamate (**A**) 14 pmol. **B:** Facilitated glutamate-evoked response after prepulse of 5-HT (**Y**) 2 pmol at the same site. **C:** Recovery 1.5 min later. **D:** Blockade of 5-HT facilitation 2 min after systemic administration of ketanserin (at arrow) 1.5  $\mu$ mol/kg, with persistence of the glutamate control response. The small deflection to the left on the P trace is due to "sighing"; note the corresponding drop in respiratory rate.



29 C). Administration of ketanserin (1-2  $\mu\text{mol/kg}$ , i.v.) reversibly blocked the facilitatory action of 5-HT without affecting the glutamate control response (Fig. 29 D).

When glutamate was ejected at higher doses (50-100 pmol) so as to evoke 5-7 pharyngeal swallows per pressure pulse, 5-HT prepulses (1-10 pmol) proved ineffective in causing any further enhancement.

With increasing doses of 5-HT (15-60 pmol), a transient, dose-related suppression of the glutamate-evoked pharyngeal responses was seen, as indicated by an increase in the latency and a decrease in amplitude and frequency. There was a partial recovery within 4-8 minutes. 5-HT pulses of 50-60 pmol totally abolished the glutamate-evoked responses, recovery remaining incomplete even after 30-60 min intervals. Intravenous administration of the 5-HT antagonists methysergide or metergoline (1-2  $\mu\text{mol/kg}$ , i.v.) failed to overcome the inhibitory effect of 5-HT. However, the latter was easily surmounted by increasing the glutamate doses 2-3 fold.

A reduction in the number and amplitude of pharyngeal responses, followed by partial or no recovery, was also observed when repeated (3-5 times) applications of glutamate at high doses were made (200-400 pmol; 1-2 nl).

Histological examination of ejection sites where glutamate was applied repeatedly in large doses showed tissue damage within the region.



### iii) Effect of chemical microstimulation of the medullary raphe nuclei

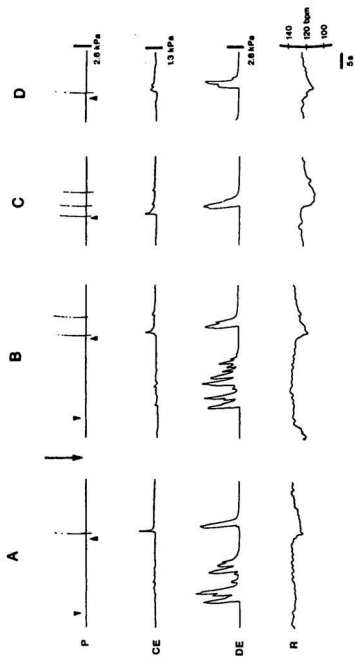
Following identification of the raphe magnus, obscurus and pontis as presumptive sources of serotonergic input to solitary deglutitive loci (see section III-1 below), these structures were explored with glutamate pulses (50-100 pmol; 100-200 pl) to determine the effects, if any, of chemical microstimulation. No deglutitive responses were produced. A low dose of quipazine (0.3-1  $\mu$ mol) was administered intravenously and the raphe nuclei were again probed with the glutamate pulses. Only weak (low amplitude), inconsistent pharyngeal responses were elicited at a latency of 3-8 sec. However, despite the quipazine pretreatment, pharyngeal responses evoked by stimulation of the raphe nuclei failed to appear more than twice.

### iv) Oesophageal responses

Prepulses of 5-HT 1-10 pmol applied at the same site or on the ependymal surface of the solitary complex (10 nmol) failed to alter the oesophageal responses evoked by ACh (Fig. 30 B) or glutamate. With higher doses of 5-HT (15-60 pmol), there was an apparent inhibition of oesophageal responses as evidenced by a decrease in their amplitude and number. The responses were completely suppressed after the highest dose of 5-HT (60 pmol), recovery again being partial or absent. The three 5-HT receptor antagonists, metergoline, methysergide and ketanserin administered intravenously (1-2  $\mu$ mol/kg i.v.) failed to overcome the suppression induced by 5-HT. Again, the responses could be restored when the

**Figure 30**

Effect of 5-HT on oesophageal and CS responses. **A:** Distal oesophageal (DE) repetitive responses to intrasolitary ACh (▼) 28 pmol resembling secondary peristalsis. Ejection of glutamate (▲) 22 pmol at the same site produced a pharyngo-oesophageal propulsive sequence. **B:** 14 s after the application of 5-HT (arrow) 10 nmol to the ependymal surface of the solitary complex, neither the ACh-evoked response nor the oesophageal component of the glutamate response, but the pharyngeal component of the glutamate-evoked CS response was facilitated. **C:** Enhanced pharyngeal response to glutamate 1.5 min later. **D:** Recovery 2 min later.



test doses of ACh or glutamate were increased 2-3 fold.

#### v) CS responses

Prepulses of 5-HT (1-10 pmol) or its prior application to the ependymal surface of the solitary complex (10 nmol; 1  $\mu$ l) enhanced the pharyngeal component of the glutamate-evoked CS (Fig. 30), causing a 2-3 fold increment in the number and amplitude of the response, without a corresponding effect on the associated oesophageal component or that evoked by ACh at the same site.

5-HT ejections at pharyngeal and CS loci sometimes caused a momentary (0.5-1 s) slowing of respiration. As a rule, this effect occurred in the absence of any overt change in pharyngeal and oesophageal intraluminal pressure and was seen irrespective of whether it was followed by facilitation or inhibition of glutamate-elicited responses. This effect was more accentuated when simultaneous swallowing responses were elicited and, unlike the latter, relatively less sensitive to ketanserin (see Fig. 25 D).

### III. Neural connections of solitary deglutitive loci

#### 1. Retrograde tracing combined with 5-HT immunocytochemistry

##### A. Injection sites

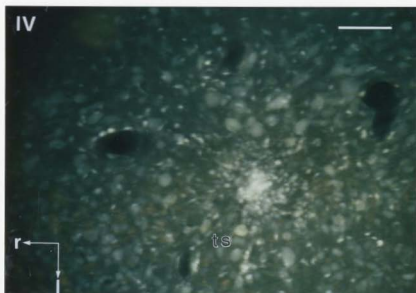
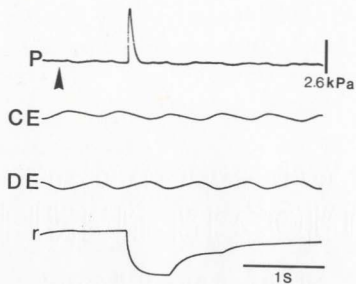
Under ultraviolet illumination, the fluorogold deposit appeared intensely white at the site of injection and consisted of dye-filled cell bodies (10-15  $\mu\text{m}$  in diameter) and fluorescent fibers and dendrites occupying a nearly spherical region (Fig. 31). The diameter of the injection sites ranged between 300  $\mu\text{m}$  and 500  $\mu\text{m}$  corresponding to an injection volume range of 1-5 nl. Each site extended through 10-15 consecutive sections indicating a dorsoventral spread of 300-450  $\mu\text{m}$  in diameter. No evidence of either the pipette track or a lesion could be detected at any of the sites. Deposits of moderate volumes of fluorogold (2.5 nl) did not show evidence of any appreciable retrograde transport when made with fine tipped (4-6  $\mu\text{m}$ ) micropipettes and in small (200-300 pl) pulses. However, when an equivalent volume was applied from larger diameter micropipette tips (8-12  $\mu\text{m}$ ) in larger pulses (500-800 pl), the dye was transported.

Rhodamine-filled latex microspheres, which were employed in one case, fluoresced red under ultraviolet illumination. This case yielded results identical to those obtained with fluorogold. The microspheres appeared as small punctate dots

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**Figure 31**

Fluorogold injection site. Case # 43 FG. **Top:** Pharyngeal response evoked by glutamate 20 pmol micropneumophoresed at a site in the solitary complex (shown below) where a 20% solution of fluorogold 3 nl was ejected from an adjacent barrel of the micropipette. **Bottom:** Photomicrograph of a horizontal section through the ventral subnucleus of the solitary complex showing the site of fluorogold injection. Abbreviations: IV fourth ventricle; ts tractus solitarius. Calibration bar: 50  $\mu$ m.



within the perikarya. However, fibre- and dendritic labeling was either sparse or difficult to discern. In addition, the fluorescence emitted by the microspheres was dimmer than that of fluorogold and thus more difficult to capture on photographic film.

### **B. Retrogradely labeled perikarya**

Retrogradely-filled cell bodies were found in many pontomedullary regions. These included: a "mirror-image" locus in the contralateral NTS, the raphe nuclei (Fig. 32), the paratrigeminal islands, the pontine and medullary reticular formations including the ventrolateral reticular area (the 'rostral ventrolateral medulla') and the periaqueductal grey. Few, if any, retrogradely-labeled cells were found within the branchiomotor nuclei or the hypoglossal nucleus. Large injections of fluorogold resulted in retrogradely labeled perikarya scattered throughout the vestibular complex.

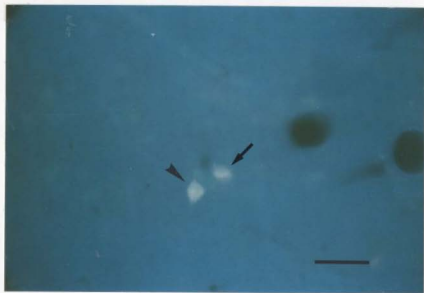
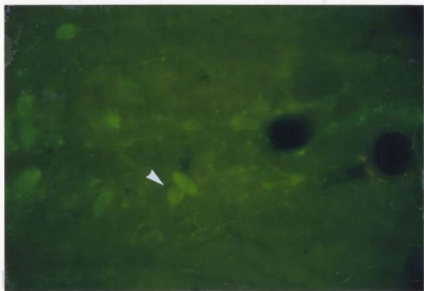
### **C. 5-HT immunoreactivity**

Extensive 5-HT immunoreactive (IR) fibre-networks, emitting fluorescein-isothiocyanate fluorescence under ultraviolet illumination, were found in all brainstem regions described previously (Steinbusch, 1981). Noteworthy in this regard was the dense network of fibres enmeshing the entire solitary complex.



**Figure 32**

Retrogradely-labeled 5-HT-immunoreactive and non-immunoreactive perikarya in the raphe obscurus nucleus. Case # 43 FG. Top: Photomicrograph under ultraviolet illumination of a horizontal section showing cells in the raphe obscurus nucleus displaying 5-HT immunoreactivity as visualised by the FITC fluorescence. Bottom: Same section under a different filter showing two cells displaying retrogradely-filled fluorogold fluorescence. Arrowheads in both pictures point to a cell containing both FITC and fluorogold labels. Arrow points to a fluorogold-labeled cell not immunoreactive to 5-HT. Calibration bar: 25  $\mu$ m.



5-HT-IR cell bodies fluoresced an intense green under ultraviolet illumination and were found within the periaqueductal grey region and in all of the raphe nuclei. In addition, scattered 5HT-IR perikarya were also observed in the pontine reticular formation, lateral to the raphe pontis; and the medullary reticular formation, lateral to the raphe magnus.

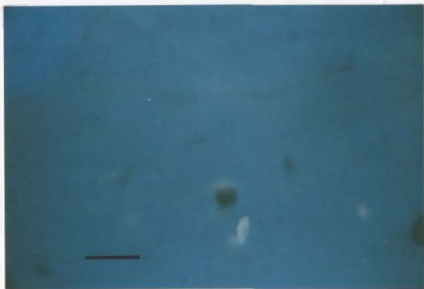
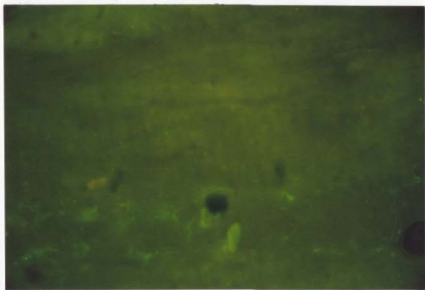
#### D. Retrogradely-labeled 5-HT-IR perikarya

Pontomedullary areas containing 5-HT-IR cell bodies were systematically scanned for the presence of fluorogold or rhodamine label. Such dual-labeled cells were found only in three regions: the raphe magnus, obscurus and pontis and varied from 2 to 9 in number in each nucleus. The highest numbers of double-labeled cells were found in the raphe obscurus (Fig. 32) and raphe magnus (Fig. 33). Typically, the double-labeled cells displayed relatively weak fluorogold fluorescence and were relatively smaller (6-8  $\mu\text{m}$  in diameter) than others in the vicinity and appeared ovoid or oblong in shape in the horizontal plane. One or two dual-labeled cells were larger (16  $\mu\text{m}$  in diameter).

The raphe pontis contained an occasional double-labeled cell similar in size and shape to the ones seen in the medullary raphe.

**Figure 33**

Retrogradely-labeled 5-HT-immunoreactive perikarya in the raphe magnus nucleus. Case # 43 FG. Photomicrographs of a horizontal section through the raphe magnus nucleus under ultraviolet illumination showing cells displaying 5-HT immunoreactivity one of which (bottom photomicrograph) also emits fluorescence resulting from retrogradely-transported fluorogold. Calibration bar: 25  $\mu$ m.



## 2. Anterograde Tracing

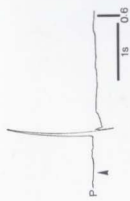
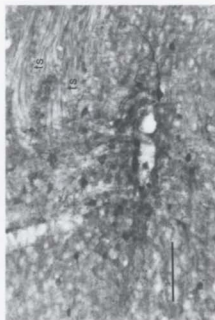
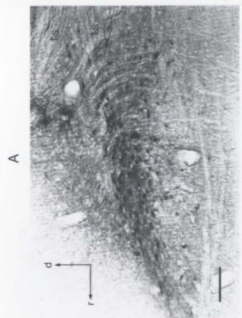
### A. Injection sites

In the sagittal plane, the PHAL injection sites appeared roughly spherical or elliptical and consisted of a central dark brown core of labeled cell bodies surrounded by a 35-100  $\mu\text{m}$  wide halo of light brown reaction product (Figs. 34, 35). Where injection sites were in the form of an ellipse, the poles were aligned in the rostrocaudal direction with the central core measuring 100-600  $\mu\text{m}$  from end to end and about 35-135  $\mu\text{m}$  in the dorsoventral axis at its widest point. Depending upon the amount of tracer applied, lectin-filled perikarya at the site of injection were seen through 5-15 consecutive sections corresponding to a diameter of 200-600  $\mu\text{m}$  in the mediolateral axis. No evidence of a pipette track or any physical damage in the form of a lesion could be observed. Lectin-filled perikarya were observed only at the site of injection.

Deposits of PHAL made with fine-tipped (4-6  $\mu\text{m}$ ) micropipettes failed to label any cell bodies and only a small patch of brown reaction product was observed at the site of injection. In such cases, no labeled fibres were observed indicating that lectin-filled perikarya were a prerequisite for anterograde transport of the tracer to occur (cf. Gerfen and Sawchenko, 1984). Large deposits of the tracer (>10 nl) yielded fibres that were darker in colour and greater in number than those resulting from relatively smaller (2-5 nl) injections.

**Figure 34**

Glutamate-responsive pharyngeal and oesophageal sites of application of PHAL in the rat solitary complex. **A:** Case # 38 PHAL. Left: Pharyngeal response evoked by glutamate pressure-ejected at a site (shown at right) in the solitary complex where PHAL was injected from an adjacent barrel of the micropipette. Right: Brightfield photomicrograph of a sagittal section through the solitary complex showing the pharyngeal site in the subnucleus intermedialis of the NTS as visualised by immunocytochemical detection of the lectin by the PAP method. **B:** Case # 10 PHAL. Left: Glutamate-evoked response from a site (shown at right) in the central subnucleus of the NTS where PHAL was injected. Right: Brightfield photomicrograph of a sagittal section showing the site of application of lectin as indicated by the presence of the DAB reaction product. Calibration bars: 100  $\mu$ m.



**CE**

Electrophysiological trace for electrode CE showing a small downward step.

**DE**

Electrophysiological trace for electrode DE showing a small downward step.

**r**

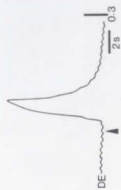
Electrophysiological trace for electrode r showing a broad downward peak.

**P**

Electrophysiological trace for electrode P showing a flat line.

**CE**

Electrophysiological trace for electrode CE showing a flat line.

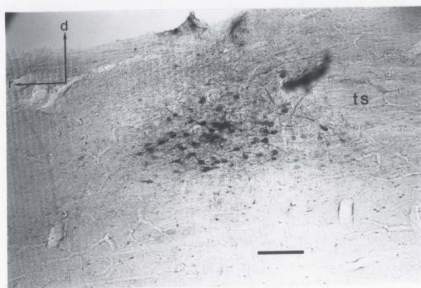
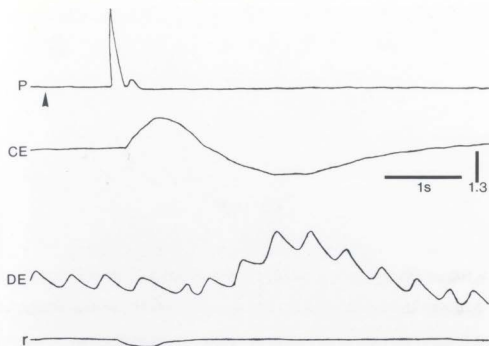




**Figure 35**

A glutamate responsive CS site of application of PHAL in the rat solitary complex.

**Case 16 PHAL.** **Top:** Complete swallow response evoked by an intrasolitary pulse of glutamate at a site (shown below) in the solitary complex where PHAL was injected from an adjacent barrel of the micropipette. **Bottom:** Brightfield photomicrograph of a sagittal section through the solitary complex showing the site of application of PHAL. Calibration bar: 100  $\mu\text{m}$ .



Within the injection sites, lectin-filled perikarya, many having labeled fibres, were densely packed, appeared round or oval in the sagittal plane, and had diameters ranging between 10 and 15  $\mu\text{m}$  (Fig. 36).

## B. Efferents from pharyngeal loci

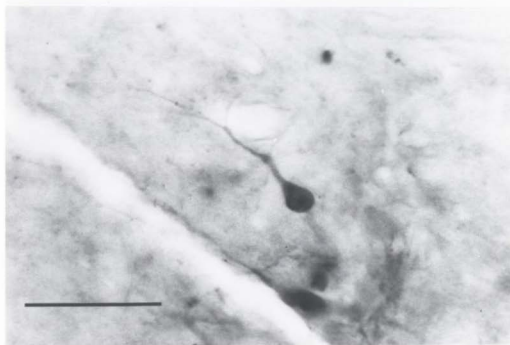
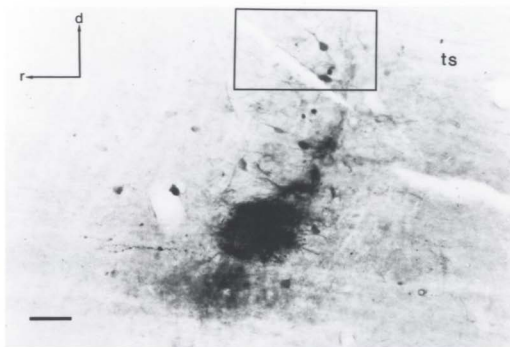
Application of PHAL at glutamate-responsive pharyngeal loci within the  $\text{NTS}_{\text{int}}$  and the  $\text{NTS}_v$  revealed distinctive projections to the branchiomotor and hypoglossal nuclei and to the general visceral efferent column. These solitary efferents to each of the motoneuronal pools are described below.

### i) The nucleus ambiguus

PHAL deposits at glutamate-responsive pharyngeal sites within the  $\text{NTS}_v$  and  $\text{NTS}_{\text{int}}$  resulted in a profusion of labeled fibres and terminals in the tip of the Amb and its semicompact and loose formations while the compact formation contained sparse label (Fig. 37). The solitary efferents descended in an oblique (ventrolateral) direction from their sites of origin in the NTS to enter the Amb from either its caudal or ventral aspects (Fig. 37). During their passage through the Amb, the fibres became varicose at frequent intervals and branched often (see Fig. 44). Evidence of terminals was seen in the form of varicosities on the soma of the Amb neurons and elsewhere.

**Figure 36**

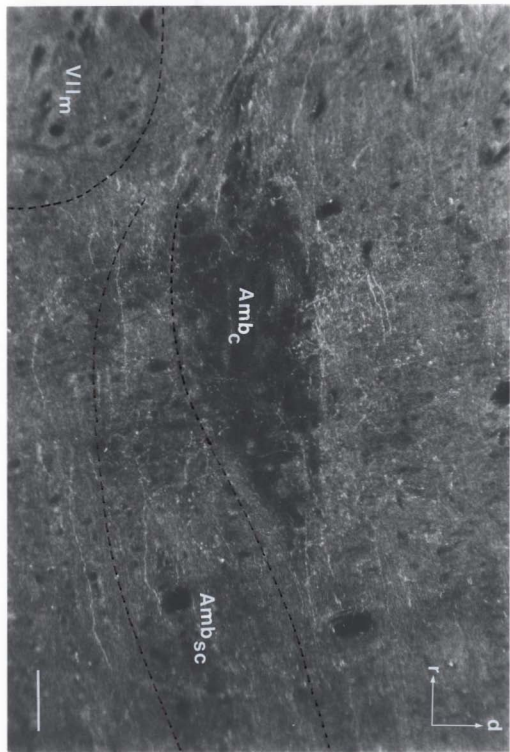
Photomicrographs showing lectin-filled neurons. **Top:** Brightfield photomicrograph of an injection site (Case # 31 PHAL) in a sagittal section through the rat solitary complex. **Bottom:** Boxed area of photomicrograph at top is shown at high magnification to demonstrate lectin-filled neurons. Calibration bars: 50  $\mu\text{m}$ .



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Figure 37

Projections to nucleus ambiguus from a solitaria pharyngeal site. Case # 38 PHAL. Darkfield photomicrograph of a sagittal section showing projections issuing from a pharyngeal site in the rat solitary complex. Projections can be seen in the tip of the nucleus and within the semicompact division (ventrocaudad). Numerous fibres can be seen continuing rostrad. Note the paucity of label in the compact formation. Compare with figure 41. Calibration bar: 100  $\mu$ m.



In addition to the pattern of labeling described above, the region immediately dorsal to the Amb<sub>c</sub> contained a dense network of fibres (Fig. 37).

Fibres coursing through the Amb exited from its tip. The rostral continuation of these fibres was difficult to trace since they intermingled with a fibre system travelling rostrally and dorsally (see below) to terminate in the parabrachial (PB) complex (see Fig. 49).

The contralateral Amb displayed a pattern of labeling similar to the ipsilateral Amb, albeit much weaker in terms of both intensity and density.

## ii) The trigeminal motor nucleus (V<sub>m</sub>)

Although the entire ventral V<sub>m</sub> was traversed by fibres, terminal labeling within the main motor nucleus was sparse.

The accessory or ventromedial subnucleus (V<sub>m</sub><sup>ac</sup>) of the trigeminal motor nucleus (Mizuno *et al.*, 1975; Sasamoto, 1979) was identified as a small cluster of neurons lying between the facial nerve and the V<sub>m</sub>, extending from the level of the Amb to about 500  $\mu$ m medially. This subnucleus of the V<sub>m</sub> contained labeled varicose fibres and terminals (see Fig. 45). Running past the facial nerve, the fibres entered the V<sub>m</sub><sup>ac</sup> from its caudal extent and branched repeatedly within the subnucleus.



### iii) The facial nuclear complex (VII<sub>l</sub>; VII<sub>o</sub>)

Although sparse label was seen scattered throughout the VII<sub>o</sub> rostrally and laterally, the motor nucleus contained a relatively higher density of fibres and terminals (Fig. 38). This portion corresponded to the dorsolateral subdivision of the VII<sub>l</sub> (Watson *et al.*, 1982). The solitary fibres followed an almost vertical course to enter the VII<sub>l</sub> through its dorsal aspects. Within the VII<sub>l</sub>, the fibers became varicose and a few of them appeared to branch.

The accessory facial subnucleus (VII<sub>o</sub>) was identified under darkfield illumination as a loose aggregate of cells lying about 0.8 mm dorsal to the main VII<sub>l</sub> at the level of the medial-most extent of the Amb and extending about 400  $\mu$ m medially. A moderate amount of label in the form of varicose fibres and terminals was observed within this subnucleus.

### iv) The hypoglossal nucleus

Under darkfield illumination, the different subdivisions of the XII<sub>l</sub> were identified in keeping with the description of Krammer *et al.* (1979). Several distinctive features characterized the pattern of labeling within the XII<sub>l</sub> (Fig. 39).

The caudalmost aspects of the dorsal subdivision contained labeled fibres and terminals. With this exception, label throughout the rostrocaudal extent of the subdivision was either sparse or absent.

**Figure 38**

Projections to the facial nucleus from a solitary pharyngeal site. Case # 38 PHAL. Darkfield photomicrographs of the facial nucleus under low (top) and high power (bottom) showing terminals and varicose fibres in the dorsolateral portion of the nucleus. Calibration bars: 100  $\mu\text{m}$  (top), 50  $\mu\text{m}$  (bottom).

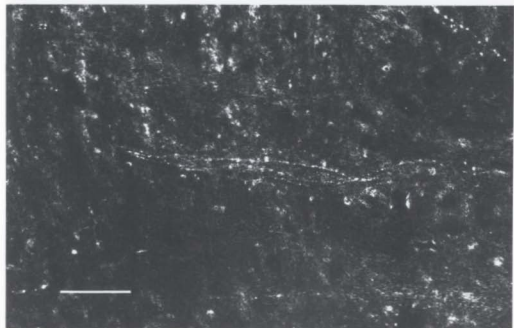
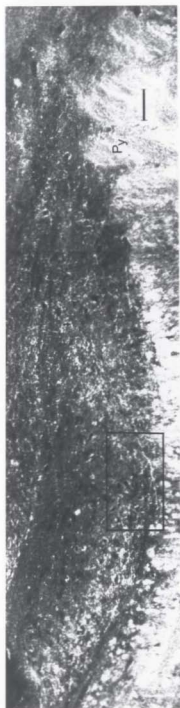


Figure 39

Projections to the hypoglossal nucleus from a solitarily pharyngeal site. Case # 38 PHAL. Top: Darkfield photomontage. Note that the bright white appearance of the pyramidal tract (Py) is artifactual since, with darkfield black-and-white photomicrographs, distinction cannot be made between the silvery appearance of the myelinated pyramidal tract and the golden colour of the fibres labeled with the DAB reaction product. The boxed area corresponds to the high magnification camera lucida drawing at bottom showing morphologic detail of the solitarily fibres. Calibration bars: 100  $\mu$ m.



The ventromedial subdivision of the XII<sub>m</sub> displayed a distinctive labeling pattern. Dense label, in the form of a network of fibres and terminals, began caudally along the dorsal aspects of the ventromedial subdivision. As this column of label continued rostrad, it sloped gradually ventrally to fringe the ventral aspects of the rostral half of the subdivision. Thus, this pattern of label left the rostrodorsal and the caudoventral halves of the ventromedial subdivision scantily labeled and separated by a diagonal column of labeled fibers and terminals.

The ventrolateral subdivision contained heavy label throughout its rostrocaudal extent. The label was pronounced along the lateral aspects of the subdivision; medial aspects displayed prominent label only in the caudal portion.

More caudally, in the spinomedullary portion of the XII<sub>m</sub>, patches of labeled fibres and terminals could be seen.

#### v) The dorsal vagal motor nucleus

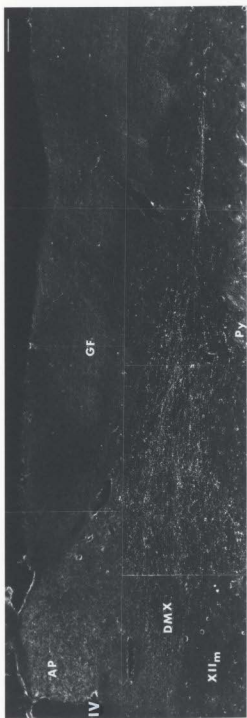
The entire DMX was filled with label (Fig. 40). Besides an abundance of terminals, a profusion of long, varicose fibres were seen streaming down the rostrocaudal extent of the nucleus. In some sections, nearly 1 mm long fibres could be traced. The bundle of labeled fibers became thinner as it continued caudally into the spinal portion of the DMX but remained intense before thinning out at the level of the cervical spinal cord. Thus, from the rostral to the caudal extent of the DMX, the labeled portion appeared funnel-shaped with the stem lying in the caudal part of the nucleus (Fig. 40).

**Figure 40**

Projections to the dorsal motor nucleus of the vagus from a solitarily pharyngeal site.

Case # 38 PHAL. Darkfield photomontage. Reaction product in AP is artifactual.

Abbreviations: **AP** area postrema; **DMX** dorsal motor nucleus of the vagus; **GF** gracile fascicle; **IV** fourth ventricle; **Py** pyramidal tract; **ts** tractus solitarius; **XII<sub>h</sub>** hypoglossal nucleus. Calibration bar: 100  $\mu$ m.





### C. Efferents from oesophageal loci

PHAL deposits at oesophageal loci within the NTS<sub>cen</sub> revealed dense projections to two motor nuclei - the nucleus ambiguus and the DMX. These solitary efferents are described below.

#### i) The nucleus ambiguus

Injections of PHAL at glutamate-responsive oesophageal sites into the NTS<sub>cen</sub> yielded a dense network of labeled fibres and terminals focussed on the Amb<sub>c</sub> (Fig. 41), while the Amb<sub>ac</sub> and Amb<sub>l</sub> contained very sparse label by comparison. The solitary efferents emerged from the NTS<sub>cen</sub> and followed a ventrolateral (oblique) course, entered the Amb<sub>c</sub> through its caudal aspects and branched extensively within the Amb<sub>c</sub>. Their subsequent course was difficult to trace since they were lost in a fibre system that coursed rostrally and dorsally to terminate within the parabrachial complex.

Identical labeling patterns were obtained after injection of tracer at cervical and distal oesophageal NTS<sub>cen</sub> sites.

The contralateral Amb<sub>c</sub> was also labeled, although the intensity and density of the label was much less than that seen in the ipsilateral Amb<sub>c</sub>.

Figure 41

Projections to the nucleus ambiguus from a solitaria oesophageal site. Case # 10 PHAL. Darkfield photomicrograph showing dense labeling within the compact formation of the nucleus ambiguus resulting from an injection of PHAL at an oesophageal site in the subnucleus centralis of the solitary complex. Note relative sparsity of label in semicompact division ventrocaudad. Compare with figure 37. Calibration bar: 100  $\mu$ m.



## ii) The dorsal vagal motor nucleus

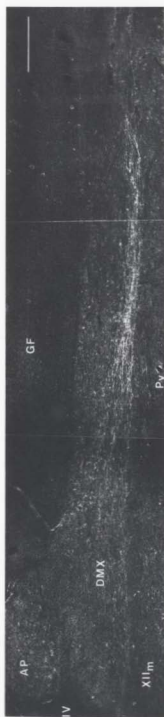
The pattern of label seen in the DMX after PHAL injections into the NTS<sub>cen</sub> was indistinguishable from that observed in pharyngeal cases, albeit somewhat heavier (Fig. 42). To ascertain the specificity of this projection from the NTS<sub>cen</sub>, a small amount of the tracer (2 nl; Case # 28 PHAL) was injected at an oesophageal site within the central subnucleus. Accuracy of targeting was confirmed by the visualisation of a small number of labeled fibres and terminals confined to the Amb<sub>c</sub>. The DMX in this particular case also contained anterograde label in the form of distinctly labeled fibres and terminals. These were seen in the middle of the rostrocaudal extent and in the spinal portion of the DMX.

## iii) Other motor nuclei

Small (3-5 nl) to medium volumes (5-10 nl) of PHAL injected into the NTS<sub>cen</sub> yielded zero to sparse label in the V<sub>m</sub>, the VII<sub>l</sub>, the VII<sub>m</sub> and the XII<sub>m</sub>. However, larger injections (>10 nl) of the tracer into the NTS<sub>cen</sub> yielded a few fibres within some of these motor nuclei. In three such cases, the XII<sub>m</sub> contained sparse but distinct anterograde label which appeared to follow the pattern of label observed in pharyngeal cases, suggesting that the former represented spillage of tracer into the adjacent pharyngeal territory. However, compared with that seen in pharyngeal cases, the density of label in the XII<sub>m</sub> resulting from large injections of the tracer into the NTS<sub>cen</sub> was extremely low. In one case where 10 nl of

**Figure 42**

Projections to the dorsal motor nucleus of the vagus from a solitariai oesophageal site. Case # 10 PHAL. Darkfield photomontage. Reaction product in AP is artifactual. Abbreviations: AP area postrema; DMX dorsal motor nucleus of the vagus; GF gracile fascicle; IV fourth ventricle; Py pyramidal tract; ts tractus solitarius; XII<sub>h</sub> hypoglossal nucleus. Calibration bar: 200  $\mu$ m.



PHAL were injected into the NTS<sub>cen</sub>, a rough estimate indicated that the density of label was about 2 orders of magnitude lower as compared with XII<sub>l</sub> labeling resulting from a medium-sized tracer injection (8 nl) into the NTS<sub>int</sub>.

#### D. Efferents from CS loci

Injections of PHAL at glutamate-responsive CS loci yielded a pattern of labeling that was a combination of both the pharyngeal and oesophageal labeling patterns. Briefly, the entire Amb was labeled (Figs. 43, 44); the V<sub>m</sub> (Fig. 45), the VII<sub>l</sub>, the VII<sub>l</sub> and the XII<sub>l</sub> displayed the labeling pattern seen in pharyngeal cases; and the pattern of labeling seen in the DMX in both pharyngeal and oesophageal cases was also evident.

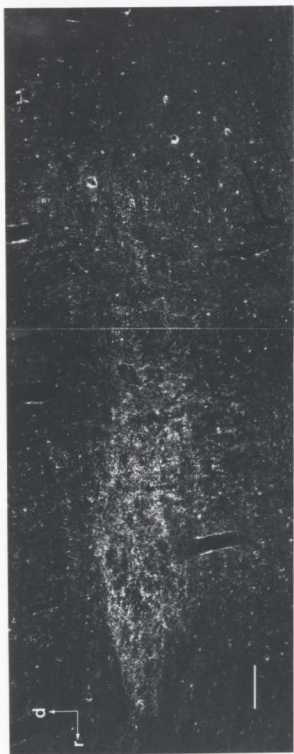
#### E. Solitary projections to other pontomedullary regions

In addition to the motor nuclei described above, solitary deglutitive efferents were observed within a number of pontomedullary regions, viz., the NTS, nuclei of the spinal trigeminal tract, the paratrigeminal islands, the principal sensory trigeminal nucleus, the parabrachial complex, and the "lacrimal" neurons rostral to the facial motor nucleus. Solitary projections to each of these structures are described below. Unless mentioned otherwise, the projections to these structures were observed in all cases *i. e.* following PHAL deposits at pharyngeal, oesophageal and CS loci.

**Figure 43**

Projections to the nucleus ambiguus from a solitaria CS site. Case # 16 PHAL. Darkfield photomontage. Dense fibre-labeling can be seen within the compact and semicompact (approx. rostral half) divisions of the nucleus and lighter label can be seen scattered in the loose formation (approx. caudal half). Calibration bar: 100  $\mu$ m.





**Figure 44**

Projections to the nucleus ambiguus from a solitary CS site. Case # 31 PHAL. Darkfield photomicrographs showing labeled fibres within the nucleus ambiguus at low (A) and high (B) magnification. C: Camera lucida drawing showing morphologic detail of fibers in the nucleus ambiguus. Note the branched fibres studded with varicosities following a tortuous course through the nucleus ambiguus. Boxed area corresponds to photomicrograph shown in A. Arrows in B and C point to the two fibres shown in both pictures. Calibration bars: 100  $\mu$ m.

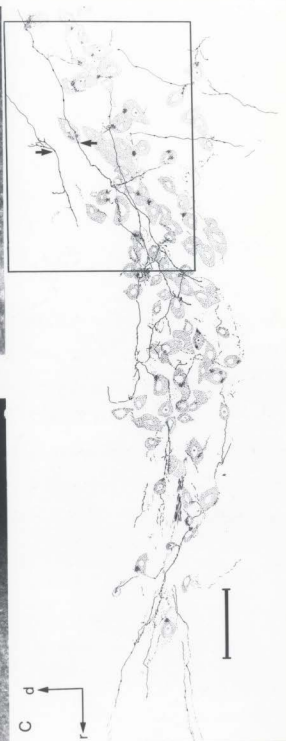
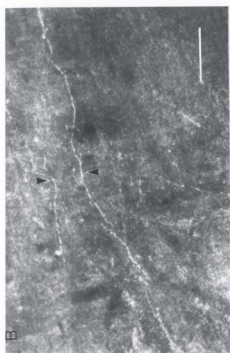
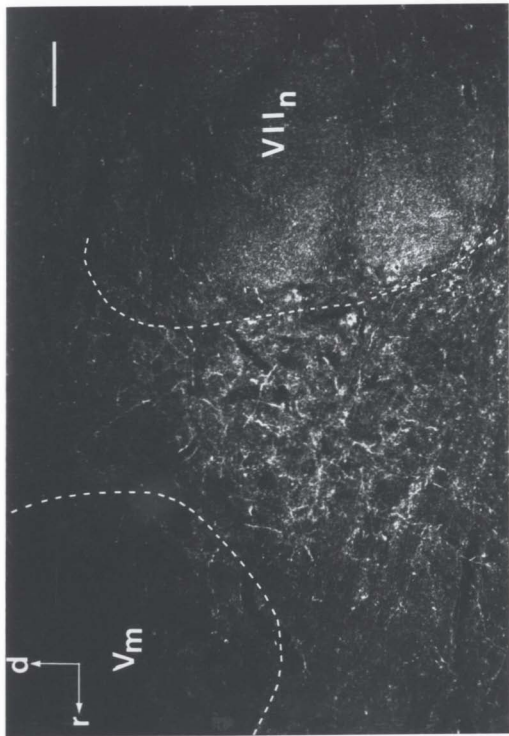


Figure 45

Projections to the accessory trigeminal motor nucleus from a solitaria CS site.

Case # 16 PHAL. Darkfield photomicrograph. Abbreviations:  $V_n$  trigeminal motor nucleus;  $VII_n$  facial nerve. Calibration bar: 100  $\mu m$ .



### i) The NTS

From pharyngeal loci, efferents to the  $NTS_{cen}$  were seen in the form of a few fibres and terminals, while around the subnucleus a moderate amount of terminal labeling was observed (Fig. 46).

Although numerous fibres ramified within the rostrocaudal confines of the NTS itself, the rostralmost (rostrolateral) portion of the NTS especially showed an aggregate of fibres and terminals (Fig. 47). This was more pronounced in pharyngeal and CS cases.

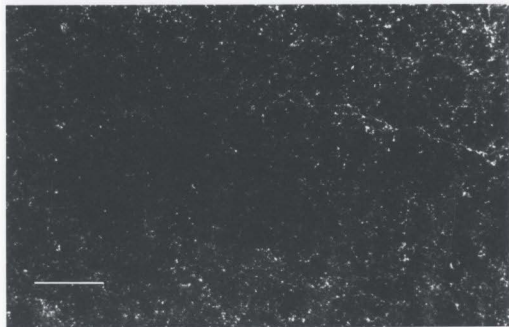
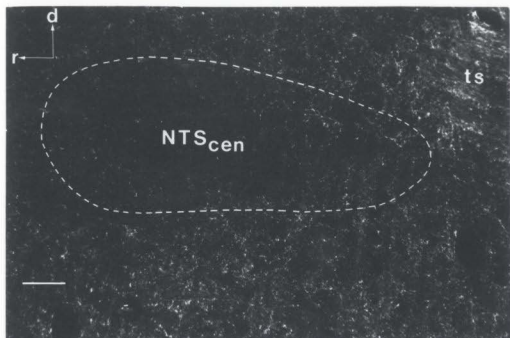
A discrete patch of labeled fibres was seen as a "mirror-image" of the injection site in the contralateral NTS (Fig. 48). This mirror-image of the injection site was very distinctly seen in those cases where large (>10 nl) injections of the tracer were made.

### ii) Nuclei of the spinal trigeminal tract, the paratrigeminal islands, the principal sensory trigeminal nucleus and the parabrachial complex

A group of solitary fibres followed a ventrolateral course before ascending dorsally to terminate in the PB complex. These fibres exited from the rostral NTS, descended ventrally at the level of the Amb, coursed along the ventral aspects of the  $VII_m$  and through the spinal trigeminal nuclei before ascending dorsally at the level of the principal trigeminal nucleus, some travelling in the tubrospinal tract to terminate in the PB complex.

**Figure 46**

Projections to the subnucleus centralis from a solitary pharyngeal site. Case # 38  
PHAL. Darkfield photomicrographs at low (top) and high (bottom) magnification.  
The dashed line in A indicates the approximate boundary of the subnucleus centralis.  
Calibration bars: 50  $\mu$ m.





**Figure 47**

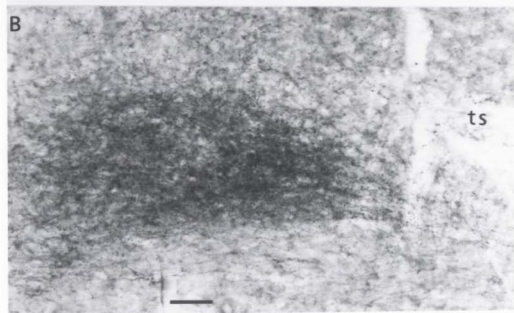
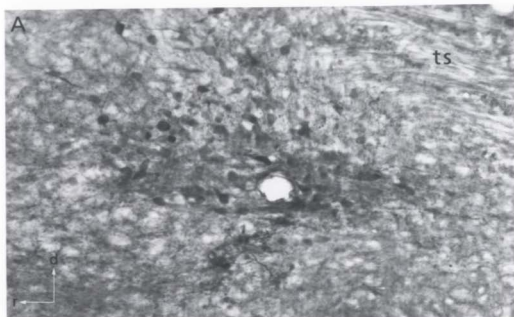
Projections to the rostrolateral NTS from a solitary pharyngeal site. Case # 38

PHAL. Darkfield photomontage. ts tractus solitarius. Calibration bar: 100  $\mu$ m.



Figure 48

Projections to the contralateral NTS ('mirror-image') from a solitary oesophageal site centered in NTS<sub>cen</sub>. Case # 10 PHAL. A: Injection site. B: Projection in contralateral NTS. Calibration bar: 50  $\mu$ m.



More medially, a contingent of this fibre system lay dorsal to the VII<sub>m</sub> and ventral to the emergence of the facial nerve and the V<sub>m</sub>.

Fibres from the solitary deglutitive loci also coursed through a dorsal route to terminate in the parabrachial complex. Following their emergence from the solitary complex, the fibres either (1) continued rostrally from the rostral NTS, running above or through the trigeminal motor nucleus to enter and terminate within the parabrachial complex or (2) ascended in the SpV and terminated in the PB.

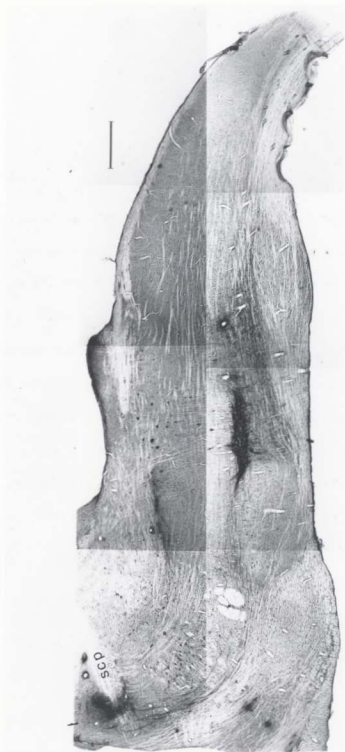
The pattern of labeling in the nSpV described above was denser and more profuse in pharyngeal cases. The contralateral nSpV showed a relatively sparser but similar pattern of labeling.

Label in the parabrachial complex was restricted to two portions lying immediately dorsal and ventral to the superior cerebellar peduncle and corresponding conceivably to the ventral lateral and medial subnuclei (Fulwiler and Saper, 1984). Large, but not small, injections of tracer at oesophageal sites duplicated this pattern of label in the PB complex suggesting that the source of the latter was the adjacent pharyngeal territory.

The paratrigeminal islands (PTI) contained both labeled fibres and terminals. The labeling was especially vivid in pharyngeal cases.

**Figure 49**

Projections to the parabrachial complex from a solitary oesophageal site. Case # 11 PHAL. Photomontage of brightfield photomicrographs showing heavy projections to the nucleus ambiguus following a large injection (20 nl) of lectin. (The injection site lies more medial.) Note the rostral course followed by the solitary efferents (the ventrolateral solitario-parabrachial pathway; see text) as they run past the facial nucleus, the facial nerve and the trigeminal motor nucleus. The heavy terminal field in the parabrachial complex is also evident. Within the nucleus ambiguus, extension of fibre labeling to parts of the semicompact division probably represents encroachment of pharyngeal territory by the tracer at the site of injection due to the large volume of lectin applied. Compare with Figure 42. scp superior cerebellar peduncle. Calibration bar: 500  $\mu$ m.



### iii) "Lacrimal" neurons

A small group of cells, lying immediately rostrolateral to the VII<sub>l</sub>, were found to receive labeled fibres and terminals (Fig. 50) from all of the deglutitive loci. These were identified as the parasympathetic "lacrimal" neurons described by Contreras *et al.* (1980).

### iv) The inferior olivary complex

In two cases involving large (20 nl; Case # 11 PHAL and # 12 PHAL) injections of the lectin, the inferior olives showed patches of labeled fibres and terminals.

## F. Unlabeled pontomedullary regions

Amongst the pontomedullary structures that contained very sparse or no terminal label, two notable regions were:

- i) The medullary reticular formation (with the exception of a few varicose fibres in the paragigantocellular portion observed only in cases (# 11 PHAL and # 12 PHAL) where large injections of the tracer were made).
- ii) The pontine reticular formation.



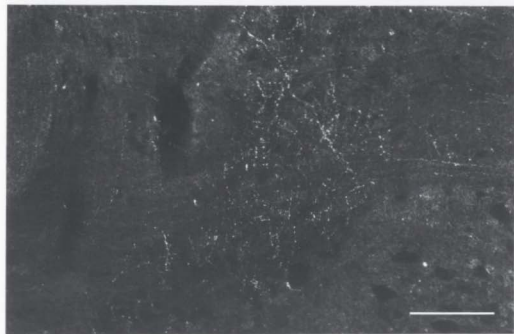
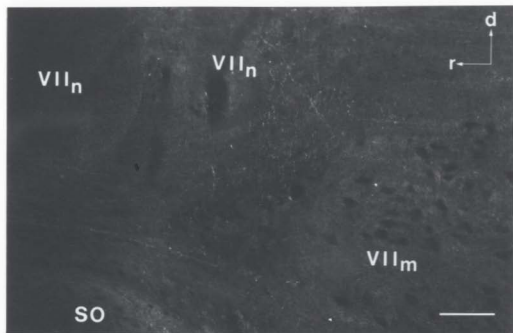
Figure 50

Projections to the "lacrimal" neurons from a pharyngeal solitarily site. Case # 38

PHAL. Darkfield photomicrographs at low (top) and high (bottom) magnification.

Abbreviations: VII<sub>n</sub> facial nucleus; VII<sub>n</sub> facial nerve; SO superior olivary complex.

Calibration bars: 100  $\mu$ m.



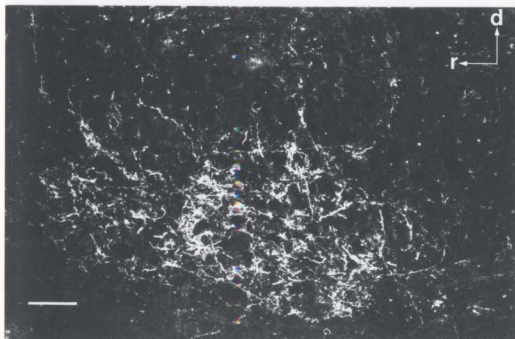
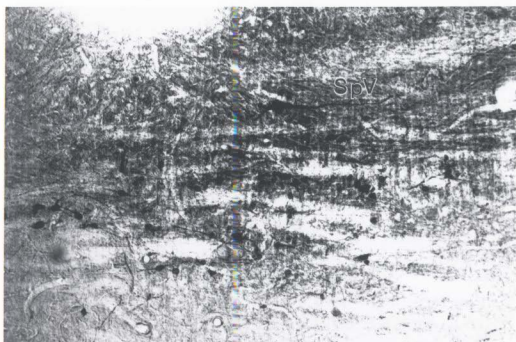
### G. Controls

As described above, PHAL injections at oesophageal sites yielded a pattern of labeling clearly distinct from those at pharyngeal sites, despite their proximity to one another (see Figs. 7, 9), thereby indicating that each served as a good control for the other. Consequently, no additional control injections were deemed necessary.

However, one control injection of PHAL was made which was targeted into the dorsal portion of the oral subnucleus of the nSpV (Fig. 51). The most prominent projection was seen within the VII<sub>m</sub> (Fig. 51; cf. Erzurumlu and Killackey, 1979). In addition, label was also seen in all subnuclei of the nSpV, the principal sensory trigeminal nucleus, the lateral portions of the parabrachial complex, the pontine reticular formation and in and around the Amb<sub>l</sub>. Label was either very sparse or absent within the Amb<sub>c</sub> and Amb<sub>ac</sub>, the V<sub>m</sub>, the XII<sub>m</sub> and the DMX.

**Figure 51**

**PHAL Control. Case # 27 PHAL. Top:** Brightfield photomicrograph of injection site in the oral subnucleus of the spinal trigeminal tract. **Bottom:** Darkfield photomicrograph showing heavy projection within the facial nucleus. **SpV** spinal trigeminal tract. Calibration bar: 100  $\mu\text{m}$ .



## CHAPTER FOUR

### DISCUSSION

#### I. Topography and functional characteristics of solitary deglutitive loci

##### 1. Response types

The non-selective deglutitive excitant, glutamate, serves as a useful tool in locating discrete deglutitive loci within the solitary complex as first shown by Bieger (1984). Owing to their ability to elicit oesophageal responses alone (Bieger, 1984), ACh or muscarine, employed in conjunction with glutamate, permits further evaluation of the deglutitive phase relationship of a particular locus.

The duration of automatically evoked pharyngeal responses in the rat averaged 200 ms in the present study. This agrees well with previous studies in the rat in which intrapharyngeal pressure measurements were made with a balloon catheter and were shown to have a duration of 200-400 ms (Bieger *et al.*, 1977; Kessler and Jean, 1985b, 1986a, 1986b).

Variations in the magnitude of the evoked responses from one experiment to the next, as seen in the present study (see Table IV), may have resulted from variations in several experimental factors, one of the most important among them being the level of anaesthesia (Meltzer, 1899; Doty and Bosma, 1956). Anaesthesia could conceivably depress the central excitatory state of the deglutitive pattern

generator resulting in a weaker response. Indeed, primary oesophageal peristalsis is especially sensitive to the level of anaesthesia, as indicated by the observation of Meltzer (1899) that, under deep anaesthesia induced by either morphine or ether, primary peristaltic activity fails to appear with a swallow triggered by mechanical stimulation of the pharynx. "This degree of anaesthesia affects the centre of deglutition in such a way as to stop the progress of the primary afferent impulse within the centre, or to prevent the transmission of its efferent impulses to the oesophagus" (Meltzer, 1899). This insight of Meltzer's is partially supported by the findings of the present investigation (section III-3-B-i) which suggest a loose coupling between the pharyngeal and oesophageal pattern generators; conceivably, decreased excitability of the interneurons under conditions of deep anaesthesia could easily dissociate the oesophageal from the pharyngeal pattern generator.

Nevertheless, the degree of variation in the amplitude of the evoked deglutitive responses in the present study lies well within the range of previous observations (Bieger *et al.*, 1977; Bieger, 1981; Bieger, 1984; Kessler and Jean, 1985b; 1986a; 1986b).

Variation in the magnitude of the evoked responses may also be attributed, in part, to the amount of agonist ejected at the deglutitive locus. Thus, although the afferent limb of the swallowing pathway was bypassed, application of different amounts of glutamate at the sites of termination of swallowing afferents (which overlap extensively with the presumptive deglutitive premotor neurons; see below)

within the NTS may be analogous to triggering of the deglutitive network through sensory input of varying intensity conveyed to the solitary complex during reflexly elicited swallowing.

## 2. Topography of the solitarial deglutitive loci

It is now well established that the NTS is a major recipient of primary afferent information from the upper alimentary tract and a variety of viscera. In addition, there is specific evidence to implicate NTS neurons in the processing of deglutitive sensory input (Jean, 1972a; 1984; Kessler and Jean, 1985a). The latter studies, however, failed to provide an accurate account of deglutitive loci within the NTS. Such a map is desirable in order to enable a precise delimitation of the deglutitive region of the solitary complex from other, functionally distinct subregions implicated in gustation (Halpern and Nelson, 1965; Travers *et al.*, 1983; Hamilton and Norgren, 1984; Sweazey and Bradley, 1986, 1988), respiration (Bianchi, 1971; Kalia and Mesulam, 1980; Cohen, 1981), cardiovascular regulation (Kalia and Mesulam, 1980) and gastrointestinal function (Leslie *et al.*, 1982; Gwyn *et al.*, 1985; Shapiro and Miselis, 1983, 1985; Norgren and Smith, 1988).

Not only are previous maps of solitarial deglutitive loci anatomically ill-defined (cf. Jean, 1984; Kessler and Jean, 1985a), use of electrophysiological methods for mapping render them inexact since stimulation of fibres of passage



is unavoidable (cf. Kessler and Jean, 1985a). Even when the maps are generated by unit recordings of "swallowing interneurons" (Jean, 1972a; Kessler and Jean, 1985a), one problem remains: a "deglutitive" discharge, as recorded in any NTS neuron, does not establish that the neuron generates output to the motoneurons; for example, it could generate collateral inhibition of other competing or antagonistic synergies such as respiration. In contrast, the technique of micropneumophoresis, refined and applied in the present study, enabled discrete deglutitive loci in the NTS to be mapped with a fair degree of precision (since glutamate is known to be a universal excitant of neurons). This technique also circumvents the problem of fiber-stimulation (Goodchild *et al.*, 1982). Thus, the map of deglutitive loci generated in the present study provided a sound neuroanatomical frame of reference for further investigations.

The location of the pharyngeal loci within the NTS<sub>v</sub> and the NTS<sub>int</sub> overlaps that of 'Group I (early or buccopharyngeal) swallowing neurons' identified in electrophysiological studies (Jean, 1984; Kessler and Jean 1985). In addition, these authors also show some 'Group I swallowing neurons' in the ventrolateral subnucleus of the NTS (their description of the location of these neurons in the 'reticular formation adjacent to the NTS' would appear to be inaccurate since, as depicted in their maps, these neurons seem to lie within the confines of the NTS); however, in the present study, failure of the chemical stimulation of the ventrolateral subnucleus of the NTS to elicit any deglutitive responses suggests that this area may participate in functions other than deglutition. In the cat, destruction

of this area does not alter reflex swallowing induced by electrical stimulation of the SLN (Weerasuriya *et al.*, 1980). In fact, the ventrolateral NTS is known to subservise respiratory function (Bianchi, 1971; Cohen, 1979; Kalia and Mesulam, 1980; Donoghue *et al.*, 1982; Berger and Averill, 1983; Backman *et al.*, 1984; Henry and Sessle, 1985; McCrimmon *et al.*, 1987).

NTS<sub>int</sub>, but not NTS<sub>v</sub>, pharyngeal loci mapped in this study overlap with the termination fields of pharyngeal afferents (Bieger and Hopkins, 1986; Altschuler *et al.*, 1989). This suggests that the NTS<sub>v</sub> may constitute an intranuclear projection site of the primary terminal fields where integration of the deglutitive sensory information occurs. The study by Altschuler *et al.* (1989) showed that certain other areas, viz., the interstitial subnucleus of the NTS and the paratrigeminal islands, were also labeled after injection of the tracer into the pharyngeal musculature. Chemical microstimulation of these structures in the present investigation failed to elicit any deglutitive responses indicating that these may not be directly involved in deglutition.

Following lesioning of the 'medial part' of the NTS, the oesophageal stage of ipsilateral SLN-induced swallowing in sheep was abolished but that of the contralateral SLN-induced swallowing remained intact (Jean, 1972b). In the rat, a circumscribed area within the intermediolateral portion of the NTS yielded oesophageal responses to chemical microstimulation with ACh, muscarine or glutamate (Bieger, 1984). The same region was retrogradely labeled as a discrete

cell group following tracer injections into the Amb<sub>c</sub> region yielding oesophageal responses to microstimulation (Bieger, 1984). This 'dense, almost circular cluster of neurons' has only recently been recognized as a distinct subnuclear group and designated the central subnucleus of the NTS by Ross *et al.* (1985) who failed to recognise its functional significance and precise relationship with the nucleus ambiguus. The NTS<sub>cen</sub> was subsequently shown (Bieger and Hopkins, 1986; Altschuler *et al.*, 1989) to receive almost all of the primary oesophageal afferent input.

Oesophageal loci mapped in the present investigations spanned the length of the NTS<sub>cen</sub> in a rostrocaudally overlapping layout with distal and cervical oesophageal loci clustered at caudal and rostral levels, respectively.

The location of the CS loci, as delineated in the present study, overlaps a portion of the termination zone of afferents carried by the superior laryngeal nerve (SLN; Altschuler *et al.*, 1989). The SLN in the rat would appear to contain the bulk of the deglutitive reflex afferents and readily elicits swallowing upon electrical stimulation (Bieger *et al.*, 1977). However, the SLN obviously must carry other types of afferents as suggested by the elaboration of a number of synergies upon stimulation of this nerve branch. Therefore, both the CS and pharyngeal loci might represent within the subnucleus intermedialis the termination zones of the deglutitive afferents carried by the SLN.

A discrepancy between the present study and an earlier one (Bieger, 1984) in the rostrocaudal extent of CS loci may be attributed in part to a correction factor of 160  $\mu\text{m}$  in the position of the obex: in the previous study, the histological identification of the obex was the merging of the dorsal lip of the central canal with the fourth ventricle. The position of the obex, as defined histologically in the present study (see methods section), lies 160  $\mu\text{m}$  caudal to that of the previous study. In addition, the previous investigation used larger ejection volumes (0.2-5 nl) compared to the present study (20-50 pl).

Data from a number of studies indicate that the endogenous excitatory amino acid neurotransmitter released by the primary afferents within the solitary complex is either glutamate or a structurally related substance. Biochemical studies first provided indirect evidence of glutamate as the transmitter of baroreceptor afferents as revealed by measurements of high-affinity uptake of glutamate prior to and after nodose ganglionectomy (Talman *et al.*, 1980; Perrone, 1981). High concentrations of glutamate were subsequently found in the medial and intermediate subnuclei of the cat NTS (Dietrich *et al.*, 1982). In addition, a depolarization-induced release of glutamate from the NTS in rat medullary slices has been reported (Kihara *et al.*, 1987). Glutamate-like immunoreactivity was detected in primary sensory neurons of rat dorsal root and trigeminal nerve (Wanaka *et al.*, 1987), implicating glutamate as a potential transmitter of primary deglutitive afferents as well. More recent and direct evidence of glutamate as a

transmitter of vagal afferents terminating in the solitary complex was provided by Lewis *et al.* (1988) by means of receptor autoradiography with L-[<sup>3</sup>H]glutamate. These authors found a reduction in the density of glutamate binding sites and an increase in receptor affinity in the NTS following nodose ganglionectomy. This study has thus attempted to resolve the earlier seemingly contradictory finding of an absence of change in high affinity glutamate uptake in the NTS following nodose ganglionectomy (Sved, 1986), the latter being attributable to the insensitivity of neurochemical markers for L-glutamate to lesions (Lewis *et al.*, 1988).

As mentioned above, deglutitive loci mapped within the NTS in this study are found to overlap with the termination fields of swallowing reflex afferents (Altschuler *et al.*, 1989) and with loci at which 'early' swallowing neurons have been reported in the rat (Jean, 1984; Kessler and Jean, 1985a). However, the techniques employed in the present investigation afford but a crude anatomical comparison between the deglutitive afferent terminal zones and the internuncial neuronal network. In order to detect the site/s within the swallowing circuitry at which the putative excitatory amino acid transmitter may be acting, a more complex experimental approach is needed. This would first require electrophysiological identification of a neuron in the nodose ganglion having a receptive field in the laryngopharynx (in the swallowing reflexogenic zone) followed by intracellular injection of a tracer such as horseradish peroxidase (HRP) to enable tracing of the central termination of the afferent fibre within the NTS<sub>int</sub>. Concurrently, HRP

would have to be injected into the  $Amb_{ac}$  in order to retrogradely label the  $NTS_{int}$  neurons. The HRP reaction products within the cell body of the  $NTS_{int}$  neuron and in the presynaptic axon terminal (of the primary afferent) may, at least in theory, be detected at either the light or electron microscopic level.

A similar approach can be taken for an 'oesophageal' primary afferent neuron by tracing its terminal into the  $NTS_{cen}$  and retrogradely labeling  $NTS_{cen}$  neurons from the  $Amb_c$ .

## II. Pharmacologic characterisation of deglutitive response types

### 1. Excitatory amino acids

The present investigation provides evidence suggesting that different excitatory amino acid receptors are involved in generating deglutitive interneuronal excitation directed to pharyngeal and oesophageal motoneurons, respectively. Since the pharyngeal response displayed a preferential sensitivity to KA, it is likely to be mediated by KA type receptors (see Fagg *et al.*, 1986, for EAA receptor nomenclature). Apart from the superior potency and efficacy of KA at pharyngeal loci, the sensitivity of glutamate-evoked pharyngeal responses to DGG but not APV and AP7, provides supportive evidence for an association of KA receptors with the pharyngeal pattern generator.

Admittedly, the potency ratios for agonists were empirically determined owing to the difficulties inherent in the *in vivo* paradigm; the affinities of the agonists for its receptor type might be different; and, in addition, the three agonists may be handled differently within the tissue. In sum, an attempt was made to distinguish between the two possible non-NMDA type receptors involved in the pharyngeal subcircuit.

In contrast to NMA/NMDA, application of KA at pharyngeal or CS loci gave rise predominantly to pharyngeal components. The absence of oesophageal components of KA-elicited responses cannot be attributed to "deglutitive inhibition"

(Meltzer, 1899; Hellemans and Vantrappen, 1967; Hellemans *et al.*, 1974) alone since swallowing activity of comparable frequency induced by the 5-HT agonist, quipazine (Bieger, 1981, 1984), often exhibits a pharyngo-oesophageal progression. Furthermore, NMA/NMDA clearly displayed a higher potency at oesophageal loci. In addition, glutamate-evoked oesophageal responses could be blocked by NMDA receptor-antagonists, APV and AP7. Taken together, these findings clearly indicate the preferential association of the NMDA receptor with presumptive oesophageal premotor neurons within the subnucleus centralis. Furthermore, this conclusion is corroborated by the inhibitory effects of the intravenously administered non-competitive NMDA receptor blocker, MK-801, observed on oesophageal components of quipazine-induced automatic swallowing as well as those of reflex swallowing elicited by electrical stimulation of the superior laryngeal nerve. The inhibitory effects of intravenous MK-801 were also evident on the pharyngeal components, albeit to a lesser extent, and especially on the amplitude rather than the rate. This suggests that NMDA receptors may also be regulating pharyngomotoneuronal excitability.

Consistent with the present findings, the NTS was found to contain all three EAA receptor types in experiments utilising *in vitro* brain-slice preparations (Miller *et al.*, 1987; Miller and Felder, 1988). Based on their data, the latter investigators have also indicated a degree of selectivity in the distribution of the different EAA receptors as well as the possibility that the same neuron may possess more than



one receptor type. Furthermore, recent reports have also shown the involvement of solitary EAA receptors in rat vagal and sympathetic baroreflexes (Guyenet *et al.*, 1987; Kubo and Kihara, 1988).

Compatibility of the NMDA receptor with the oesophageal pattern generator is easily conceptualised given the plasticity of this receptor type (for review of NMDA receptors see Dingledine, 1985; Fagg *et al.*, 1986; Cotman *et al.*, 1988) and the variability and diversity of oesophageal motility patterns (e.g. Vanek and Diamant, 1987; see Roman, 1982 for review). In contrast, as reflected in the precision and celerity of the act (Doty and Bosma, 1956), the pharyngeal neurocircuit is probably "hard-wired" to a greater extent than is the oesophageal subcircuit and, conceivably, best served by conventional fast synaptic transmission as that provided by the kainate receptor.

The well-documented excitotoxic nature (Olney *et al.*, 1971; Olney, 1978; Mayer and Westbrook, 1987 for review) of the three EAA agonists, in particular, that of NMDA, was evident in this study as well. Moreover, unlike their excitatory effects, the toxic action of the three agonists was a relatively slower and spatially more restricted process since repeated applications of these substances yielded consistent, reproducible responses for a considerable length of time; yet, subsequent histological examination revealed a discrete lesioning of the ejection site.

In summary, the present findings provide evidence for a preferential association of KA and NMDA receptors, respectively, with NTS internuncial

neurons forming part of the presumptive pharyngeal and oesophageal pattern generators (see section III-2). Furthermore, these findings suggest that, with their potential as therapeutic agents (Stone, 1987; Foster *et al.*, 1987), clinical use of NMDA receptor antagonists may result in dysphagia (i. e. impaired deglutition), a side-effect which might be exacerbated in stroke victims.

## 2. Serotonergic mechanisms

Previous work (Bieger, 1981) has shown that 5-HT-mimetics, upon systemic administration, will exert a powerful facilitatory effect on the swallowing reflex and induce automatic swallowing (Bieger *et al.*, 1972; Bieger, 1974; 1977; Menon *et al.*, 1976a; 1976b; 1986; Clineschmidt, *et al.*, 1977; Clineschmidt and McGuffin, 1978; Tseng *et al.*, 1978; Tseng, 1978; 1979). Although basal forebrain structures were shown to be involved in both catechol- and indoleaminergic actions on automatic swallowing (Bieger, 1974; Hockman *et al.*, 1979 for review), the lower brainstem was identified as the principal locus of 5-HT action (Bieger, 1981). Moreover, comparison with two other deglutitive excitants, ACh and glutamate (Bieger, 1984), disclosed the selective action of 5-HT on the pharyngeal stage of swallowing. The present investigation corroborates the postulated involvement of a medullary substrate and provides further information concerning the anatomical structure and the type of 5-HT receptor mediating deglutitive stimulation with serotonin. As will be discussed in section III-3, the neurons concerned are premotor elements which

form part of the buccopharyngeal deglutitive pattern generator and which receive a propriobulbar serotonergic input (see section III-2). A principal feature of this input appears to be its synergistic interaction with glutamate analogous to that reported for facial (VanderMaelen and Aghajanian, 1980) or spinal (White and Neuman, 1980) motoneurons. Insofar as glutamate may be a transmitter candidate for vagal sensory afferents mediating the swallowing reflex (see discussion section I-2), a similar mechanism could underlie the facilitatory effects of 5-HT on reflex swallowing described in the cat (Hockman *et al.*, 1979) and the rat (Bieger, 1981).

The present findings provide strong evidence that the NTS is the site where 5-HT exerts an excitatory action on deglutition as indicated by the elicitation of deglutitive responses upon pneumophoretic application of 5-HT at pharyngeal sites. The pharmacologic specificity of this action was demonstrated by blockade of the effect of the amine by the 5-HT<sub>2</sub> and 5-HT<sub>1c</sub> receptor antagonist, ketanserin. Since the 5-HT mimetic, quipazine, was administered intravenously, it does not preclude the possibility of excitatory actions of 5-HT taking place outside the NTS also, e. g. on the motoneurons. A rich serotonergic innervation is found within the branchiomotor nuclei (Fuxe, 1965; Steinbusch, 1981).

Mediation of the excitatory effects of 5-HT by 5-HT<sub>3</sub> receptors can be ruled out since administration of 5-HT<sub>3</sub> receptor antagonists, ICS 205-930 and BRL 43694 neither produced any effect on their own nor on quipazine-induced swallowing. Moreover, 5-HT<sub>3</sub> receptor participation can be discounted because,

5-HT receptors of an M subtype (5-HT<sub>3</sub>; Richardson and Engel, 1986) are reported to mediate depolarization of small but not large caliber vagal fibres (Richardson *et al.*, 1985; Round and Wallis, 1986), and C-fibres are not thought to serve as swallowing reflex afferents (Andrew, 1956; Agostoni *et al.*, 1957).

The inhibition by 8-OH-DPAT of quipazine-induced swallowing suggests that 5-HT<sub>1A</sub> receptors may play a role in mediating the depressant effects of 5-HT; however, the exact site of action of 8-OH-DPAT remains to be determined. Participation of other subtypes of the 5-HT<sub>1</sub> receptor is unlikely because of the failure of a number of agents having an action at different subtypes of the 5-HT<sub>1</sub> receptor *viz.*, TFMPP, pizotifen, cyanopindolol and yohimbine, to produce any effect. However, since none of these agents are selective, the possibility of involvement of other subtypes of the 5-HT<sub>1</sub> receptor, in addition to 5-HT<sub>1A</sub>, remains.

Involvement of a 5-HT<sub>1</sub> receptor subtype in mediating the inhibitory effects of serotonin on deglutition is also indicated by the blockade of this effect by the non-selective 5-HT receptor antagonist, metitepine (methiothepin), in the study by Kessler and Jean (1985b; 1986a; see below). However, metitepine is a non-specific antagonist of 5-HT receptors (see Bradley *et al.*, 1986) and possesses neither greater selectivity nor higher potency at subtypes of the 5-HT<sub>1</sub> receptor than do the antagonists employed in the present study (see Fozard, 1987). It is, therefore, difficult to reconcile the antagonism of the inhibitory effect of 5-HT on reflex

swallowing by methiothepin (Kessler and Jean, 1985b; 1986a) with the results of the present investigation showing the inability of a number of 5-HT receptor antagonists to block the inhibitory effects of either 5-HT or 8-OH-DPAT on automatic swallowing.

It should also be pointed out that, unlike the facilitatory effect, the suppression by 5-HT occurring after application of high doses of the amine, would appear to be nonselective insofar as the stage of swallowing (pharyngeal or oesophageal) is concerned. Therefore, two other explanations may be considered: neuronal desensitization secondary to overstimulation; or a non-specific artifact.

The present findings emphasise the need for caution in defining neural 5-HT actions owing to the exquisite sensitivity of certain receptor systems (VanderMaelen and Aghajanian, 1980). As shown previously, 5-HT will give rise to membrane hyperpolarization and decreased neuronal excitability (Phillis *et al.*, 1968) when applied in amounts 1-2 orders of magnitude higher than those required to produce excitation (McCall and Aghajanian, 1979; White and Neuman, 1980). In the present study, upon continuous stimulation with 5-HT and/or application of a high dose, an apparent inhibition of both the pharyngeal and the oesophageal responses was observed, which could, however, not be overcome by the three 5-HT receptor antagonists, methysergide, metergoline or ketanserin. Work on spinal motoneurons (Neuman and White, 1982; White and Neuman, 1983) has similarly shown that facilitation, but not suppression, caused by 5-HT or quipazine, of glutamate-evoked

responses can be antagonised by methysergide and metergoline. It should also be recalled that 5-HT precursor loading in rats treated with monoamine oxidase inhibitors results in a mixture of excitatory-inhibitory effects on automatic swallowing (Bieger, 1981).

The foregoing considerations might explain the inhibitory effect of 5-HT on reflex swallowing (Kessler and Jean, 1985b; 1986a) demonstrated in ketamine-anaesthetised rats subjected to submaximal repetitive electrical stimulation of the superior laryngeal nerve, at one minute intervals, so as to elicit as many as 8 pharyngeal swallows per train of pulses; the effect of 5-HT agonists, pressure-injected in the dose-range of 300-5000 pmol (i.e. 2 or more orders of magnitude greater than that used in the present study) was then found to be purely inhibitory. It would appear that, in these experiments, excitatory input delivered to the NTS was of sufficient intensity to produce a near-maximal swallowing response (cf. Bieger *et al.*, 1977); hence, additional excitation by chemical stimulation with 5-HT, if any, would have been difficult to detect. Indeed, when maximal chemical stimulation with large glutamate doses was applied in the present study, suppressant effects by 5-HT were readily demonstrable. As well, the suppression by high doses of 5-HT resembled that following repeated application of large glutamate doses, sufficient to result in massive edema and physical destruction of brain tissue. In part at least, the inhibitory effect of 5-HT could therefore be an experimental artifact secondary to injurious stimulation.

Antagonism by DGG of the excitatory effect of the 5-HT-mimetic, quipazine, suggests that the central serotonergic input converges on the same KA receptor-activated subcircuit that receives the primary deglutitive afferent input. The failure of ketanserin to affect the glutamate-evoked pharyngeal responses indicates that an excitatory 5-HT input is not absolutely required for responses mediated by the solitary KA type receptors. This situation contrasts with that for the solitary NMDA receptors where an intact cholinergic input would appear to be essential for the responses they mediate (see section II-3).

The present findings emphasise the importance of a critical choice of anaesthetic for physiopharmacological investigations. Unlike urethane (Maggi and Meli, 1986), ketamine, employed in some investigations (Kessler and Jean, 1985b, 1986a, 1986b), would be unsuitable for neuropharmacological studies of deglutitive mechanisms since i) it has been reported to block the neuronal and extraneuronal uptake of monoamines (Lundy *et al.*, 1985) and ii) it behaves as an antagonist of NMDA-receptor mediated functions (Anis *et al.*, 1983; Thomson *et al.*, 1985; MacDonald *et al.*, 1987). Regarding i) catecholaminergic and serotonergic influences have been demonstrated on both reflex and automatic swallowing (Bieger, 1974, 1981; Bieger *et al.*, 1972; Bieger *et al.*, 1977; Bieger *et al.*, 1978; Kessler and Jean, 1985b, 1986a, 1986b; Menon *et al.*, 1986); and regarding ii), as suggested by the present findings, it could impair the oesophageal component of automatic swallowing. Urethane, on the other hand, as used in the present

investigation, is considered a preferred anaesthetic agent owing to its ability to induce a surgical plane of anaesthesia "without affecting neurotransmission in various subcortical areas and the peripheral nervous system" (Maggi and Meli, 1986).

As regards the 5-HT effects on breathing, it is conceivable that transmitter substances common to the two overlapping circuits controlling respiration (see Mueller *et al.*, 1982 for review) and deglutition would have opposite effects on these two functions since during swallowing, owing to a common passageway, respiration must cease. This hypothesis is borne out by previous findings (Bieger, 1981) showing local application of 5-HT to the ependymal surface of the solitary complex to result in lowering of respiratory rate as well as the demonstration of an inhibitory action of 5-HT on respiratory reflex interneurons localized in the NTS (Sessle and Henry, 1985).

In conclusion, the evidence obtained confirms a central excitatory action of serotonin on deglutition. The specific neural substrate involves premotor neurons of the NTS concerned with the activation of the pharyngeal stage. The excitatory action of serotonin is mediated by a receptor belonging to either the 5-HT<sub>2</sub> and/or the 5-HT<sub>1c</sub> subtype. Furthermore, although it can be facilitated by 5-HT, the glutamate-evoked responses (presumably mediated by KA receptors) at the pharyngeal neuronal loci does not appear to be dependent upon an intact 5-HT input.



### 3. Interaction of Glutamatergic and Cholinergic mechanisms

In light of previous investigations in the rat (Bieger, 1984), a central muscarinic cholinergic input appears to be required for generating the neural impulse pattern for oesophageal peristalsis. Since glutamate-induced responses elicited from the subnucleus centralis of the NTS were not fully blocked by muscarinic cholinergic antagonists (Bieger, 1984; present investigation), it is probable that at least one other synaptic pathway contributes to the generation of oesophageal activity. Based on the present findings, it is now proposed that activation of this pathway depends on NMDA receptors. This neuron system, comprising the presumptive oesophageal pattern generator circuitry, projects ventrally to the rostral oesophagomotor compact formation of the nucleus ambiguus (Bieger, 1984; Stuesse and Fish, 1984; Ross *et al.*, 1985; Cunningham and Sawchenko, 1989; see section III below).

As yet, one can only speculate on the precise manner in which the parallel processing of the NMDA-EAAergic and muscarinic cholinergic routes is accomplished so as to generate two partially-interdependent, yet distinct, oesophageal motility patterns since the anatomical identity of the deglutitive pattern generator at the single neuron level remains to be determined. However, the actions of cholinergic agents and their interactions with glutamate within the NTS<sub>cen</sub> yielded clues which may now permit the formulation of a hypothesis that

would explain the *in vivo* activation of the NMDA receptor-mediated pathway at physiological concentrations of  $Mg^{++}$  (Nowak *et al.*, 1984). As suggested by Klockgether *et al.*, (1987) and based on the results obtained in the present study, it is conceivable that excitatory impulse activity (provided by the muscarinic pathway in this case) might be sufficient to overcome the  $Mg^{++}$  blockade of NMDA receptor-mediated mechanisms *in vivo*. Inherent in this proposal is a model of a neuron at the level of the  $NTS_{cen}$  receiving a primary afferent signal via NMDA receptors and a central cholinergic input. The latter maintains the cell at a certain level of excitation through a tonic input which, consequently, relieves the  $Mg^{++}$  block of the NMDA receptor channel, thus allowing the NMDA receptors to be activated through an EAAergic input delivered primarily via activation of primary deglutitive reflex afferents.

The presence of intrinsic cholinergic impulse activity is suggested by the ability of intravenous physostigmine alone to evoke oesophageal contractile responses indistinguishable from those elicited by muscarine applied into the  $NTS_{cen}$ . Conceivably, ACh accumulates at the receptor site and activates the neuronal circuit.

This model can also account for the partial blockade of the glutamate-evoked oesophageal response by muscarinic antagonists (cf. Bieger, 1984): blockade of the muscarinic cholinceptors would remove the excitation needed to overcome the  $Mg^{++}$  block, thus preventing the participation of NMDA receptors. Since

blockade of muscarinic receptors greatly diminishes the glutamate-evoked response, it is possible that the NMDA receptor is incapable of mediating a sustained depolarization in the absence of the cholinergic excitation. It is also conceivable that intrinsic inhibitory inputs, if present, would further prevent NMDA receptor activation.

Loss of propulsiveness of the glutamate-evoked peristaltic oesophageal responses by muscarinic cholinoreceptor blockade suggests that muscarinic cholinoreceptors may also play a critical role in producing timing cues within the presumptive oesophageal pattern generating circuit during oesophageal peristalsis. Such a conclusion derives support from studies (Dodds *et al.* 1981) that showed suppressant effects of atropine on the incidence and progression of primary oesophageal peristalsis throughout the oesophagus except the proximal (2 cm) which is generally believed to be the striated portion. However, a recent study has indicated that an additional 2-3 cm below the striated muscle portion contains an equal mixture of smooth and striated muscle (Meyer *et al.*, 1989). Thus, in the study by Dodds *et al.* (1981), it may be surmised that, although peripheral muscarinic receptor blockade (e.g. on oesophageal smooth muscle) may have been responsible for impairment of oesophageal function to a large extent, the central effects of atropine cannot be ruled out insofar as loss of peristaltic progression is concerned. Admittedly, this conclusion is rather speculative and, for substantiation, requires further work in the human or other primates.

Some parallels between the neural paradigm of the present study and other systems exist. For example, NMDA sites in rat brain have been found associated with sensory systems (Monaghan and Cotman, 1986) and are a major component of the thalamic response to natural somatosensory stimulation (Salt, 1986). Synergism between muscarinic cholinceptors and EAA receptors has recently been reported in cat somatosensory cortical neurons (Metherate *et al.*, 1988) and between nicotinic cholinceptors and EAA receptors in the rat ambiguous complex (Wang and Bieger, 1989).

In sum, based on the interaction of muscarinic and EAA agents within the NTS<sub>cen</sub>, it is proposed that an excitatory cholinergic input converges on the same neuron that receives the primary EAAergic afferent input. The cholinergic input appears essential not only for activation of the NMDA receptors through relief of the Mg<sup>++</sup> block, but also for a sequential spread of excitation through these presumptive oesophageal premotoneurons. Although the presence of a cholinergic input to the NTS<sub>cen</sub> has been postulated for some time (Bieger, 1984), its source remains to be determined.

### III. Neural connections of the NTS

#### 1. Methodological considerations

##### A. Retrograde tracing and 5-HT immunocytochemistry experiments

To evaluate the spread of tracer outside the solitary complex in cases where large (>4 nl) injections of fluorogold had been made, a possible index was the presence of retrogradely-labeled cell bodies in the vestibular complex. This finding suggests damage to the vestibulofugal fibres and uptake of tracer into damaged fibres of passage. It is also noteworthy that the vestibular nuclei do not project to the NTS (Mehler and Rubertone, 1985). In addition, if each half of the deglutitive neurocircuit or "half-centre" projects to its counterpart, as suggested by the split-brainstem experiments (Ishihara, 1906; Doty *et al.*, 1967) the extent of the labeled "mirror-image" on the contralateral side could also serve as a useful index of the injection site in cases involving large injection volumes of the tracer.

Failure of the tracer to be transported following its application from fine-tipped micropipettes (i.e. in the absence of overt neuronal damage) indicated that some mechanical damage is a prerequisite to retrograde transport. This finding also indicates the suitability of the dye for marking loci for subsequent histological identification in mapping and pharmacological studies.

The specificity of the 5-HT antiserum had been established earlier in the laboratory by the test of preadsorption of the antibody with the antiserum and subsequent immunocytochemistry. Furthermore, the specificity of the immunocytochemical procedure was confirmed by the presence of 5-HT-IR perikarya confined only to the brainstem structures known to contain 5-HT cell bodies (Dahlström and Fuxe, 1964; Steinbusch, 1981; Sano *et al.*, 1982; Descarries *et al.*, 1982).

#### B. Anterograde tracing experiments

Previous attempts at tracing efferents of the NTS relied heavily on degeneration techniques and autoradiographic detection of tritiated amino acids (e.g. Cottle and Calaresu, 1975; Loewy and Burton, 1978; Norgren, 1978). However, these procedures lacked the precision and the resolution needed to study efferents issuing from a circumscribed region. In the words of Loewy and Burton (1978) in the cat "...it was not possible to restrict the injection mass exclusively to one of the nuclei of the solitary complex or entirely within the complex itself..." and "This injection heavily labeled all the subnuclei of the solitary complex ... and the margins of the injection mass extended over the ventral part of the dorsal column nuclei, the dorsal part of the reticular formation, the dorsal motor nucleus of the vagus, the intercalatus nucleus and the dorsal portion of the hypoglossal nucleus". In contrast, in the present study, i) a specific anterograde tracer (Gerfen

and Sawchenko, 1984) was utilised which was far superior to conventional autoradiographic tracing methods in terms of both precision and resolution (ter Horst *et al.*, 1984; ter Horst and Luiten, 1986) and ii) small amounts of the tracer were applied at NTS loci that had first been located by their short latency deglutitive responses to glutamate. Thus, the projections from these sites can be considered to issue mainly from the solitary deglutitive areas. Moreover, by repeating each experiment with different quantities of tracer, an evaluation of tracer spread into adjacent areas could be made: label in the inferior olives was seen only in two cases where the largest injections (20 nl) of the tracer were made. In the rat, the inferior olivary complex does not receive projections from the NTS (Flumerfelt and Hrycihyn, 1985). Therefore, label in the inferior olives may be an index of the spread of the lectin outside the NTS, presumably to the dorsal column nuclei (Beckstead *et al.*, 1980). Even though fairly large injections (>10 nl) of PHAL into the NTS<sub>cen</sub> qualitatively replicated the pattern of label in the XII<sub>m</sub> resulting from tracer injections in the NTS<sub>int</sub>, the hypoglossal projection (see section 3-iv below) was extremely sparse by comparison, reflecting the limited encroachment of tracer on adjacent pharyngeal territory. Perhaps the strongest piece of evidence showing the precision of tracing (i.e. transport of the lectin from only a discrete area corresponding to the injection site) in the present study consists in dramatically different patterns of labeling resulting from injection of tracer into the pharyngeal and oesophageal loci, which were separated from each

other by a distance of, in some cases (e.g. rostral cervical oesophageal and pharyngeal sites), only about 150  $\mu\text{m}$ . This also indicates that appreciable transport of the tracer occurs only from the centre of the injection site where lectin-filled perikarya were visible.

Contrary to other reports (McFarland and Lee, 1987; Shu and Peterson, 1988), the present study revealed that PHAL is transported only in the anterograde direction (barring the immediate vicinity of the injection site). The reason for this discrepancy is unclear. It may be that, to some extent, the mode of application (iontophoresis versus pneumophoresis) plays a role (Gerfen and Sawchenko, 1984; Shu and Peterson, 1988). That stimulation with glutamate would have affected the transport of the lectin is unlikely because the control injection in the nSpV was done in the absence of glutamate stimulation.

The presence of lectin-filled perikarya at the site of injection suggests that either an uptake or some other process (e. g. phagocytosis) must occur in and around the site of injection. Presumably, the tracer has to be sequestered within the cell body before it can be anterogradely transported (cf. Gerfen and Sawchenko, 1984).

Lack of labeled fibers in the absence of lectin-filled perikarya at the site of injection supports the observation of Gerfen and Sawchenko (1984) that PHAL is not anterogradely transported by fibres of passage.



## 2. Serotonineric afferents to solitary deglutitive loci

The present study suggests that the 5-HT input to solitary pharyngeal loci arises from the raphe obscurus, magnus and pontis nuclei and generally confirms purely anatomical studies showing that 5-HT innervation of the solitary complex originates from neurons in the pontomedullary raphe nuclei (Palkovits, 1985; Thor and Helke, 1987; Schaffar *et al.*, 1988). However, only in the present study, efforts were made to restrict the injection of the tracer to physiologically defined sites. In this regard, it should be pointed out that this is the first investigation which has attempted to correlate deglutitive function to neuroanatomical data.

The present findings are in partial agreement with those of Thor and Helke (1987) who showed that serotonineric projections to the NTS arise from the raphe magnus, obscurus and pallidus nuclei and from a group of 5-HT-IR neurons termed the paraolivary nucleus by Hökfelt *et al.* (1978). In agreement with the findings of the present study, Thor and Helke (1987) also found the greatest numbers of both tracer-labeled and 5-HT-IR perikarya in and around the raphe magnus. However, these authors failed to detect double-labeled perikarya within the raphe pontis. This failure may be attributed to retrograde transport of only a small percentage (2%) of the latex microspheres used in that study since optimal transport requires a mean microsphere diameter of 20 nm (Holländer *et al.*, 1989) while the commercially available suspension contains a mixture of microspheres

ranging in diameter between 20 and 200 nm with a mean of 50 nm. That the raphe pontis does project to the NTS is corroborated by the study of Schaffar *et al.* (1988) who observed retrogradely labeled 5-HT-IR cell bodies in the raphe pontis when tracer was applied to the NTS. Although, as revealed by the present study, the contribution from the raphe pontis to the 5-HT innervation of the solitarius deglutitive area may be small, its involvement cannot be ruled out. Electrical stimulation around this region has been reported to facilitate reflex swallowing in the cat (Rupert, 1979).

The presence of a dense meshwork of serotonergic fibres throughout the solitary complex (Fuxe, 1965; Steinbusch, 1981; Maley and Elde, 1982; Pickel *et al.*, 1984; Leslie, 1985; Bieger, 1985; present study) coupled with the multiplicity of function of the NTS necessitates a careful targeting of tracer into the area of interest in order to arrive at any definitive conclusions regarding the source/s of the serotonergic innervation of a particular region subserving a specific function. In the present study, the injection sites were confined to a relatively small area compared to those of other studies (Thor and Helke, 1987; Schaffar *et al.*, 1988) and in addition were centered at NTS sites from which deglutitive responses were evoked. However, even the most judicious placement of tracer cannot exclude the possibility of uptake by damaged fibres of passage in a region as densely filled with fibers as the NTS. Furthermore, assignment of function to a specific portion of the solitarius serotonergic fibre network solely on the basis of its proximity to a

functionally distinct subregion cannot be made with absolute certainty due to the "rete nervosa diffusa" structure of the 5-HT neuron system (Sano *et al.*, 1982). Originally proposed by Golgi (1890), rete nervosa diffusa refers to a reticular system formed by long, frequently anastomosing axons. According to the Japanese workers, axons of serotonin neurons not only branch frequently, but form a true network by frequent anastomosis and typically possess "en passant" type synapses (Sano *et al.*, 1982). The anastomosis is said to occur not only between branches of the same axon but also between axons from different serotonin neurons. Thus, in view of the foregoing, it is likely that the 5-HT terminal plexuses ignore well-defined boundaries between "subregions" of the NTS identified on the basis of a particular function.

Nevertheless, despite the technical limitations and difficulties outlined above, the present findings suggest that cells within the raphe magnus, obscurus and pontis contribute to the serotonergic fibre-network within the NTS<sub>v</sub> and the NTS<sub>int</sub>, at least partially. The weakness of the retrograde label within the 5-HT immunoreactive cells probably reflects the extensive branching of the axons since only a part of the branched axonal network may have been exposed to the tracer injection.

If true, the rete nervosa diffusa structure of the serotonergic system might also explain the failure of chemical microstimulation of these raphe nuclei to evoke deglutitive responses. Technically, this failure may also be attributed to the

inability to stimulate selectively and concurrently the small number of 5-HT cells within the three raphe nuclei that contribute to the serotonergic input to the NTS.

The absence of serotonergic input to the NTS from other presumptive 5-HT sources *viz.*, the medial lemniscal (B9) cell group, the ventromedial paragigantocellular and pontine reticulotegmental nuclei, and the raphe dorsalis, medianus and pallidus nuclei, contrary to the observations of Thor and Helke (1987) and Schaffar *et al.* (1988), suggests that these may project to regions of the NTS involved in visceral functions other than deglutition.

It should be noted that retrogradely labeled, but non 5-HT-IR, perikarya were present in the periaqueductal grey as well as all of the pontomedullary raphe nuclei, in general agreement with previous reports (Thor and Helke, 1987; Schaffar *et al.*, 1988). The type of neurotransmitter/s contained in these neurons remains to be determined. One candidate is thyrotropin-releasing hormone which is present in cells of the raphe pallidus and obscurus nuclei (Palkovits *et al.*, 1986). In addition, the presence of retrogradely-labeled perikarya in the 'rostral ventrolateral medulla' indicates catecholaminergic inputs to the NTS deriving from the C1 catecholaminergic cell group. Indeed, this cell group, among others, has been reported to project to the solitary complex (Thor and Helke, 1988).

In conclusion, by means of a three-fold approach *viz.*, combining functional identification of solitary deglutitive loci, retrograde tracing and 5-HT

immunocytochemistry, the source of the serotonergic input, or at least a part of it, to the  $NTS_{int}$  and  $NTS_v$  was found to lie mainly in the raphe magnus and obscurus with a minor contribution from the raphe pontis. In addition, the periaqueductal grey and pontomedullary raphe nuclei were found to project to the deglutitive NTS sites via non-serotonergic pathways.

### **3. Efferents of the NTS**

#### **A. Solitariai deglutitive efferents to motoneuronal pools**

The present investigation has revealed the presence of efferents from solitariai deglutitive loci to the motoneuronal pools involved in deglutition. Due to the close proximity of the fibres and terminals to the presumptive motoneurons, it is unlikely that additional interneurons are interposed between the solitariai efferents and the motoneurons. Thus, solitariai deglutitive efferents probably make synaptic contact with the deglutitive motoneurons. Hence, the deglutitive loci of the NTS can be considered to consist of true premotoneuronal elements involved in coordinating the sequential firing of the different motoneurons during swallowing.

In the following sections, solitariai projections to different areas are discussed.

#### **i) The nucleus ambiguus**

The nucleus ambiguus (Amb) is a major pool of motoneurons supplying the striated pharyngo-laryngo-oesophageal musculature (Lawn, 1966; Bieger and Hopkins, 1987) including most of the muscles that are active during swallowing (Doty and Bosma, 1956). The tip of the Amb represents the stylopharyngeus muscle; the cricothyroid muscle representation overlaps the rostral portion of the

semicompact division of the Amb ( $Amb_{sc}$ ); the  $Amb_{sc}$  represents the pharyngeal constrictors and the loose division of the Amb ( $Amb_l$ ), the laryngeal muscles other than the cricothyroid (Bieger and Hopkins, 1987). Efferents from pharyngeal loci were found at the tip of the Amb and the  $Amb_{sc}$  and  $Amb_l$ .

The projection resulting from injections of PHAL at pharyngeal sites, lying immediately dorsal to the  $Amb_c$  in the ventrolateral reticular formation, could, conceivably, be directed to the "ventral group of swallowing interneurons" identified in electrophysiological studies in the sheep and the rat (Jean, 1972a; 1984; Kessler and Jean, 1985a) and postulated to be under control of "swallowing neurons of the dorsal group" (Jean, 1984). In the sheep, the ventral, but not the dorsal, group of "swallowing interneurons" was found to project to the  $V_{\bullet}$  (Jean *et al.*, 1983; Amri *et al.*, 1984) implying an absence of solitary efferents to the  $V_{\bullet}$ . This finding is in striking contrast with the results of the present investigation in the rat (see below).

The  $Amb_c$ , which contains the oesophagomotoneurons, received projections only from the oesophageal loci in the  $NTS_{cen}$  confirming previous reports (Bieger, 1984; Stuesse and Fish, 1984; Ross *et al.*, 1985; Cunningham and Sawchenko, 1989) and providing anatomical detail as well as functional data. However, no difference in the pattern of labeling within the  $Amb_c$  was seen when comparing projections issuing from cervical and distal oesophageal NTS loci. This may be attributed to

the extensive overlap within the  $Amb_c$  of the representation of upper, middle and lower levels of the oesophagus (Bieger and Hopkins, 1987).

The close proximity of the solitario-ambigual projections to the presumptive motoneurons, the frequent varicosities and the actual visualization of fine, terminal bouton-like reaction product on the  $Amb$  perikarya are all strongly indicative of direct synaptic contacts of the solitarily fibres with the ambigual (presumptive) motoneurons. (The exception may be the patch of labeled fibres dorsal to the  $Amb$  seen in pharyngeal cases). In this regard, the description of Cunningham and Sawchenko (1989) that the terminal varicosities 'appeared to wrap around the ambigual motoneurons' is very apt. Furthermore, extensive fibre branching within the  $Amb$  suggests the possible existence of axon collaterals contacting the  $Amb$  motoneurons.

Cunningham and Sawchenko (1989) have recently provided immunocytochemical evidence that the  $NTS_{cen}$ - $Amb_c$  projection is partly somatostatinergic. However, with its putative role as the organizer of oesophageal motility patterns, it is unlikely that the  $NTS_{cen}$  would transmit its frequent commands to the  $Amb_c$  motoneurons via peptide messengers that require synthesis within the perikaryon and subsequent axoplasmic transport to the terminal sites. Hence, although somatostatin may serve a role as neuromodulator, it may not be the main transmitter of this solitario- $Amb_c$  pathway. Ongoing pharmacological investigations (Wang and Bieger, 1989; Bieger and Wang, 1989) confirm the results



of previous work (Bieger, 1984) indicating that these projections may be glutamatergic and/or cholinergic.

Finally, it should be noted that, although the bulk of the solitario-ambigual projections is ipsilateral, a small proportion of these do project to the contralateral Amb.

In conclusion, as originally postulated by Bieger (1984), the NTS<sub>cen</sub> constitutes the premotoneuronal stage and, therefore, the internuncial neuronal network involved in the organization of oesophageal peristalsis.

## ii) The trigeminal motor nucleus

The most noteworthy finding was that the accessory or ventromedial division of the V<sub>n</sub> (V<sub>n</sub><sup>vm</sup>) received projections from solitarily pharyngeal and CS sites but not from oesophageal loci. This subnucleus contains motoneurons supplying the anterior belly of the digastric muscle and the mylohyoid (Mizuno *et al.*, 1975; Sasamoto, 1979; Holstege *et al.*, 1983). Both these muscles are active during swallowing (Doty and Bosma, 1956; Bieger *et al.*, 1977; see below). The close proximity of the terminals and fibers to the presumptive motoneurons strongly suggests that the solitario-trigeminal projections impinge directly on the V<sub>n</sub><sup>vm</sup> motoneurons.

As mentioned above, the present findings of direct solitario-trigeminal connections in the rat are at variance with previous reports in sheep (Jean *et al.*,

1983; Amri *et al.*, 1984). On the basis of antidromic mapping and neuronal tracing, Jean *et al.* (1983) and Amri *et al.* (1984) showed that the  $V_{\text{m}}^{\text{sol}}$  receives projections, not from the solitary deglutitive neurons, but from deglutitive neurons located in the "ventral region of the medullary swallowing centre" in the reticular formation above the nucleus ambiguus. The discrepancy between the present findings and those of Jean *et al.* (1983) and Amri *et al.* (1984) may be attributed to: i) spread of tracer outside the intended site of injection in the latter study or ii) species differences (sheep versus rat). The former possibility is supported by the finding that the anterograde tracer placed in the ventral group of swallowing neurons (in and around the Amb) resulted in label in the parabrachial complex (Jean *et al.*, 1983); hence, the ventrolateral solitario-parabrachial projection originating from the solitary complex may have been labeled (see below). In any case, the large size of their injection (Fig. 1 of Jean *et al.*, 1983) would appear to render it difficult to pinpoint the precise source of the resultant efferents. However, it is noteworthy that, in the same study, injections of the retrograde tracer (HRP) placed within the  $V_{\text{m}}$  did yield labeled perikarya within the NTS 1-2 mm rostral to the obex in the sheep; the HRP-injected region of the  $V_{\text{m}}$  probably included the  $V_{\text{m}}^{\text{sol}}$  since the site of injection was stereotaxically predetermined by locating the antidromic action potential produced by electrical stimulation of the mylohyoid nerve. However, the resultant retrogradely labeled solitary cells were not believed by the authors to lie in the swallowing region of the NTS (Jean *et al.*, 1983). Regarding

point ii) above, the sheep has several neuroanatomical features which are not seen in the rat (Car *et al.*, 1975; see below). Studies have indicated an absence of solitario-trigeminal projections in cat and monkey as well (Morest, 1967; King, 1980; Beckstead *et al.*, 1980), although Loewy and Burton (1978) found label around the border of the  $V_m$ , but not over 'large motoneurons' of the  $V_m$ . Thus, assuming that these investigators would not have failed to detect the small proportion of solitarily fibres projecting to the relatively smaller cell group of the  $V_m^m$ , the only other explanation would be that the pathway is present in the rat, but not in sheep, cat or monkey. In the rat, a solitario-trigeminal projection has previously been described by means of tritiated amino acid autoradiography (Norgren, 1978).

### iii) The facial nuclear complex

Two conspicuous regions of the facial nucleus contained label following tracer injections at pharyngeal, but not oesophageal, loci: the rostralateral portion of the main  $VII_m$  and throughout the  $VII_a$ . The labeled portion of the main  $VII_m$  corresponds to its lateral subdivision and is, presumably, the source of innervation of orofacial muscles including the buccinator (Papez, 1927; Greene, 1963; Martin and Lodge, 1977; Watson *et al.*, 1982; Travers, 1985), a facial muscle that is recruited during swallowing (Blanton *et al.*, 1970). Consistent with the present

findings, deposits of HRP into the VII<sub>m</sub> at its 'intermediate and lateral' divisions yielded retrograde label within the NTS (Travers and Norgren, 1983).

The VII<sub>sp</sub>, also termed the suprafacial nucleus, contains motoneurons supplying the the posterior belly of the digastric muscle (Mizuno *et al.*, 1975; Watson *et al.*, 1982; Shohara and Sakai, 1983) and the stylohyoid muscle (Székely and Matesz, 1982; Shohara and Sakai, 1983). During swallowing, both bellies of the digastric are active (Munro, 1972; Bieger *et al.*, 1977). The activity of the stylohyoid during swallowing is well established (Doty and Bosma, 1956). In fact, it forms part of the "leading complex" during deglutition (Doty and Bosma, 1956). Label was seen within the accessory facial nucleus in the present study following tracer injections at pharyngeal and CS sites. Again, the close proximity of the label to the presumptive motoneurons suggests that the solitario-facial projections impinge directly on the motoneurons.

Absence of NTS<sub>cen</sub> projections to the trigeminal and facial motor nuclei is corroborated by Cunningham and Sawchenko (1989).

#### iv) The hypoglossal nucleus

The somatotopic organisation within the XII<sub>m</sub> of branches of the hypoglossal nerve innervating the tongue musculature in the rat, as revealed by the HRP method, has been described by Krammer *et al.* (1979) and the myotopic

representation of the geniohyoid and thyrohyoid muscles in the XII<sub>m</sub> by Kitamura *et al.* (1983). The dorsal subnucleus of the XII<sub>m</sub> is found in the rostral two-thirds of the motor nucleus and contains cells of origin of the styloglossus and hyoglossus branches, and is, therefore, the presumptive source of innervation of the respective tongue-retractor muscles. The representation of these muscles overlaps considerably within the dorsal subnucleus, however, the styloglossal representation extends to the rostral end and the hyoglossal to the caudal end of the subdivision. In the present study, label within the dorsal subnucleus of the XII<sub>m</sub> and preferentially near its caudal boundary was indicative of direct solitarily efferents to the hyoglossal and styloglossal motoneurons, with a much higher proportion of projections to the cells of origin of the ramus of the hypoglossal nerve supplying the hyoglossal muscle.

The ventromedial subnucleus is the largest division of the XII<sub>m</sub>, extending throughout the entire length of the motor nucleus, and contains motoneurons retrogradely labeled after application of tracer to the genioglossus branch of the hypoglossal nerve (Krammer *et al.*, 1977). In the present study, following injections of lectin at pharyngeal and CS sites, a very specific pattern of labeling was observed within the XII<sub>m</sub>: the ventral margin of the rostral half and the dorsal aspects of the caudal half were found labeled as was the oblique band which was continuous with the labeled portion and which roughly separated the rostral and caudal halves of the subnucleus (see Fig. 39).

The ventrolateral subnucleus of the XII<sub>n</sub> is seen only at caudal levels of the nucleus and contains cells of origin of the geniohyoid branch of the hypoglossal nerve. These tongue-protruder motoneurons within the lateral aspects of the subnucleus were found to receive solitaria projections in the present study.

Recruitment of both the tongue protruders and retractors during the buccopharyngeal stage of swallowing is well-documented (Doty and Bosma, 1956; for reviews, see Doty, 1968; Miller, 1982; Hiiemae and Crompton, 1985). Indeed, in the cat, the genioglossus muscle was found to be active very early in swallowing, frequently preceding the mylohyoid (Lowe and Sessle, 1973). Both the styloglossus and the geniohyoid constituted part of the "leading complex" during deglutition (Doty and Bosma, 1956). During the pharyngeal stage of swallowing, elevation of the hyoid bone requires the synchronous activity of the mylohyoid and the hyoglossal muscles while anterior movement of the hyoid requires the synergistic activity of the geniohyoid and the mylohyoid muscles (Matsumoto, 1977).

The thyrohyoid is a laryngeal elevator that participates in swallowing (Andrew, 1956; Doty and Bosma, 1956). The representation of this muscle is at caudal, spinomedullary levels of the XII<sub>n</sub> (Kitamura *et al.*, 1983). This region was also labeled in pharyngeal and CS cases.

The remarkable pattern of label along specific areas of each of the hypoglossal subnuclei, concomitant with a conspicuous sparsity or absence of label in other areas, is suggestive of recruitment of specific sub-populations of the

tongue-retractor and -protruder motoneurons during swallowing. Such a conclusion receives support from other investigations. Car and Amri (1987) found that only a subgroup of XII<sub>n</sub> motoneurons were active during deglutition in the sheep. Travers and Jackson (1988), recording from single cells of the XII<sub>n</sub> in the awake rat during licking, swallowing and gaping (rejection), found that, during elicitation of the three synergies, different motoneurons were active.

The present findings of direct projections from the NTS to the hypoglossal nucleus in the rat are consistent with previous reports (Norgren, 1978) but are at variance with those of Amri and Car (1988) in the sheep who, on the basis of retrograde tracing and antidromic mapping, found that only an insignificant proportion of XII<sub>n</sub> motoneurons receive projections from the NTS while the bulk of the hypoglossal input came from the ventrolateral reticular formation. As mentioned above, this discrepancy could be due to species differences (sheep versus rat). Indeed, the sheep has other neuroanatomical features such as bifurcating SLN fibres with primary terminations in both the NTS and the pons (Car *et al.*, 1975) which are not found in the rat (Altschuler *et al.*, 1989). Apart from the species differences, another explanation for the discrepancy would be that, since the solitary deglutitive loci constitute a small fraction of the entire NTS, the solitary cells projecting to the hypoglossal may have escaped detection. The same reason may explain the presence of solitario-hypoglossal efferents seen in one anterograde tracing study utilising tritiated amino acid autoradiography (Norgren,

1978) but sparsely in another HRP tracing study (Travers and Norgren, 1984). Other anterograde tracing studies could not provide unequivocal data regarding solitario-hypoglossal projections in the rat since the amount of injected tracer was so large that it infiltrated the XII<sub>n</sub> itself (e.g. Ross *et al.*, 1985). The latter group did observe efferents from the NTS to the XII<sub>n</sub> but, owing to the problem of the large size of the injection site, concluded that these may have arisen from outside the NTS. Results of the present investigation are inconsistent with such a conclusion insofar as the solitario-hypoglossal efferents are concerned.

In summary, pharyngeal solitatorial loci were found to project directly to specific areas of the XII<sub>n</sub> containing different subpopulations of tongue-protruder and -retractor motoneurons controlling muscles that participate in deglutition. The absence of these projections from oesophageal loci is corroborated by the study of Cunningham and Sawchenko (1989).

#### v) The dorsal motor nucleus of the vagus

Besides an intrinsic innervation from cells in the myenteric plexus, the smooth muscle of the oesophagus has a rich extrinsic nerve supply whose exact origin and course has remained controversial. Data from a number of histological and tracing studies (e. g. Marinesco and Parhon, 1907; Niel *et al.*, 1980; Hudson and Cummings, 1985; Vyas *et al.*, 1987) have implicated the DMX as being the source of preganglionic supply to oesophageal smooth muscle. In the rhesus



monkey or the opossum, deviation of a swallowed bolus at the level of the cervical (striated muscle) oesophagus did not impede the progression of peristalsis in the thoracic (smooth muscle) oesophagus (Janssens *et al.*, 1976; Janssens, 1978). Furthermore, in the cat (which has striated muscle in only the cervical oesophageal region), distal oesophageal motility was impaired following lesions of the DMX (Higgs *et al.*, 1965). It is, therefore, conceivable that motor commands to the smooth muscle component of the oesophagus arise from preganglionic cells in the DMX. Although projections from the NTS to the DMX have been reported in the cat (Morest, 1965; Cottle and Calaresu, 1975; Loewy and Burton, 1978), rat (Norgren, 1978; Ross *et al.*, 1985), and monkey (Beckstead *et al.*, 1980), no documentation of the DMX receiving a circumscribed projection from the deglutitive loci of the NTS exists. An exception is a recent anatomical study, in which evidence of direct efferents from the NTS<sub>cen</sub> to the DMX was attributed to spread of tracer outside the subnucleus (Cunningham and Sawchenko, 1989).

In the present study, following lectin injections at both pharyngeal and oesophageal loci, labeled fibres and terminals were found within the DMX. These findings have several implications. First, they indicate that, during swallowing, the pharyngeal pattern generator exerts some degree of control over the smooth muscle of the oesophagus since it has direct access to the putative source of its preganglionic supply.

Second, the spinal portion of the DMX is the presumptive source of parasympathetic innervation of the rat lower oesophageal sphincter (Vyas *et al.*, 1987; Vyas, Bieger and Hopkins, in preparation). Thus, the solitary projections may represent the neuroanatomical pathway responsible for relaxation of the lower oesophageal sphincter beginning with the pharyngeal stage of swallowing.

Finally, deposits of PHAL at NTS<sub>cen</sub> sites yielding oesophageal responses also resulted in an abundance of label throughout the DMX. This suggests that during peristalsis, not only does the NTS<sub>cen</sub> organize the striated muscle contractions, but it may also play a role in simultaneous coordination of oesophageal smooth muscle activity via vagal preganglionic fibers. This aspect may be of considerable importance in species that have smooth muscle in distal portions of the oesophagus. It remains to be seen what possible role the NTS<sub>cen</sub> might play in gastric function: it is noteworthy in this regard that the DMX is the main source of gastric preganglionic fibres and that relaxation of the proximal stomach occurs during deglutition (the so-called 'receptive relaxation').

## B. Solitary efferents to other pontomedullary structures

### i) The solitary complex

The presence of projections, albeit sparse, from the pharyngeal loci to the NTS<sub>cen</sub> indicates that the coupling of pharyngeal and oesophageal stages occurs at

the level of the NTS itself. The pattern of label further suggests that the pharyngeal solitary efferents may make synaptic contact on both the soma and dendrites of the NTS<sub>cen</sub> neurons; however, this issue needs further clarification. In addition, the sparsity of label suggests that the coupling between the pharyngeal and the oesophageal subcircuits is not very tight. Absence of label in the contralateral NTS<sub>cen</sub> indicates that the pharyngeal neural subcircuit is coupled mainly to the ipsilateral NTS<sub>cen</sub>.

The rostrally focussed projection from the solitary deglutitive loci, in particular the CS and pharyngeal sites, confirms previous reports (Norgren, 1978) while providing information regarding its precise source. The rostral NTS has been implicated in gustatory function (Halpern and Nelson, 1965; Travers *et al.*, 1983; Hamilton and Norgren, 1984). The present findings, therefore, indicate direct links between deglutitive and gustatory substrates at the level of the solitary complex.

In the present study, the anterograde tracing confirmed the results of retrograde tracing experiments *viz.*, evidence of reciprocal connections between the deglutitive loci of each NTS (see Fig. 48). These connections may represent anatomical links between the two presumptive "half-centers" for swallowing (Ishihara, 1906; Doty *et al.*, 1967).

- ii) Nuclei of the spinal trigeminal tract, the principal trigeminal sensory nucleus, the paratrigeminal islands and the parabrachial complex

The present investigation confirmed the presence of a projection from the NTS that swept ventrolaterally through the (interpolary and oral) nuclei of the spinal trigeminal tract (nSpV) and through the principal sensory trigeminal nucleus before ascending dorsally to terminate in the parabrachial complex as described in the cat (King, 1980) and reported in the rat (Beitz *et al.*, 1987). The sensory trigeminal nuclei are involved in the processing of orofacial sensory information (for review, see Bannister, 1976; Kelly, 1981; Tracey, 1985) and are the primary recipients of such sensory input. Within the nuclei of the spinal trigeminal tract, terminations from primary afferents of the trigeminal nerve are somatotopically arranged such that the mandibular division is represented most dorsally and the ophthalmic division most ventrally (Torvik, 1956). The oral subnucleus receives projections from the nasal and oral cavities in the cat (Wall and Taub, 1962) and from the soft palate in the rat (Altschuler *et al.*, 1989). The interpolar subnucleus and the principal trigeminal nucleus receive a major proportion of sensory afferents from the vibrissae in the rat (Belford and Killackey, 1979; Arvidsson, 1982). Since the NTS is an important relay of visceral afferents, direct solitario-trigeminal links, as seen in the present study, may constitute part of a system of reciprocal pathways between the nSpV and the NTS involved in integrating somatic and visceral afferent inputs from wide areas of the body, as suggested by Ménétrey and Basbaum (1987).

The solitary efferents to the paratrigeminal islands also represents a reciprocal projection pathway (Menétrey and Basbaum, 1987). The significance of these, however, is unclear. It may be that the solitario-paratrigeminal connection represents inhibitory inputs that suppress pharyngeal pain during swallowing since the paratrigeminal islands receive heavy primary afferent projections from the soft palate, pharynx, and larynx via the glossopharyngeal nerve and are involved in chemosensory, probably pharyngeal nociceptive, function (Altschuler *et al.*, 1989). In the rat, the NTS efferents to the sensory trigeminal nuclei were reported to be partly enkephalin-immunoreactive (Beitz *et al.*, 1987).

The parabrachial complex is known to receive projections from the solitary complex and is the primary relay for many ascending medullary pathways in the cat and rat (Norgren, 1978; Loewy and Burton, 1978; Ricardo and Koh, 1978; King, 1980; Fulwiler and Saper, 1984; Maley and Panneton, 1988). The present investigation found the parabrachial complex the most rostral structure in the rat brainstem on which solitary deglutitive efferents terminated indicating its role as the primary synaptic relay for ascending solitary deglutitive fibres. However, this cannot be ascertained since more rostral levels of the neuraxis were not examined in the present study.

In agreement with the present investigation, Cunningham and Sawchenko (1989) also concluded that the NTS<sub>cen</sub>-parabrachial projection may have originated from adjacent structures in the NTS. This is supported by the finding that, large,

but not small, injections at oesophageal sites produced the pattern of label in the parabrachial complex seen after pharyngeal injections.

In contrast to the rat, in the sheep, SLN fibres bifurcate and terminate at two sites in the brainstem - the NTS and a pontine area above the  $V_{\text{H}}$ ; the latter constitutes the primary synaptic relay for ascending laryngeal pathways (Car *et al.*, 1975). A similar situation exists in the cat and the monkey for ascending gustatory pathways (Morest, 1967; Rhoton, 1968; Bernard and Nord, 1971).

In the rat, the solitario-parabrachial projection was found to exhibit immunoreactivity to several peptides and catecholamine-synthesizing enzymes (Milner *et al.*, 1984; Kawai *et al.*, 1988) and has been implicated in various visceral functions.

### iii) "Lacrimal neurons"

The small neuronal group, rostralateral to the main  $VII_{\text{H}}$ , was retrogradely labeled following application of HRP to the greater superficial petrosal component of the facial nerve. This neuronal group is believed to be involved in lacrimation (Contreras *et al.*, 1980). However, the postganglionic fibres of the sphenopalatine (pterygopalatine) ganglion innervate not only the lacrimal glands but also the pharyngeal, palatine and nasal glands (Williams *et al.*, 1989). Therefore, the "lacrimal" neurons of Contreras *et al.* (1980) probably also contribute parasympathetic efferents to other secretory glands in the oropharyngeal mucosa.

irect projections to these neurons from solitary loci may be indicative of: i) interaction between neurons involved in deglutitive and in oropharyngeal secretory/gustatory function at the level of the NTS ii) pathways actually arising from gustatory substrates within the NTS. Regarding i) the solitary deglutitive neurons would appear to have direct access to secretomotor neurons involved in salivation and pharyngeal mucous secretions; and conditions such as increase in salivary and mucous secretion would appear to facilitate swallowing (see review by Kennedy and Kent, 1988).

Regarding ii) it is noteworthy that gustatory and deglutitive substrates overlap and intermingle extensively within the NTS as judged by the afferent terminations of primary gustatory and deglutitive afferents (Hamilton and Norgren, 1984; Altschuler *et al.*, 1989). Thus, projections from solitary deglutitive loci to areas implicated in gustatory function may be indicative of the limit of resolution of the technique employed in the present study.

### C. Unlabeled pontomedullary regions

#### i) The medullary reticular formation

On the basis of lesion experiments, Doty *et al.*, (1967) postulated that the "swallowing centre" organizing the buccopharyngeal stage of swallowing lies in the medullary reticular substance at a level between the rostral pole of the inferior

olive and the posterior pole of the VII<sub>e</sub>. Subsequent electrophysiological experiments in cat also supported this hypothesis (Manchanda and Aneja, 1972). If this were true, then, since the swallowing afferents terminate within the NTS (Altschuler *et al.*, 1989), efferents from the solitary deglutitive loci should have impinged on either i) the medullary reticular formation or ii) areas other than the deglutitive motoneuronal pools. In the absence of significant terminal label in the medullary reticular substance, the present study fails to support the existence of a swallowing centre in the rat medullary reticular formation and is consistent with similar conclusions based on more recent electrophysiological data (Kessler and Jean, 1985a). It is, therefore, conceivable that in the study by Doty *et al.* (1967), lesions to the medullary reticular formation may have damaged the solitary efferent pathways that course through this region.

#### ii) The pontine reticular formation

Based on neuroanatomical tracing studies in the cat (Holstege *et al.*, 1983), it was postulated that the swallowing centre may be located in the caudal pontine tegmentum in an area dorsal to the superior olivary complex. However, in the present study, a conspicuous absence of label was noted in this region following tracer injections in solitary deglutitive loci. Hence, it is unlikely that, at least in the rat, the pontine reticular formation is involved in swallowing. Electrical stimulation of this region also failed to elicit any deglutitive responses (Kessler and Jean, 1985a).



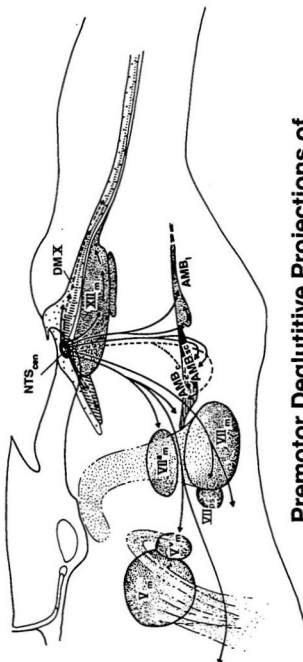
#### D. Proposed circuit model

In summary, the present study revealed that efferents from the deglutitive region of the solitary complex are distributed to motor nuclei of cranial nerves V, VII, IX, X, and XII and are myotopically organized within each nucleus (Fig. 52). Pharyngeal sites in the  $NTS_{int}$  and  $NTS_v$  give rise to efferents to pharyngomotor ( $Amb_{sc}$ ), laryngomotor ( $Amb_l$ ), divisions of the Amb, stylopharyngeal and cricothyroid motoneurons (tip of Amb and  $Amb_{sc}$ ), mylohyoid and anterior belly of digastric motoneurons ( $V_n^m$ ), stylohyoid and posterior belly of digastric motoneurons ( $VII_n^m$ ), motoneurons of certain oral muscles (specific subregions of  $VII_n$ ), tongue protruder and retractor motoneurons (different subdivisions of the  $XII_n$ ) while efferents to the oesophagomotor division ( $Amb_e$ ) of the Amb issue from the  $NTS_{cen}$ . The source of gastric preganglionic fibres and the putative extrinsic source of innervation of the oesophageal and gastroesophageal smooth muscle, the DMX receives projections from both pharyngeal and oesophageal loci.

Thus, these solitary efferents constitute true premotor deglutitive projections. Pharmacological data from this study have demonstrated the presence of distinct receptor-mediated processes within the solitary deglutitive loci which, when activated, are capable of generating the swallowing sequence. On the other hand, the pattern of distribution of the solitary deglutitive efferents is consistent with a role of the NTS premotor neurons as generators and coordinators of the pharyngeal ( $NTS_{int}$  and  $NTS_v$ ) and oesophageal ( $NTS_{cen}$ ) motor pattern during swallowing. Coordination between the two solitary 'half centres' for swallowing

**Figure 52**

Summary diagram of solitary deglutitive efferents. Schematic of a sagittal slab of the rat brainstem depicting efferents arising from pharyngeal sites (solid lines) within the subnuclei intermedius and ventralis of the NTS and those originating from oesophageal sites (broken lines) in the subnucleus centralis. Abbreviations: **Amb<sub>c</sub>** compact formation of nucleus ambiguus; **Amb<sub>l</sub>** loose formation of nucleus ambiguus; **Amb<sub>sc</sub>** semicompact formation of nucleus ambiguus; **DMX** dorsal motor nucleus of the vagus; **NTS<sub>cen</sub>** subnucleus centralis of the NTS; **V<sub>3</sub>** trigeminal motor nucleus; **V<sub>3</sub><sup>am</sup>** ventromedial (accessory) subnucleus of trigeminal motor nucleus; **VII<sub>3</sub>** facial nucleus; **VII<sub>3</sub><sup>a</sup>** accessory facial nucleus; **VII<sub>3</sub>** facial nerve; **VII<sub>3</sub><sub>pa</sub>** source of parasympathetic preganglionic fibres of VII<sub>3</sub>; **XII<sub>3</sub>** hypoglossal nucleus.



**Premotor Deglutitive Projections of  
Solitarius Complex in the Rat**

is probably effected via direct reciprocal connections between them. The distinctive identities of the pharyngeal and the oesophageal pattern generators, as indicated by both neuroanatomical and pharmacological data of the present study, is consistent with the functional divisions (pharyngeal and primary and secondary oesophageal peristalsis) of the act of swallowing. As suggested by the low density of  $NTS_{int}$ - $NTS_{cen}$  projections, the two stages of swallowing appear to be loosely coupled ipsilaterally at the level of the NTS. This loose coupling may explain the association (normal swallowing) and dissociation (deglutitive inhibition) of the oesophageal stage with the pharyngeal stage of swallowing.

Finally, the region dorsal to the  $Amb_c$  may represent an interneuronal zone whose possible involvement in the buccopharyngeal stage of swallowing remains to be elucidated.

## SUMMARY AND CONCLUSIONS

Functional, pharmacological and neuroanatomical properties of premotor deglutitive substrates within the solitary complex were investigated in order to elucidate the role of the solitarily neurons in organizing the motor pattern of swallowing. Deglutitive loci were defined in terms of their activation at short latency by appropriate chemical microstimuli. Pharmacological studies addressed receptor mechanisms underlying the deglutitive excitant effects of (i) glutamate, with a view to distinguishing between the excitatory amino acid receptor types activating the pharyngeal and oesophageal stages of swallowing; and (ii) ACh/muscarine and serotonin as putative mediators of two postulated central inputs to the NTS with selectivity for oesophageal and pharyngeal stages of swallowing, respectively. Neuroanatomical analyses aimed at determining the position of the NTS with respect to the swallowing network as demonstrated by efferents from and certain afferents to the solitarily deglutitive loci. The techniques used included micropneumophoresis, neuronal tract tracing and immunocytochemistry.

The results of this research are summarized as follows:

1. Pharyngeal loci extend from the level of the obex to 900  $\mu\text{m}$  rostrad and overlap the subnuclei intermedialis and ventralis of the NTS.

2. Cervical and distal oesophageal loci are coextensive with the subnucleus centralis of the NTS and display a rostrocaudal organotopic pattern.
3. Complete swallow loci occupy a discrete zone 200-550  $\mu\text{m}$  rostral to obex and lie between the subnuclei centralis and intermedialis in the mediolateral axis.
4. Pharyngeal loci respond preferentially to activation of kainate receptors as do oesophageal loci to NMDA receptor activation.
5. Kainate receptor-mediated activation of the pharyngeal loci is facilitated by 5-HT<sub>2</sub> and/or 5-HT<sub>1c</sub> receptors, but is independent of the excitatory 5-HT input.
6. In contrast, NMDA receptor-mediated activation of the oesophageal loci is dependent upon an intact cholinergic input of unknown origin and presumably converging on muscarinic receptors on the same neuron.
7. The serotonergic innervation of the pharyngeal solitary loci is contributed, at least in part, by 5-HT cells of the raphe magnus, obscurus and pontis nuclei.

8. Pharyngeal loci project to motoneuronal pools controlling muscles that are active during the pharyngeal stage of swallowing. These include the semicompact and loose formations and the tip of the nucleus ambiguus, the ventromedial trigeminal motor nucleus, the dorsolateral division of the facial nucleus, the accessory facial nucleus and specific subregions of the hypoglossal nucleus.
9. Oesophageal loci project to the compact oesophagomotor division of nucleus ambiguus.
10. Both pharyngeal and oesophageal loci project to the dorsal vagal motor nucleus, the putative source of preganglionic innervation of the oesophageal smooth muscle and the lower oesophageal sphincter.
11. Direct projections to motoneuronal pools from the solitary deglutitive loci demonstrate that the latter contain true premotoneuronal elements.
12. The organotopic arrangement of projections from solitary deglutitive loci supports the hypothesis that the internuncial neural network contained within the subnuclei intermedialis, ventralis and centralis is responsible for generating the pharyngo-oesophageal motor pattern of swallowing.

13. Coupling between the pharyngeal and oesophageal stages of swallowing probably occurs, albeit weakly, at the level of the NTS via direct projections from the pharyngeal to the oesophageal locus.

#### Future studies

The present research has raised several issues:

1. What is the organization of the solitary deglutitive neural circuitry at the single neuron level? This is an issue whose resolution can be expected to yield important insights into the exact layout of the deglutitive premotor efferents.
2. What transmitter and receptor mechanisms are utilised by solitary premotoneurons?
3. What does the apparent diversity of the serotonergic projections to solitary deglutitive substrates imply?
4. If the NTS<sub>cen</sub> organises the oesophageal motility patterns, exactly how is this accomplished? This question may be answered if the source and the significance of the cholinergic input could be determined. The interaction of NMDA receptors



and muscarinic cholinceptors on NTS<sub>cen</sub> neurons would have to be studied at the single neuron level.

5. The tongue protruders and retractors obviously participate in swallowing, but what is the exact significance of the remarkable pattern of distribution of the solitario-hypoglossal projections? Does it indicate that different populations of motoneurons are recruited during swallowing as opposed to those during the execution of other synergies? What other implications does this finding have?

6. Where does the DMX come into the picture as regards swallowing? Available data indicate that the extrinsic innervation of the oesophageal smooth muscle as well as that of the lower oesophageal sphincter arises from this motor nucleus. Does the dual input from the pharyngeal and oesophageal premotor areas in the NTS to the DMX indicate primary and secondary peristaltic central control of the oesophageal smooth muscle?

Answers to these questions may be expected to advance our understanding of the principles governing the neural organisation of deglutition.

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