MORPHOLOGICAL AND NEUROCHEMICAL ANALYSIS OF SENSORY AFFERENTS OF THE VAGUS NERVE.

Rachel Mary Sykes

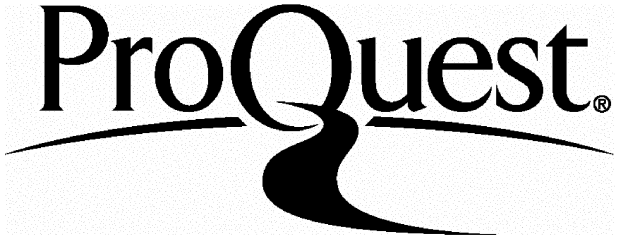
Thesis submitted for the degree of Doctor of Philosophy to the Faculty of Science at the University of London.

Department of Physiology,

The Royal Free Hospital School of Medicine,

Rowland Hill Street,

London, NW3 2PF.



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ABSTRACT

A detailed morphological analysis of sensory afferents throughout the different subnuclei of the rostrocaudal extent of the rat nucleus tractus solitarius (nTS) is described. Vagal afferents were labelled using neuroanatomical tracing techniques and examined at the electron microscope level. Labelled boutons were characterized as containing round, clear synaptic vesicles and occasionally dense core vesicles and forming asymmetric synaptic contacts with predominantly small to medium calibre dendrites. This anatomical organization was observed throughout the entire nTS. Only one possible vagal afferent was observed forming an axo-axonic contact suggesting modulation of vagal afferent input occurs by interactions on a common post-synaptic target.

Further studies were undertaken to investigate the neurochemical content of vagal afferents. Glutamate, has been detected in a variety of afferent projections throughout the CNS but evidence for this amino acid in vagal afferents is equivocal. Initially the distribution of glutamate-immunoreactive structures in the nTS was examined using post-embedding immunocytochemical techniques. Immunoreactive perikarya were sparse at the more caudal and rostral extremes of the nTS but were more numerous at intermediate levels where distinct groups of glutamate-containing neurones were observed in the tractus solitarius and medial subnucleus. The nTS was densely innervated with glutamate-immunoreactive boutons which had a homogeneous distribution throughout its rostrocaudal extent. Ultrastructural analysis revealed that these immunolabelled boutons had an identical morphological profile to vagal afferents. When anterograde tracing and immunocytochemistry were combined in the same tissue immunolabelled boutons further identified as vagal afferents were detected, establishing

that the sensory neurones of the nodose ganglia contribute to the glutamatergic innervation of the nTS.

Substance P (SP), calcitonin gene-related peptide (CGRP) and 5-hydroxytryptamine (5-HT) had previously been demonstrated in nodose ganglia perikarya but there was no direct evidence for their presence in central vagal afferent terminations. A technique was developed that allowed the simultaneous detection of both the anterogradely labelled vagal afferents and putative neurotransmitter at the light microscope level using antibodies labelled with different fluorochromes. Problems encountered with identifying double-labelled structures with a standard fluorescent microscope led to the development of a method of analysis that ultilized a laser scanning confocal microscope and image capture system. Direct evidence for the presence of 5-HT, SP and CGRP immunoreactivity in the central terminals of sensory vagal afferents was obtained. Very few SP-immunoreactive vagal afferents were observed suggesting that SP may play a greater role in the periphery as opposed to the CNS. Vagal afferents containing 5-HT were detected in regions that receive mainly gastrointestinal tract afferents whilst vagal afferents immunoreactive for CGRP were concentrated in areas receiving a general visceral innervation. The differential distribution of vagal afferents immunoreactive for these neurochemicals suggests that the chemical expression of nodose ganglia perikarya may be related to their target tissues or the functional class of the afferent fibres.

ABBREVIATIONS

ABC	streptavidin biotinylated complex
ACE	central nucleus of the amygdala
ADN	aortic depressor nerve
AH	anterior hypothalamus
ANS	autonomic nervous system
AP	area postrema
BNST	bed nucleus of the stria terminalis
CB	carotid body
CCK	choleocystokinin
CGRP	calcitonin gene related peptide
ChAT	choline acetyltransferase
CL	lateral cuneate nucleus
CSN	carotid sinus nerve
CT	chorda tympani
CT-B	cholera toxin subunit B
CVLM	caudal venttrolateral medulla
DAB	3'3'diaminobenzidine
dlnTS	dorsolateral subnucleus of the nucleus tractus solitarius
DVN	dorsal motonucleus of the vagus nerve
EAA	excitatory amino acid
E.M.	electron microscope
EPSP	excitatory post-synaptic potential
FITC	fluoroscein isothiocyanate
GABA	gamma aminobutyric acid
GAD	glutamic acid decarboxylase
GPN	greater petrosal nerve
H_2O_2	hydrogen peroxide
5-HT	5-hydroxytryptamine
HYP	hypothalamus
III	oculomotor nerve
ILC	infralimbic cortex
io	inferior cortex
I.P.	inttraperitoneal

IPSP	inhibitory post-synaptic potential
IGS	immunogold silver intensification
IX	glossopharyngeal nerve
K.F	Kolliker-Fuse nucleus
LC	locus coeruleus
LDTg	laterodorsal tegmental nucleus
LHA	lateral hypothalamic area
LT-IX	lingual-tonsilar branch of IX nerve
MLF	medial longitudinal fasiculus
mnTS	medial subnucleus of the nucleus tractus solitarius
NA	nucleus ambiguus
ncomm.	commissural subnucleus
NGS	non-immune goat serum
ninTS	interstitial subnucleus of the nucleus tractus solitarius
nInTS	intermediate subnucleus of the nucleus tractus solitarius
nInTS	intermediate subnucleus of the nucleus tractus solitarius
NKA	neurokinin A
NRA	nucleus retroambiguus
NRD	nucleus raphe dorsalis
NRM	nucleus raphe magnus
NRO	nucleus raphe obscurus
NRP	nucleus raphe pallidus
nTS	nucleus tractus solitarius
nVII	facial motonucleus
nXII	hypoglossal motonucleus
OrG	orbital gyrus
P	pyramidal tract
PAG	periaqueductal grey
PB	phosphate buffer
PBL	lateral parabrachial nucleus
PBM	medial parabrachial nucleus
PBS	phosphate buffered saline
PFC	prefrontal cortex
PGi	paragigantocellular nucleus
PHA-L	*Phaseolus vulgaris* leuco-agglutinin
PH-IX	pharyngeal branch of the IX nerve
PR	paramedian reticular formation

PVN	paraventricular nucleus
RAR	rapidly adapting receptor
RFN	retrofacial nucleus
rPCTt	rostral parvocellular formation
RTN	retrotrapezoid nucleus
RVLM	rostral ventrolateral medulla
SAR	slowly adapting receptor
SLN	superior laryngeal nerve
SG	subnucleus gelatinosus
SP	substance P
SpV	spinal trigeminal complex
TMB	tetramethylbenzidine
TNC	caudal trigeminal nucleus
TPBS	Tris/phosphate buffered saline
TS	tractus solitarius
V	facial nerve
VII	trigeminal nerve
vlnTS	ventrolateral subnucleus of the nucleus tractus solitarius
vnTS	ventral subnucleus of the nucleus tractus solitarius
VPMpc	ventral posteromedial thalamic nucleus
VPN	vagal preganglionic neurone
X	vagus nerve
XII	hypoglossal motonucleus

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CHAPTER 1

General introduction.

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1:1 INTRODUCTION.

The autonomic nervous system (ANS) maintains a constant internal environment by regulating and co-ordinating the functions of the viscera. It consists of two major divisions, the parasympathetic and sympathetic nervous systems, whilst the enteric nervous system makes up a third, separate division. In the past there was controversy about whether the ANS included the sensory (afferent) as well as the motor (efferent) branch. This confusion arose from the interpretation of a paper by the famous physiologist, Langley, who in 1921 defined the ANS as "the nerve cells and fibres by means of which efferent impulses pass to tissues other than multinuclear striated muscle". Some workers took this statement to mean that the ANS should be considered as purely a motor system although in an earlier textbook (1900), "Schafers Textbook of Physiology", Langley clearly stated that he did consider visceral sensory afferent fibres to be an integral part of the ANS. However, today it is well established that the ANS includes an afferent sensory as well as a motor component.

The efferent branch of the ANS is usually composed of pre- and postganglionic neurones. The preganglionic neurones of the sympathetic division are situated in the thoracic and upper lumbar cord and their axons synapse in the sympathetic chain or pass through the ganglia to synapse in collateral ganglia e.g. coeliac ganglia situated near the viscera. One exception is the adrenal medulla which is directly innervated by sympathetic neurones located in the spinal cord. The preganglionic fibres of the parasympathetic division originate from neurones situated mainly in brainstem nuclei and to a lesser extent in the sacral spinal cord. These fibres exit in either the oculomotor (III), facial (VII), glossopharyngeal (IX) or vagus (X) cranial nerves or 2nd, 3rd or 4th ventral roots and synapse in parasympathetic ganglia on or within the walls of various organs. Most viscera are innervated by both sympathetic and

parasympathetic divisions which generally have opposing functions, although some structures e.g. some blood vessels and sweat glands are innervated by one division only (Langley, 1921).

The sensory afferent pathways arise from receptors located in the walls, parenchymal and serosal membranes of the internal organs and blood vessels. These receptors respond to a variety of stimuli e.g. mechanical, chemical, thermal or irritant to provoke both conscious and unconscious sensation. The cell bodies of the sympathetic afferents are located in the dorsal root ganglia at the thoracic and upper lumbar spinal levels whilst those of the sacral parasympathetic division are situated in S2-S4 dorsal root ganglia (Ranson, 1921). The geniculate, petrosal and nodose ganglia contain the cell bodies of the VII, IX and X cranial parasympathetic nerves respectively. Information from the afferent system is relayed by mono- and polysynaptic pathways in the CNS and modulates autonomic outflow to the end organ.

1:2 ORGANIZATION OF VAGAL NERVE PREGANGLIONIC NEURONES.

The efferent fibres of the vagus nerve arise mainly from two medullary nuclei, the dorsal motonucleus of the vagus nerve (DVN) and the nucleus ambiguus (NA; Conteras et al., 1980; Gwyn et al., 1985; Hamilton et al., 1987; Hopkins et al., 1984; Kalia, 1981; Kalia & Mesulam, 1980a; Karim & Leong, 1980; Micelis & Malsbury, 1985; Stuesse, 1982). The DVN is an anatomically distinct nucleus situated in the dorsomedial portion of the medulla, close to the floor of the fourth ventricle and extending caudally to the first cervical segment. In contrast, the nucleus ambiguus is an ill-defined nucleus located in the ventrolateral portion of the medullary reticular formation extending rostrally to the facial nucleus and caudally to the C1 level of the

spinal cord (Fig. 1:1). The DVN provides the major innervation to subdiaphragmatic structures but also innervates cervical and thoracic structures whilst the nucleus ambiguus innervates predominantly the thoracic and cervical viscera. Some vagal motoneurones situated in the nucleus ambiguus directly innervate the striated muscle of the upper airway. Neuronal tracing techniques have demonstrated that vagal motoneurones in both the DVN and nucleus ambiguus are organized viscerotopically (Altschuler et al., 1989, 1991; Bieger & Hopkins, 1987; Fox & Powley, 1985; Kalia & Mesulam, 1980b).

1:3 ORGANIZATION OF THE CENTRAL AFFERENT PROJECTIONS OF THE VAGUS NERVE

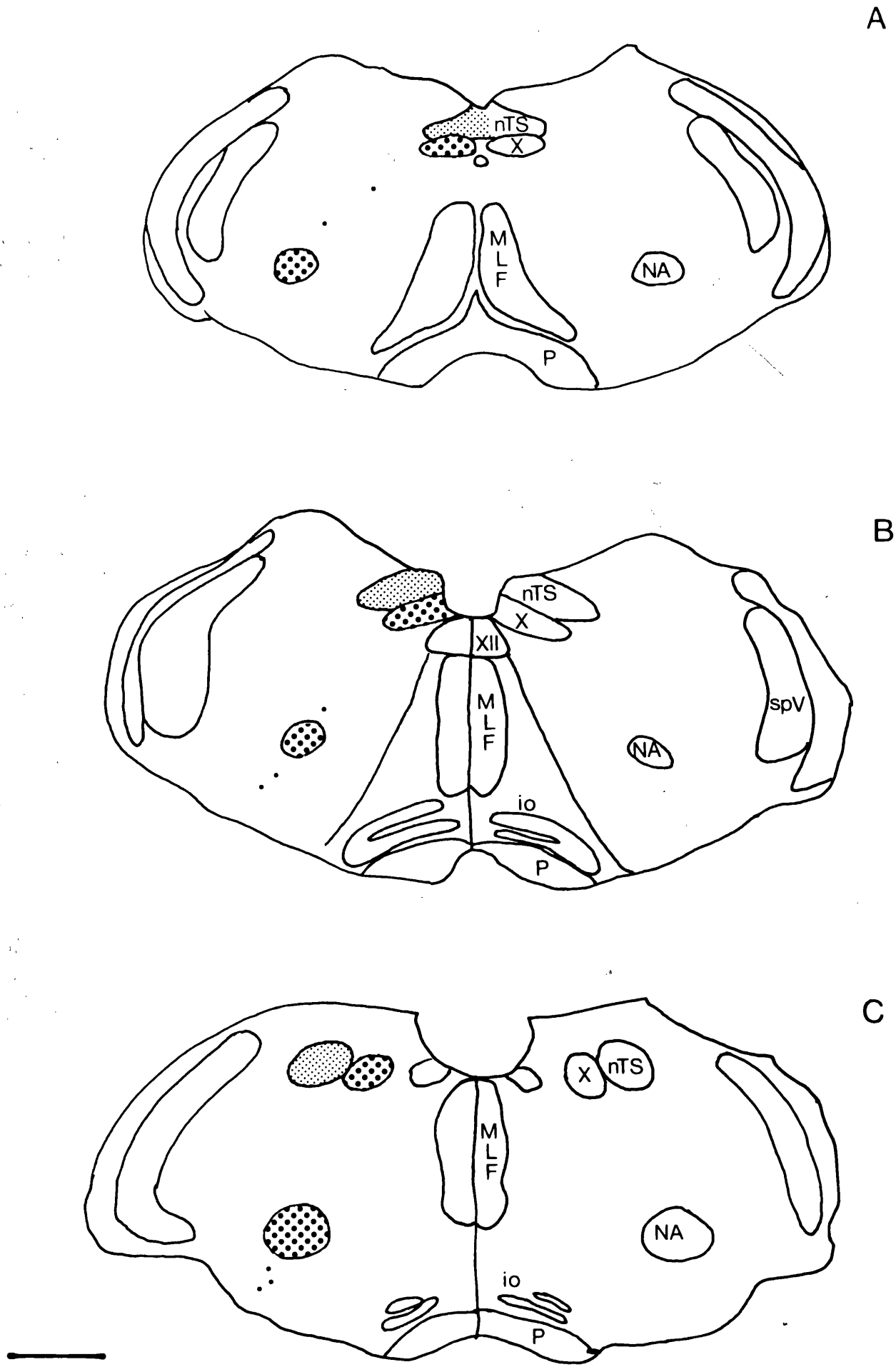
The afferent neurones of the vagus nerve are contained within the nodose (inferior) and jugular (superior) ganglia, the location and gross anatomy of which varies slightly between species. Relatively little is known regarding the central projections and functions of the jugular ganglia neurones. These neurones are associated classically with general somatic afferents and terminate mainly in the trigeminal brainstem nuclear complex (Foley & DuBois, 1934; Keller et al., 1987). However, the studies in this thesis are focused on vagal afferents whose parent cell bodies are situated in the nodose ganglia. These vagal sensory afferents terminate in the dorsomedial medulla, predominantly in the nucleus tractus solitarius (nTS) with a smaller projection to the area postrema (AP) and DVN. Before describing the organization of vagal afferent projections a brief description of the nTS and area postrema will be given.

The nTS forms a Y-shaped structure that extends from the spinomedullary junction to the level of the seventh cranial nerve in the rostral medulla. Due to its length

FIGURE 1:1.

Series of coronal sections through the rostrocaudal extent of the rat medulla illustrating the unilateral projection of vagal sensory afferents (small circles) and the location of vagal motoneurones (large circles). A) caudal to obex B) obex C) rostral to obex. Scale bar = 1mm.
(Adapted from Paxinos & Watson)

ABBREVIATIONS: io, inferior olives; MLF, medial longitudinal fasciculus; NA, nucleus ambiguus; nTS, nucleus of the tractus solitarius; P, pyramidal tract; SpV, spinal trigeminal complex; X, dorsal motonucleus of the vagus nerve; XII, hypoglossal motonucleus.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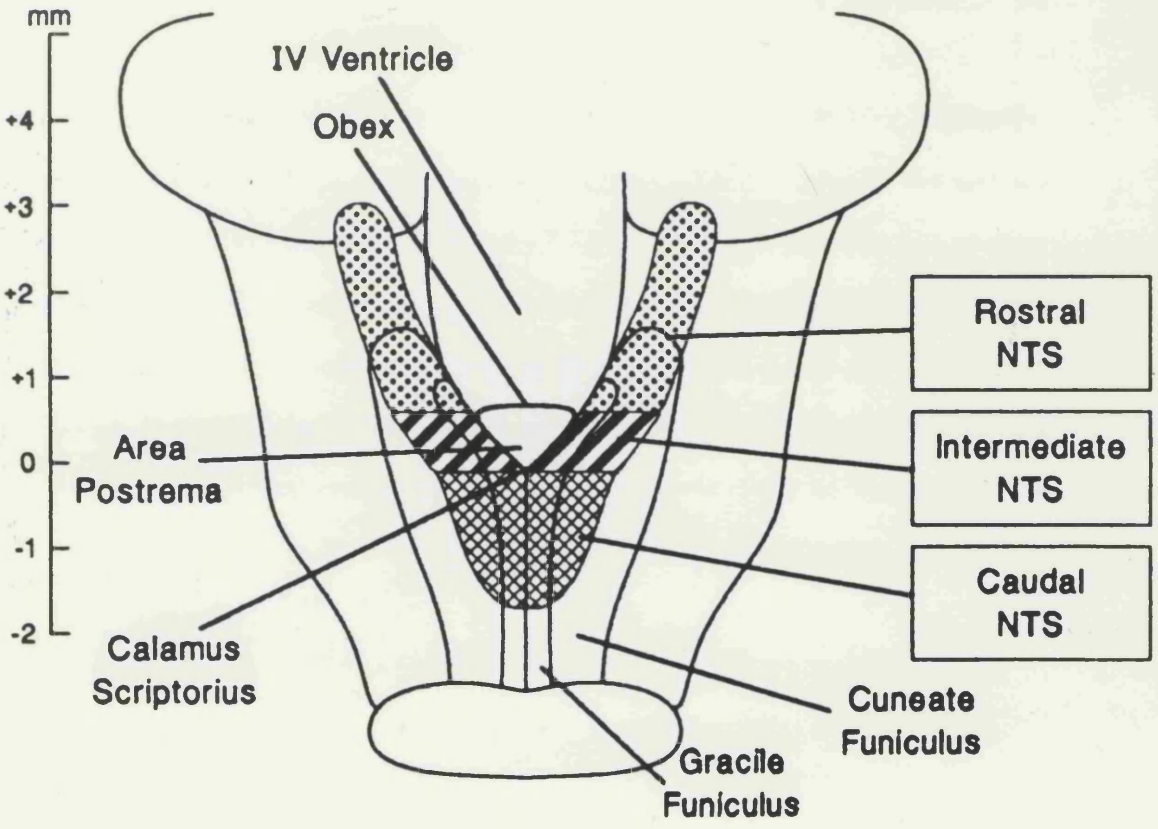
it is convenient to divide the nucleus into three zones, relative to their position to the area postrema e.g. rostral, intermediate and caudal (Fig. 1:2). The nTS has a complex organization and there have been numerous and conflicting systems for subdividing this area based on its anatomy and efferent organization (guinea pig- Allen, 1923, rat- Herbert et al., 1990a; Kalia & Sullivan, 1982; Torvik, 1956; cat- Brodal et al., 1956; Cajal, 1909; Cottle, 1964; Loewy & Burton, 1978; Taber, 1961; dog-Estes et al., 1989; rabbit- Meesen & Olszewski, 1949; hamster- Whitehead, 1988; human- Cajal, 1909; Hyde & Miselis, 1992; McRitchie & Tork, 1993). This confusion has arisen because the borders of the nTS subnuclei of laboratory animals are rather indistinct and vary between species and because studies have used different techniques for defining the area e.g. Nissl-stained sections, Golgi-stained sections, degeneration methods or patterns of connectivity. The most widely used nomenclature for the cat nTS is probably the system proposed by Kalia and Mesulam (1980a) based on a combination of the cytoarchitecture observed with Nissl-stained sections and the connectivity patterns as demonstrated by horseradish peroxidase (HRP) histochemistry. They divided the nTS into 9 subnuclei identified by their relative position to the tractus. A similar system was suggested by Kalia and Sullivan (1982) for the rat nTS and their nomenclature will be used in this thesis with the addition of the subnucleus gelatinosus (see Fig. 1:3; Rinaman et al., 1989).

The area postrema is a circumventricular organ and is situated at the caudal end of the fourth ventricle. In the rat and rabbit the area postrema is a unilateral structure whilst in other species e.g. cats, monkeys and man it is bilateral. The area postrema is highly vascular with fenestrated capillaries that lack tight junctions between the endothelial cells and therefore lies "outside" the blood-brain barrier (Leslie, 1986). Neurones of the area postrema and afferent neurones terminating within this structure are exposed to both the blood plasma and the cerebrospinal fluid.

FIGURE 1:2.

Diagramatic representation of the dorsomedial medulla illustrating the 3 zones of the rat nTS in relationship to the area postrema; caudal, intermediate and rostral.
(Reproduced from Loewy & Spyer, 1990).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IV Ventricle
Obex
Area
Postrema
Calamus
Scriptorius
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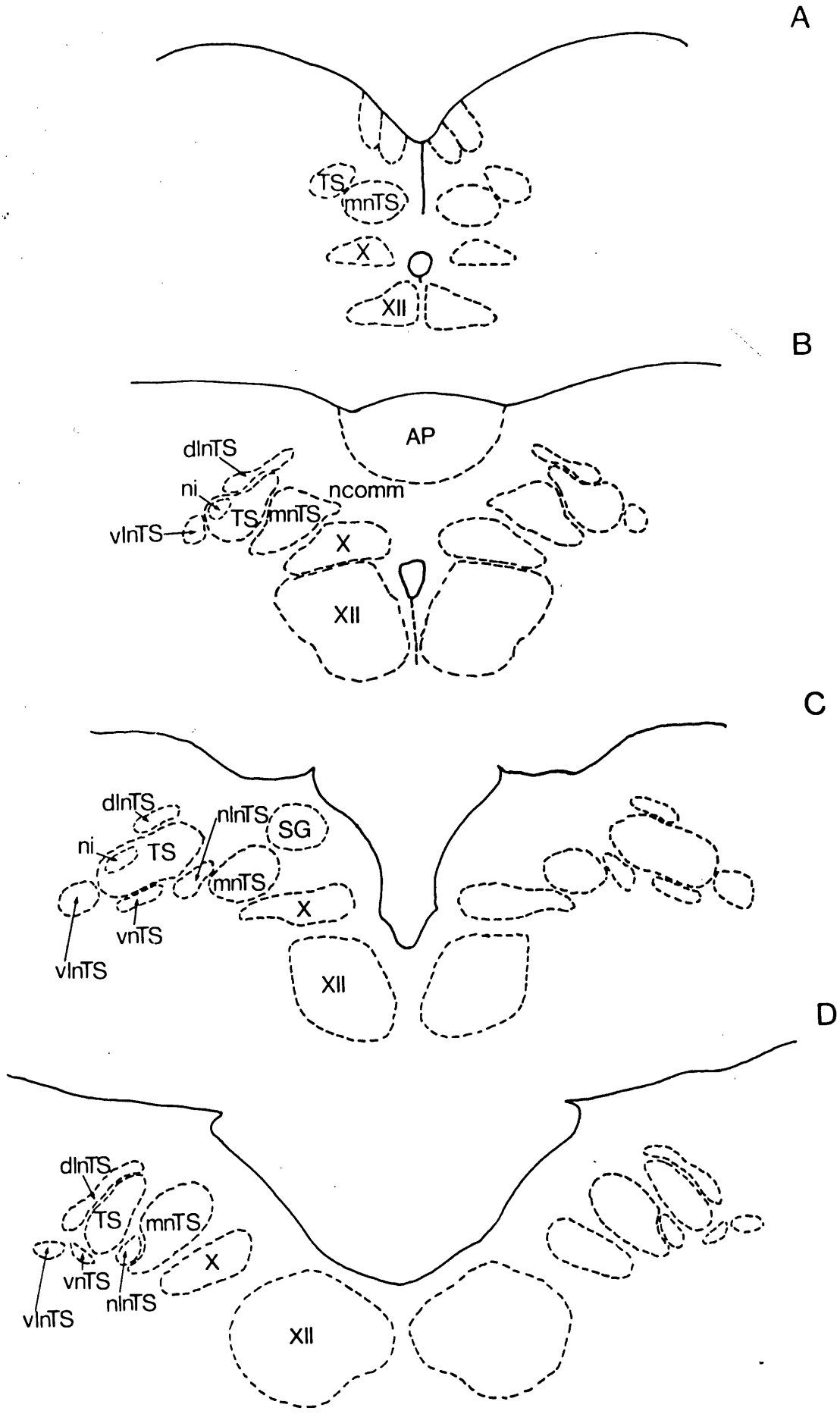
NTS
Intermediate
NTS
Caudal
NTS
Cuneate
Funiculus
Gracile
Funiculus

FIGURE 1:3.

Series of coronal sections though the dorsomedial medulla defining the subdivisions of the rat nTS (adapted from Kalia & Sullivan, 1982). Distance from obex; A) -0.7mm B) -0.3mm C) 0.1mm D) 1.0mm.

ABBREVIATIONS: AP, area postrema; dlnTS, dorsolateral subnucleus of the nucleus tractus solitarii; mnTS, medial subnucleus of the nucleus tractus solitarii; ncomm, commissural subnucleus; ninTS, interstitial subnucleus of the nucleus tractus solitarii; nInTS, intermediate subnucleus of the nucleus tractus solitarii; SG, subnucleus gelatinosus; TS, tractus solitarius; vnTS, ventral subnucleus of the nucleus tractus solitarii; vlnTS, ventrolateral subnucleus of the nucleus tractus solitarii; X, dorsal motonucleus of the vagus nerve; XII, hypoglossal motonucleus.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Cajal (1909) was one of the earliest neuroanatomists to study the central projections of the vagus nerve using the Golgi technique. Later investigators ultilized degeneration methods (Astrom, 1953; Cottle, 1964; DuBois, 1929; Foley & DuBois, 1934; Ingram & Dawkins, 1945; Schwartz et al., 1951; Torvik, 1956) to ascertain the central terminations of vagal afferents in a variety of species. However, these methods are relatively insensitive and often failed to reveal unmyelinated afferents that make up a large proportion of vagal afferents.

The development of sensitive neuroanatomical tracing methods using tritiated amino acids or HRP and its conjugates, combined with very sensitive histochemical reactions to reveal the peroxidase reactivity (Mesulam, 1978), confirmed and extended the information obtained from previous degeneration and electrophysiological experiments. Generally these new techniques demonstrated that the central terminations of vagal afferents had a more widespread distribution than had previously been thought. Neuronal tracing techniques have been used to trace the central projections of the vagus nerve in a variety of species including the cat (Kalia & Mesulam, 1980a; Nomura & Mizuno, 1982), rat (Conteras et al., 1982; Kalia & Sullivan, 1982), monkey (Beckstead & Norgren, 1979; Gwyn et al., 1985; Hamilton et al., 1987), dog (Chernicky et al., 1984), mouse (Funakoshi et al., 1989), golden hamster (Micelis & Malsbury, 1985), ferret (Ranson et al., 1993) and mink (Ranson et al., 1993).

Vagal afferent fibres enter the medulla laterally, either in multiple fascicles e.g. rat and cat (Conteras et al., 1982; Kalia & Mesulam, 1980a; Kalia & Sullivan, 1982; Nomura & Mizuno, 1983) or in 1 or 2 bundles e.g. monkey and dog (Beckstead & Norgren, 1979; Chernicky et al., 1984; Gwyn et al., 1985) and pass dorsomedially towards the tract. In some species the afferent and efferent fibres enter the medulla in the same fibre bundles e.g. cat whilst in the dog and rat the afferent and efferent fibres enter separately. Some axons terminate at this level whilst the majority travel in a

caudal direction and give off axon collaterals at various levels of the nTS. Vagal afferents project to all nTS subnuclei throughout its rostrocaudal extent with the heaviest labelling just rostral to obex. Fibres cross the midline in the commissural subnucleus (ncomm.) and extend rostral for several millimeters in the contralateral nTS. There is also a smaller projection of vagal afferents to the area postrema and ipsi- and contralateral DVN. Although the general pattern of vagal innervation of the nTS is similar for most species there are some differences regarding the density of innervation of individual subnuclei. For example, in the monkey there is a paucity of vagal afferent labelling dorsal and dorsolateral to the tractus solitarius (TS; Beckstead & Norgren, 1979; Gwyn et al., 1985).

1:4 VISCEROTOPIC ORGANIZATION OF VAGAL NERVE AFFERENTS.

A major advantage of HRP and its conjugates is that it can be transported transganglionically allowing the central projection of sensory vagal afferents from various branches of the vagus and individual viscera to be mapped. However, neuronal tracers applied to cut nerves or end organs label sensory afferent fibres indiscriminately and only neurophysiological techniques are able to elucidate the central projection of individual, functionally identified afferent fibres (Berger & Averill; 1983; Davies & Kubin, 1986; Donoghue et al., 1982, 1984; Kalia & Richter, 1985a, 1988a; Kubin et al., 1991).

Neuronal tracing techniques have indicated that the central projection of nodose ganglia neurones may be organized in two ways. The first involves an organ-specific projection to individual nTS subnuclei. For example gastrointestinal afferents project to the subnucleus gelatinosus whilst pulmonary receptors terminate in ventral and

ventrolateral subnuclei. The second involves an overlapping projection of vagal afferents innervating most end organs to the commissural subnucleus and its rostral counterpart, the medial subnucleus.

1:4:1 CARDIOVASCULAR AFFERENTS.

The vagus nerve plays a major role in the control of cardiovascular activity. Therefore, the central projections of sensory vagal afferents that innervate the great vessels and run in the aortic depressor nerve (ADN) have been intensively studied in a variety of species using both neuroanatomical and electrophysiological techniques. Although there are some discrepancies between the neuroanatomical studies e.g. whether the DVN or area postrema also receive an afferent projection, most studies agree that the major projection of the ADN is to the medial, commissural and dorsolateral subnuclei, or the equivalent areas in pigeon and rabbit, in the caudal two-thirds of the nTS (cat- Ciriello, 1983; Ciriello et al., 1981; Ciriello & Calaresu, 1981; Kalia & Welles, 1980; rat- Garcia et al., 1979; Kumada & Nakajima, 1972; Wallech & Loewy, 1980; pigeon- Katz & Karten, 1979). Earlier electrophysiological studies had suggested a wider field of termination that extended outside the nTS but no tracing studies have detected these projections and possible reasons for this discrepancy are that fibres of passage were activated or recordings were of polysynaptically evoked potentials (Crill & Reis, 1968; Donoghue et al., 1977; Jordan & Spyer, 1978).

Some progress has also been made in elucidating the central projection of specific types of aortic baroreceptors, namely the myelinated fibres, using antidromic mapping techniques (Donoghue et al., 1982). This technique involves recording from single afferents in the nodose ganglion, identified by their spontaneous discharge and determining their brainstem projection using medullary stimulation. The shape of the depth-threshold contour allows regions of branching and the presumed terminals of

individual afferents to be plotted. However, this technique has relatively low resolution and does not allow the projection of fine axons to be discriminated. Donoghue and co-workers (1982) reported that in rabbits myelinated aortic baroreceptors have branches mainly in the lateral and caudal regions and more rarely in medial regions. Results were slightly different in the cat as the identified afferents had branches in either the lateral or caudal region but not usually in both.

Sensory vagal afferents innervating the heart terminate in similar areas to the aortic depressor nerve afferents (Kalia & Mesulam, 1980b). Other cardiovascular afferents travel in the carotid sinus nerve, a branch of the glossopharyngeal nerve. Interestingly, these afferents also project to the "cardiovascular area" although their terminal field is rather more extensive than that of the aortic depressor nerve afferents (Berger, 1979; Ciriello et al., 1981; Panneton & Loewy, 1980). Antidromic mapping techniques revealed that both myelinated and unmyelinated baroreceptors running in the carotid sinus nerve have a similar nTS projection to aortic baroreceptors (Donoghue et al., 1984) but differed markedly to the chemoreceptor projection. Recent neuroanatomical studies in the cat have reported the existence of a novel pathway from the carotid sinus projecting to the nTS via the vagus nerve (Torrealba & Claps, 1988b), although there is no evidence that this pathway exists in other species.

1:4:2 RESPIRATORY TRACT AFFERENTS.

Vagal afferents from receptors located in the larynx, trachea and bronchi project predominantly to the interstitial subnucleus (ninTS) with the medial, ventrolateral and commissural subnuclei all receiving a weaker innervation (Altschuler et al., 1989; Kalia & Mesualm, 1980b; Patrickson et al., 1991; Sweazey & Bradley, 1986). The major projection of vagal afferents innervating the lungs is to ventrolateral and dorsolateral regions of the nTS with a smaller projection to ventral, medial and commissural

subnuclei (Kalia & Mesulam, 1980b).

There are three main groups of receptors distributed throughout the tracheobronchial tree and lungs; the slowly adapting stretch receptors (SARs), the rapidly adapting "irritant" stretch receptors (RARs) and the bronchopulmonary C-fibre receptors. Pulmonary rapidly and slowly adapting stretch receptors are of particular interest due to their potent effects on cardiovascular and respiratory systems. The projection of individual stretch receptors have been investigated with antidromic mapping techniques (Davies & Kubin, 1986; Donoghue et al., 1982), spike-triggered averaging (Berger & Averill, 1983) and intra-axonal labelling with the neuronal tracer HRP after physiological identification (Kalia & Richter, 1985a, 1988a). Although there are a few discrepancies between studies in determining their exact terminal fields the major projection of SARs is to medial regions with a smaller innervation of ventral and ventrolateral areas (Berger & Averill, 1983; Donoghue et al., 1982; Kalia & Richter, 1985a) whilst medial, dorsal and lateral regions receive the major RAR projection (Davies & Kubin, 1986; Kalia & Richter, 1988a). Antidromic mapping techniques have also been used to examine the central projections of bronchial and pulmonary C-fibre receptors (Kubin et al., 1991). Caudal to obex the most dense branching was detected in the commissural subnucleus whilst rostral to obex there were projections to the medial nTS, the area postrema and around the border of the parvocellular nucleus. This distribution was observed for both C-fibre receptors innervating the bronchus and the lungs.

Further studies have used intra-axonal injection of HRP into individual fibres to visualize the central projection of single afferents running in the superior laryngeal nerve (SLN; Bellingham & Lipski, 1992). Labelled afferent fibres were reported to arborize mainly in ventral and ventrolateral regions of the nTS with a smaller projection to dorsal areas.

1:4:3 GASTROINTESTINAL AFFERENTS.

The vagal afferent projection from individual organs that make up the gastrointestinal tract have also been examined anatomically although electrophysiological data mapping the precise projection of functionally identified fibres is lacking. Gastrointestinal tract vagal afferents appear to be more discretely organized than other vagal afferents and project to distinct nTS subnuclei with only minimal overlap between the terminal fields of individual gastrointestinal tract viscera, particulary at the level of the area postrema. The major projections are: pharynx- interstitial subnuclei, oesophagus- medial subnuclei, stomach- subnucleus gelatinosus (SG) and caecum- commissural subnucleus (Altschuler et al., 1989, 1991; Gwyn et al., 1985; Hamilton & Norgren, 1984; Kalia & Mesulam, 1980b; Leslie et al., 1982b; Norgren & Smith, 1988; Rogers & Herman, 1983; Scharoun et al., 1984; Shapiro & Miselis, 1985, Zhang et al., 1992).

Other viscera associated with gastrointestinal function also have a vagal innervation. The vagal innervation of the rat pancreas appears to be very small as no afferent terminals were detected in the dorsomedial medulla after injection of neuronal tracer into this organ although a few cells were detected in the nodose ganglia (Rinaman & Miselis, 1987). The hepatic branch of the rat vagus has a dense projection to the subnucleus gelatinosus, particulary the left side, with a smaller projection to the medial nTS and area postrema. At the intermediate level of the nTS the medial portion of the commissural subnucleus receives a dense innervation (Norgren & Smith, 1988, Rogers & Herman, 1983).

1:5 VISCEROTOPIC ORGANIZATION OF SENSORY NEURONES IN THE NODOSE GANGLIA.

The perikarya of the nodose ganglia are organized in a loose topographical manner. Neurones innervating abdominal visceral structures e.g. stomach and pancreas tend to be situated in the caudal and mid regions whilst thoracic structures i.e. oesophagus and ADN are innervated by mid and rostral areas of the ganglion (Altschuler et al., 1989, 1991; Donoghue et al., 1982; Garcia et al., 1979; Green & Dockray, 1987; Hopkins & Armour, 1989; Kalia & Mesulam, 1980b; Kalia & Welles, 1980; Norgren & Smith, 1988; Portalier & Vigier, 1979; Sharkey et al., 1984). Perikarya innervating the heart are distributed throughout the ganglia in the dog (Hopkins & Armour, 1989) and cat (Kalia & Mesulam, 1980b).

1:6 MORPHOLOGY OF VAGAL AFFERENTS.

Neuroanatomical tracing and degeneration techniques have been used to examine the morphology of vagal afferents at the ultrastructural level. Only a few areas of the nTS i.e. commissural subnucleus (Chiba & Doba, 1976), subnucleus gelatinosus (Leslie et al., 1982; Rinaman et al., 1989) and interstitial subnucleus (Chazal et al., 1991) have been examined. Vagal afferents predominantly form synaptic contacts with small to medium calibre dendrites and rarely with soma (Gywn et al., 1982; Kalia & Ritchter, 1985b, 1988b; Leslie et al., 1982; Sumal et al., 1983). Terminals contain round, clear vesicles and occasional dense core vesicles. Neuronal labelling of individual afferent fibres has demonstrated that single afferent fibres arborize over considerable distances rostrocaudally in the nTS and terminate as hundreds of bouton terminals (Bellingham & Lipski, 1992; Kalia & Richter, 1985b, 1988b).

1:7 PUTATIVE NEUROTRANSMITTERS OF THE VAGAL AFFERENT SYSTEM.

Evidence is available supporting the presence of a wide variety of putative neurotransmitters and neuromodulators in the vagal afferent system. Generally these studies have concentrated on examining the neurochemical content of sensory cell bodies in the nodose ganglia.

1:7:1 SUBSTANCE P AND CALCITONIN GENE-RELATED PEPTIDE.

Both SP and CGRP have been demonstrated in the sensory neurones of the nodose ganglia and peripheral vagus nerve using radioimmunoassay, immunocytochemistry and *in situ* hybridization techniques (Czyzyk-Krzeska et al., 1991; Funakoshi et al., 1989; Gamse et al., 1979; Helke & Hill, 1988; Katz & Karten, 1980; Ling et al., 1992; Lundberg et al., 1978; Torrealba, 1992). As in other sensory afferent systems (Gibbins et al, 1985; Helke & Niederer, 1990; Lee et al., 1985; Lundberg et al., 1985; Wiesenfeld-Hallin et al., 1984) CGRP and SP are extensively co-localized in nodose ganglia perikarya (Helke & Niederer, 1990; Ju et al., 1987; Lundberg et al., 1985). Using double-labelling immunocytochemical techniques the majority of SP-immunoreactive cells were found to contain CGRP immunoreactivity with a separate population of cells that contain CGRP immunoreactivity alone. In the rat SP and CGRP-containing cells are situated mainly towards the rostral pole of the ganglion and to a lesser extent in the mid-region but in other species e.g. monkey SP-positive cells are scattered throughout the ganglion (Ling et al., 1992). Both immunocytochemical and *in situ* hybridization techniques have noted variability in the intensity of labelling but whether this reflects the activity of the cell is not known. Immunoreactive fibres have also been observed in the nodose ganglion in colchicine-

treated rats and were most heavily labelled near or caudal to the entrance of the superior laryngeal nerve (SLN; Helke & Hill, 1988). In pigeon, rabbit and monkey nodose ganglia SP immunoreactivity has been observed in the pericellular fibre plexi that encircle individual SP-negative neurones but examination of the material at the ultrastructural level has failed to reveal any synaptic contacts with ganglion cells (Katz & Karten, 1980; Ling et al., 1992). The function of these pericellular arborizations is unclear but Katz and Karten (1980) have suggested that they may modulate nodose ganglia perikarya that are not SP-immunoreactive.

CGRP and SP immunoreactivity has been detected in peripheral vagal sensory endings, predominantly in the airways (Cadieux et al., 1986; Dey et al., 1990) and oesophagus (Green & Dockray, 1987; Lundberg et al., 1983) and to a lesser extent in the heart (Urban & Papka, 1985), stomach, pancreas and guinea pig pylorus (Chery-Croze et al., 1988; Green & Dockray, 1988; Lindh et al., 1983; Su et al., 1987).

1:7:2 OTHER NEUROPEPTIDES.

A variety of studies have provided evidence that support the presence of a variety of other neuropeptides in the vagal afferent system including galanin, neuropeptide Y, neurokinin A (NKA), vasoactive intestinal peptide, gastrin, somatostatin and choleocystokinin (CCK; Calingasan & Ritter, 1992; Czyzyk-Krzeska et al., 1991; Helke & Hill, 1988; Lundberg et al., 1978; Mantyh & Hunt, 1984). With the possible exception of NKA and galanin these other neuropeptides appear to be present in fewer nodose ganglia perikarya than SP or CGRP. Although detected immunocytochemically CCK was not revealed in nodose ganglion perikarya using mRNA probes (Czyzyk-Krzeska et al., 1991) and it is possible that the antibodies to CCK used in the immunocytochemical studies were cross-reacting with CGRP (Hokfelt et al., 1988; Ju et al., 1986).

1:7:3 5-HYDROXYTRYPAMINE.

5-hydroxytryptamine (5-HT) has been demonstrated in the perikarya of both the cat and the rat nodose ganglia using biochemical (Gaudin-Chazal et al., 1978), [^{3}H]5-HT uptake (Gaudin-Chazal et al, 1981,1983) and immunocytochemical techniques following the treatment with a monoamine oxidase inhibitor (Gaudin-Chazal et al., 1982; Nosjean et al., 1990). Other approaches have involved injection of [^{3}H]5-HT into the nTS combined with autoradiography to detect labelled cells in the nodose ganglia (Gaudin-Chazal et al., 1982). However, it should be noted that the use of radiolabelled 5-HT is based on the assumption that this compound is taken up exclusively by cell bodies and terminals in which they are normally utilized. With this method at least 3% of cells are reported to be heavily labelled in the cat (about 300 per ganglion) with a similar percentage in the rat (Gaudin-Chazal et al., 1982; Nosjean et al., 1990). The degree of perikaryal labelling varied, within the same preparation, when either immunocytochemical or [^{3}H]5-HT uptake techniques were used but the reason for this phenomenon is unclear. It is possible that it reflects the different functional origins of the labelled neurones or alternatively may reflect a difference in the uptake capability of the neuronal membrane. Labelled cells were scattered throughout the ganglia and the majority were either medium-sized (Gaudin-Chazal et al., 1982a; Nosjean et al., 1990).

1:5:4 EXCITATORY AMINO ACIDS.

Biochemical, pharmacological and transmitter specific retrograde tracing studies (see chapter 3 for refs.) have investigated the possibility that the excitatory amino acid (EAA), glutamate, is a neurotransmitter of sensory vagal afferents. However, despite a large body of literature this question has not been answered conclusively due to the indirect nature of the techniques used.

1:7:5 ACETYLCHOLINE.

Whether acetylcholine is present in the vagal afferent system was a matter of controversy for a number of years. Studies on the cholinergic system in the dorsal medulla following nodose ganglionectomy yielded conflicting results (Helke et al., 1983; Simon et al., 1985). Biochemical techniques e.g. measurement of choline acetyltransferase (ChAT) activity, choline uptake and endogenous acetylcholine had indicated the presence of cholinergic neurones but it is possible that there was contamination by cholinergic efferent fibres (Falempin et al., 1989; Palouzier, et al., 1987; Palouzier-Paulignan et al., 1991; Ternaux et al., 1989). Some further evidence comes from studies that have observed a functional reinnervation of the superficial somatic muscle by some vagal afferent fibres following cross-anastomosis with the possible formation of cholinergic synapses (Coget & Rousseau, 1983; Falempin et al., 1989; Falempin & Rousseau, 1983). The immunocytochemical detection of ChAT has provided confirmation of a population of cholinergic neurones in the rat and rabbit nodose ganglia (Palouzier et al., 1987; Ternaux et al., 1989).

1:7:6 CATECHOLAMINES.

Formaldehyde-induced catecholamine fluorescence and immunoreactivity for the catecholamine biosynthetic enzyme tyrosine hydroxylase has been detected in perikarya located throughout the nodose ganglia of the rat (Helke & Niederer, 1990; Katz et al., 1983). The absence of dopamine B-hydroxylase and phenylethanolamine N-methyltransferase suggests that these neurones synthesize dopamine but not adrenaline or noradrenaline.

1:7:7 NITRIC OXIDE.

Recent studies have provided evidence that sensory neurones in the rat nodose ganglia synthesize nitric oxide as demonstrated by the presence of nicotinamide adenine dinucleotide phosphate diaphorase activity (Aimi et al., 1991). This enzyme was detected in almost a third of nodose ganglia perikarya. Double-labelling studies further showed that the majority of nitric oxide synthesizing neurones also contained CGRP whilst a smaller proportion also contained SP (Aimi et al., 1991).

1:8 POST-SYNAPTIC TARGETS OF VAGAL AFFERENTS.

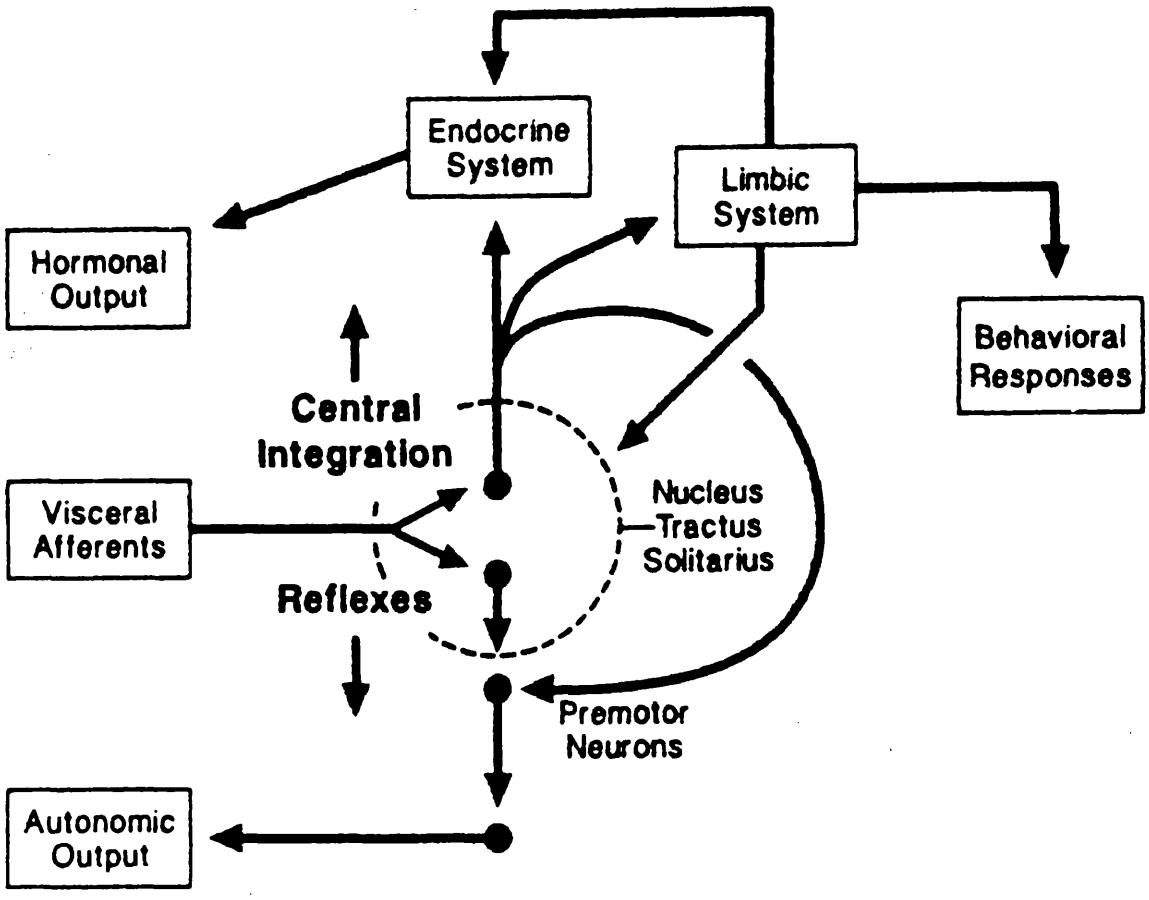
Vagal afferents make synaptic contacts with neurones (denoted as 2nd order neurones) in the dorsomedial medulla and by direct and indirect pathways modulate sympathetic and parasympathetic outflow (see Fig. 1:4). Recent developments in neuroanatomical methodology have resulted in a greater understanding of the neurochemistry of these neurones, the origin and variety of their synaptic inputs and their afferent projections.

1:8:1 NEUROCHEMISTRY OF 2ND ORDER NTS NEURONES.

The nTS contains neurones immunoreactive for a wide variety of neuropeptides, amino acids, monoamines and classical neurotransmitters (for review see van Giersbergen et al., 1992). However, as yet only limited information is available regarding the neurochemistry of neurones receiving sensory vagal input. Sumal and co-workers (1983) demonstrated synaptic contacts between some vagal afferents and catecholaminergic neurones in the rat medial nTS. There are also suggestions that gamma aminobutyric acid (GABA ; Maqbool et al., 1991), SP (Kawai

FIGURE 1:4.

Diagram illustrating how primary afferent information transmitted in the cranial nerves is processed either for reflex responses or as part of a central autonomic circuit that can affect autonomic, hormonal and behavioural activities.
(Reproduced from Loewy & Spyer, 1990).


Endocrine System
Limbic System
Hormonal Output
Behavioral Responses
Central Integration
Visceral Afferents
Nucleus Tractus Solitarius
Reflexes
Premotor Neurons
Autonomic Output

et al., 1989) and met-enkephalin-immunoreactive (Velley et al., 1991) neurones receive synaptic input from vagal afferents.

1:8:2 VAGO-VAGAL PATHWAYS.

Neuroanatomical studies have provided evidence that a small proportion of vagal afferents make synaptic contact with VPNs in the rat DVN (Neuheuber & Sandoz, 1986; Rinaman et al., 1989). The physiological function/s of this short monosynaptic pathway is still unknown although recently evidence has been provided that this pathway may be involved in the regulation of gastric motility and secretion as numerous synaptic contacts were observed between vagal afferents and VPNs innervating the stomach in the rat subnucleus gelatinosus (Rinaman et al., 1989).

1:9 OTHER AFFERENT PROJECTIONS TO THE DORSOMEDIAL MEDULLA.

The dorsomedial medulla receives a wide range of peripheral and central inputs some or all of which may converge on the same neurones as those receiving sensory vagal input (Ciriello et al., 1981; Mifflin et al., 1988b). In general, sensory afferents of the VII, IX and X cranial nerves terminate in the nTS in a roughly topographical fashion with the rostral one-third of the nucleus receiving the majority of afferents concerned with gustation and the remaining two-thirds receiving afferents mainly involved with cardiovascular, respiration and general visceral function (see Loewy, 1990). In addition, the nTS receives afferent projections from the trigeminal nuclei and spinal cord (see Table 1. for refs.). As these projections originate from areas that receive somatic and visceral input it has been postulated that the nTS acts to relay somatic and visceral information from the whole body (Menetrey & Basbaum, 1987).

TABLE 1. OTHER PROJECTIONS TO THE NTS.

AUTHORS	SPECIES	ORIGIN
Altschuler et al. (1989)	rat	IX
Bandler & Tork (1987)	cat	PAG
Berger (1979)	cat	CSN
Ciriello et al. (1981)	cat	CSN
Claps & Torrealba (1988)	cat	CB
Conteras et al. (1982)	rat	V, VII, IX
Cornwall et al. (1990)	rat	LDTg
Davies & Kalia (1981)	cat	CSN
Finley & Katz (1992)	rat	CB
Fulweiler & Saper (1984)	rat	PB, K.F
Grelot et al. (1989)	cat	PH-IX
Hamilton & Norgren (1984)	rat	CT, GPN, LT-IX
Holstege (1987)	cat	AH, LHA, PVN
Hosoya & Sugiura (1984)	rat	GPN
Housley et al. (1987)	rat	IX, CSN
Hurley et al. (1991)	rat	ILC
Jacquin et al. (1982)	rat	V
Marfurt & Rajchert (1991)	rat	V
Menetrey & Basbaum (1987)	rat	TNC, spinal cord
Morest (1967)	cat	AP
Nomura & Mizuno (1982)	cat	CT
Onai et al. (1987)	cat	PFC, BNST, ACE, PVN
Panneton & Loewy (1980)	cat	CSN
Pfaller & Arvidsson (1988)	rat	V
Roder & Ciriello (1992)	cat	CVLM

Schaffer et al. (1988)	rat	NRM, NRD, NRP, PGi
Shapiro & Miselis (1985)	rat	AP
Sieders & Stuesse (1984)	rat	CSN
Thor & Helke (1987)	cat	NRM, NRO, NRP
Thor & Helke (1988)	cat	A1, A2, A5, C1, C2, C3, LC
Torrealba & Claps (1988)	cat	CSN
van der Kooy et al. (1984)	rat	PFC, BSNT, ACE, HYP
Yasui et al. (1991)	cat	OrG, ILC

Abbreviations: ACE, central nucleus of the amygdala; AH, anterior hypothalamus; AP, area postrema; BSNT, bed nucleus of the stria terminalis; CB, carotid body; CSN, carotid sinus nerve; CT, chorda tympani; CVLM, caudal ventrolateral medulla; GPN, greater petrosal nerve; HYP, hypothalamus; ILC, infralimbic cortex; IX, glossopharyngeal nerve; LC, locus coeruleus; LDTg, laterodorsal tegmental nucleus; LHA, lateral hypothalamic area; LT-IX, lingual-tonsilar branch of IX nerve; NA, nucleus ambiguus; NRD, nucleus raphe dorsalis; NRM, nucleus raphe magnus; NRO, nucleus raphe obscurus; NRP, nucleus raphe pallidus; OrG, orbital gyrus; PAG, periaqueductal grey; PB, parabrachial nucleus; PBL, lateral parabrachial nucleus; PFC, prefrontal cortex; PGi, paragigantocellular nucleus; PH-IX, pharyngeal branch of the IX nerve; PVN, paraventricular nucleus; TNC, caudal trigeminal nucleus; V, trigeminal nerve; VII, facial nerve;

Various areas of the CNS involved in the control of autonomic function also project to the nTS and DVN. These include the ventrolateral medulla and pons, medullary raphe nuclei and parabrachial nuclei and forebrain projections from the prefrontal cortex, bed nucleus of stria terminalis, amygdala and lateral anterior and arcuate areas of the hypothalamus. Interestingly, these forebrain projections may have some topographical organization as the prefrontal cortex innervates dorsal regions of the nTS whilst in contrast subcortical projections innervate more ventral regions (Yasui et al., 1991).

As well as receiving primary afferent input from the vagus nerve the area postrema also receives a small projection from the glossopharyngeal (Ciriello et al., 1981), carotid sinus (Davies & Kalia, 1981; Panneton & Loewy, 1980) and trigeminal nerves (Jacquin et al., 1982). The major central input is from the paraventricular and dorsomedial hypothalamic nuclei (Shapiro & Miselis, 1985; van der Kooy & Koda, 1983) with a smaller projection from the parabrachial nucleus (Morest, 1967; Shapiro & Miselis, 1985). There is also possibly a small projection from the caudal nTS (Shapiro & Miselis, 1985) but this projection is hard to distinguish due to the close proximity of the two areas.

1:10 EFFERENT PATHWAYS OF THE DORSOMEDIAL MEDULLA.

Many of the CNS areas that send afferent projections to the nTS are reciprocally connected and receive direct neuronal inputs from this nuclei possibly providing a feedback function. Information carried in the vagus nerve is transmitted to autonomic motoneurons in the intermediolateral cell column, nucleus ambiguus and DVN in direct pathways that project from the nTS or indirectly in polysynaptic pathways via areas that

include A5 noradrenergic cell group, caudal raphe nucleus, rostral ventrolateral medulla, the paraventricular and lateral hypothalamic areas. There are also long, direct projections to forebrain areas, primarily the central nucleus of the amygdala, subfornical organ, the bed nucleus of the stria terminalis, insular cortex and a number of nuclei in the hypothalamus (see Table 2 for refs.).

The nTS has extensive connections with the parabrachial nucleus, a region that plays a major role in transmitting information to forebrain areas involved in autonomic regulation e.g. the amygdala, the bed nucleus of the stria terminalis and a number of hypothalamic nuclei. The use of discrete injections of neuronal tracer has provided evidence that the projection from the nTS to the parabrachial nucleus has a viscerotopic organization. For example, the rostral nTS that receives gustatory input projects to the medial parabrachial area whilst the commissural subnucleus that receives general visceral information project to the lateral parabrachial area. In turn, these regions project to higher areas of the brain in a topographical fashion (Herbert et al., 1990b).

Neurones of the area postrema project to similar areas as nTS neurones. The heaviest projection is to the parabrachial nucleus with smaller projections to the nTS and A1 cell group (Herbert et al., 1990a; King, 1980; Loewy & Burton, 1978; Mehler, 1983, Shapiro & Miselis, 1985; van der Kooy & Koda, 1983). Other projections to the tegmental nucleus and the ventrolateral medulla have also been reported (Shapiro & Miselis, 1985, van der Kooy & Koda, 1983).

TABLE 2. EFFERENT PROJECTIONS FROM THE NTS.

AUTHORS	SPECIES	PROJECTIONS
Bandler & Tork (1987)	cat	PAG
Beckman & Whitehead (1991)	hamster	nXII, nV, nVII, PRF
Beckstead et al. (1980)	monkey	NA, PBL, PBM, VPMpc
Berk et al. (1993)	pigeon	PB
Cornwall et al. (1990)	rat	LDTg
Cunningham & Sawchenko (1989)	rat	NA
Herbert et al.,(1990)	rat	K.F, PB
Herbert & Saper (1990)	rat	PB
Kapp et al. (1989)	rabbit	ACE, BSNT, PBL, PBM, K.F
King (1980)	cat	PBL, K.F
Loewy & Burton (1978)	cat	PB, K.F., PAG, NA, A1, CL, spinal cord
Morilak et al. (1989)	rabbit	RVLM
Norgren, (1978)	rat	nXII, nVII, TNC, PBN
Otake et al. (1992)	cat	PBL, PBM, K.F, A5, RTN,RFN,NA,LC,DVN
Ricardo & Koh (1978)	rat	VLM, PB, HYP, ACE
Riche et al. (1990)	rat	PB, HYP, BSNT, ACE
Ross et al. (1985)	rat	RVLM, CVLM, NRA
ter Horst et al., (1989)	rat	HYP
Whitehead (1990)	hamster	PB
Zardetto-Smith & Gray (1990)	rat	ACE

Abbreviations: ACE, central nucleus of the amygdala; BSNT, bed nucleus of the stria terminalis; CL, lateral cuneate nucleus; CVLM, caudal ventrolateral medulla; DVN, dorsal motonucleus of the vagus nerve; HYP, hypothalamus; K.F, Kolliker-Fuse; LC, locus coeruleus; LDTg, laterodorsal tegmental nucleus; NA, nucleus ambiguus; NRA, nucleus retroambiguus; nV, trigeminal motonucleus; nVII, facial motonucleus, nXII, hypoglossal motonucleus; PAG, periaqueductal grey; PB, parabrachial nucleus; PBL, lateral parabrachial nucleus; PBM, medial parabrachial nucleus; PFC, prefrontal cortex; PGi, paragigantocellular nucleus; PR, paramedian reticular formation; PRF, parvicellular reticular formation; RFN, retrofacial nucleus; RTN, retrotrapezoid nucleus; RVLM, rostral ventrolateral medulla; TNC, caudal trigeminal nucleus; VPMpc, ventral posteromedial thalamic nucleus

CHAPTER 2

A morphological analysis of vagal afferents in the nTS.

CONTENTS

2:1 INTRODUCTION.

Since the development of techniques utilising sensitive neuronal tracers there has been a multitude of studies that have ascertained the heterogeneous distribution of vagal sensory afferent terminations of the whole vagus nerve to the various subnuclei of the nTS, area postrema and DVN in a number of species. Further studies have investigated the central projection of vagal afferent fibres innervating individual viscera in a variety of species (see General Introduction for refs.). Despite this intense interest in the central distribution of vagal sensory afferents information of their synaptic organization is limited. Most ultrastructural descriptions have been relatively brief (Gywn et al., 1982) have concentrated on one nTS subdivision e.g. the cat interstitial subnucleus (Chazal et al., 1991), substantia gelatinosa (Leslie et al., 1982) and rat commissural subnucleus (Chiba and Doba, 1976). Other studies have been slanted towards the examintion of one neurochemical class of post-synaptic target (Maqbool et al., 1991; Sumal et al., 1983).

Electrophysiological studies have indicated that certain classes of vagal afferents e.g. pulmonary slowly adapting receptors and superior laryngeal nerve afferents can be modulated presynaptically by other vagal afferents (Barillot, 1970; Jordan & Spyer, 1979; Richter et al., 1986; Rudomin, 1967) although other classes of vagal afferents e.g. aortic depressor nerve afferents and myelinated cardiac afferents do not appear to exhobit presynaptic inhibition (Jordan & Spyer, 1979: Richter et al., 1986). The presumed anatomical substrate for presynaptic inhibition are axo-axonic synaptic contacts (Gray, 1962) but neuroanatomical evidence for the presence of axo-axonic synaptic contacts between vagal afferents and other vagal and non-vagal afferents is

equivocal (Chiba & Doba, 1976; Kalia & Richter, 1985b, 1988b; Sumal et al., 1983).

In vivo intracellular studies of nTS neurones have reported that stimulation of either the vagus or carotid sinus nerve can evoke excitatory post-synaptic potentials (EPSPs), inhibitory post-synaptic potentials (IPSPs) or EPSP/IPSP sequences with the IPSP usually having a longer latency (Felder & Mifflin, 1988; Mifflin et al., 1988a,b). The same sequences have also been observed using *in vitro* brain slices with stimulation of the tractus (Brooks et al., 1992). A difficulty with electrophysiological studies is that it is not always possible to distinguish between neurones which process primary afferent input (i.e 2nd order neurones) and higher order neurones. However, as no evidence been presented, so far, to suggest that primary afferent input provides inhibitory input it is assumed that the IPSP sequence represents activation of an inhibitory neurone.

The present study was undertaken to provide a comprehensive description of the ultrastructure of vagal afferents, their synaptic organization and the identity of their post-synaptic targets throughout the various subnuclei of the rostrocaudal extent of the rat nTS. In addition, the nTS was examined for possible anatomical substrates that might mediate presynaptic control of vagal afferents. These aims were accomplished by labelling vagal afferents with the neuronal tracer HRP combined with electron microscopic analysis.

2:2 METHODS

2:2:1 SURGICAL PROCEDURES.

Twelve Sprague-Dawley rats (200-320g) were anaesthetized with an intra-peritoneal (I.P) injection of pentobarbitone (Sagital, 60mg/kg) and either the tail or femoral vein was cannulated so that 10% Sagatal, diluted with saline, could be administered during the surgical procedure to maintain anaesthesia. An incision was made along the ventral surface of the neck and the muscle layers parted by blunt dissection. The right vagus nerve was separated from the carotid artery and traced back past the carotid bifurcation to the nodose ganglion with the aid of a dissecting microscope. The ganglion was identified as a slight swelling lying just proximal to the entry of the superior laryngeal nerve. Multiple pressure injections of 20% HRP (w/v: dissolved in dist. H_2O; Boehringer) were slowly made into various sites in the ganglion so that approximately 15µl was injected in total using an injection system that was an adaption of the system described by Saper (1983). The wound was sutured in layers and the animal kept warm while it recovered from the anaethesia. All surgical procedures were carried out under aseptic conditions.

In some experiments (n=2) the cervical vagus was cut, a piece of Parafilm (American National Can) was placed under the central portion of the nerve and HRP crystals applied to the severed end. After half an hour the Parafilm was removed and the animals recovered as before.

Sham controls (n=2) received 15µl of saline injected into the nodose ganglia. In addition control experiments were performed to identify reaction product resulting

from the spread of HRP within the surgical field. In 2 animals 15µl of 20% HRP (w/v) was placed around the nodose ganglia.

2:2:2 TISSUE PREPARATION.

All animals were perfusion fixed to preserve ultrastructural detail. Rats were deeply anaesthetized with an I.P. injection of Sagatal and the chest cavity opened. The blood was first flushed out with isotonic saline (at room temperature) using a peristaltic pump. The tissues were then fixed by perfusing through the ascending aorta with a fresh solution of 2% paraformaldehyde and 2% glutaraldehyde in 0.1M phosphate buffer (PB; pH7.4) at 0-4°c. This concentration and mixture of aldehydes gives good ultrastructure and also retains HRP activity. The medulla was removed and post-fixed overnight in the same solution. Coronal sections were cut at 80µm on a vibrating microtome (Oxford Instruments) and washed in PB (pH7.4) buffer to remove any remaining aldehydes.

2:2:3 HRP HISTOCHEMISTRY.

Sections were reacted using either the *o*-tolidine method (Somogyi & Smith, 1979) or the tetramethyl benzidine method (TMB; Mesulam, 1978) to detect transported HRP.

a. Tetramethyl benzidine method.

After washing twice in ice cold PB (pH6.0) sections were preincubated for 20 minutes at room temperature in a TMB medium. The incubation medium was prepared by making up 2 solutions, A and B, and adding 2.5mls of B to A. Solution A consisted of 92.5mls of distilled H_2O, 250mg ammonium molybdate and 5mls of 0.2M

PB (pH6.0) whilst B was a solution of 25mg TMB (Sigma) dissolved in 12.5mls of absolute alcohol heated to 40°c. To initiate the reaction 10µl of 1% hydrogen peroxide was added to the reaction medium. After 20 minutes the reaction was stopped with 2 brief washes in cold 0.1M PB (pH6.0).

b. *O*-tolidine method.

Sections were initially washed for 30 minutes in 0.1M citrate buffer (pH5.5) before being transferred to the incubation medium for 2 hours in the dark on a rocker table. The incubation medium consisted of 30mg *o*-tolidine (Sigma) in 100mls of the citrate buffer with 1ml of 5% sodium nitroprusside and 1ml of 1% H_2O_2. The reaction was stopped after 20 minutes by rinsing in citrate buffer.

c. Stabilization procedure.

The reaction product of both methods was stabilized and intensified (Rye et al., 1984) by preincubating for 10 minutes in a solution of 0.05% 3'3' diaminobenzidine tetrahydrochloride (DAB; Sigma) and 0.05% cobalt chloride in 0.1M PB (pH7.4). The reaction was started by adding 10µl of 1%H_2O_2/1ml DAB solution, monitored in the light microscope to judge when an optimum signal to noise ratio was achieved, and finaly washed in buffer.

2:2:4 ELECTRON MICROSCOPY.

Sections were post-fixed in 1% osmium tetroxide in 0.1M PB in a fume hood for 30 minutes and rinsed in distilled H_2O. The tissue was dehydrated using 50%,

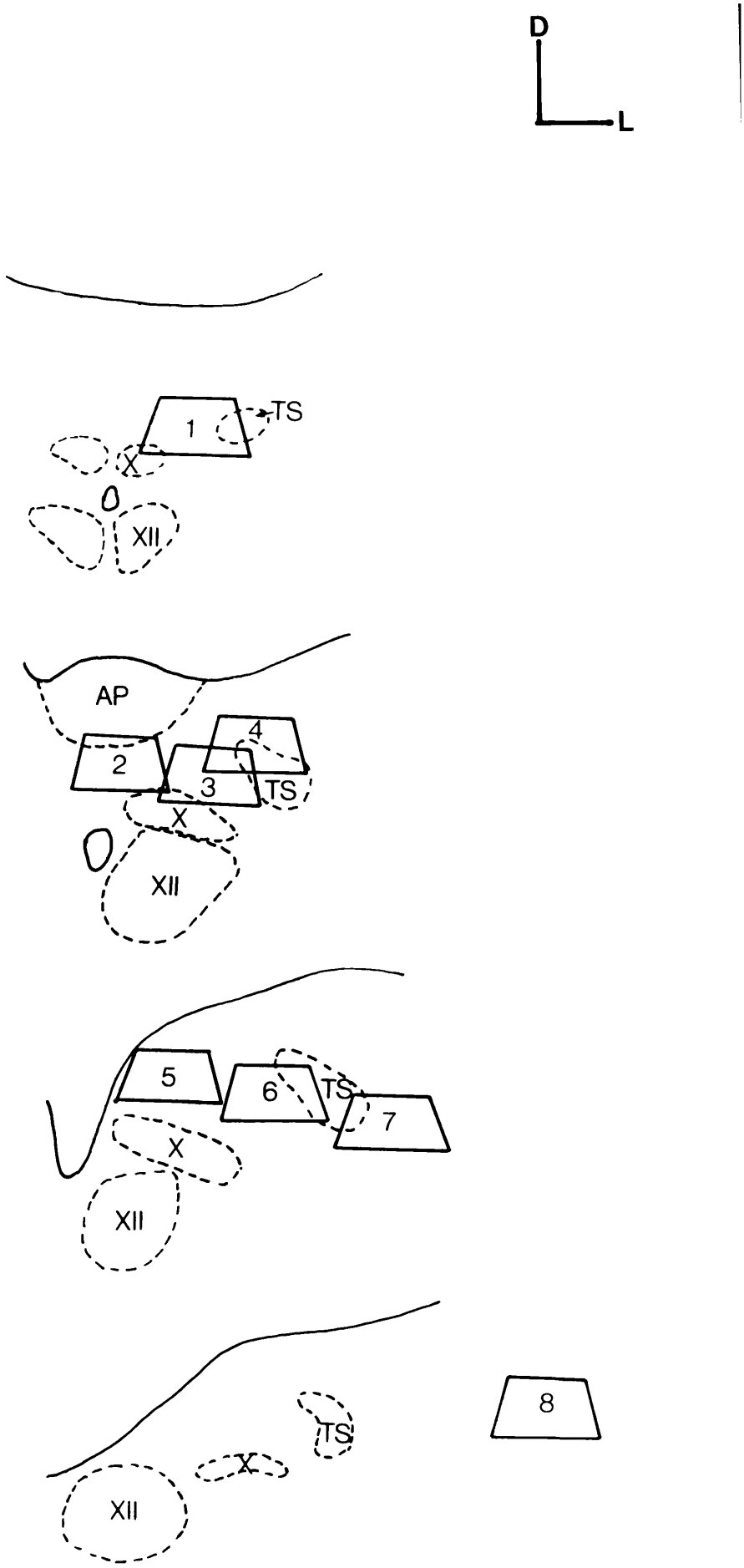
70%, 90% and 2 changes of 100% ethanol. Sections were left for 40 minutes in 70% and 15 minutes in each of the other grades. The degree of contrast was enhanced by adding 1% uranyl acetate to 70% ethanol. The dehydration procedure was followed by 2 x 15 minute changes in propylene oxide, a solvent that aids penetration of the resin into the tissue. The sections were left overnight in the embedding medium (Durcapan; Fluka Chemicals) before mounting onto glass microscope slides and coverslipping. To cure the resin slides were placed in an oven at 60°c for 12-24 hours. Slides were examined on a light microscope and the distribution of vagal afferents in medullary sections was plotted with the aid of a drawing tube using material from animals that demonstrated the heaviest labelling (Fig. 2:2).

Areas from different subdivisions of the nTS (see Fig. 2:1) were taken from well-perfused animals that had heavy vagal afferent labelling and were further examined in the electron microscope (E.M.). Blocks were prepared by removing the coverslip and gently warming the slide on a hotplate to soften the resin. Using a sharp razor a piece of resin containing the area of interest was carefully peeled off the slide and 'superglued' onto the flat end of a small cylindrical resin block. Blocks were trimmed into a trapezoid shape and excess resin cut from the surface using a glass knife and Reichert Jung ultramicrotome. A diamond knife was used to cut gold/silver ultrathin sections (about 70nm). Ribbons containing 5-8 sections were picked up from the surface of the distilled H_2O filled boat on plastic-coated (1% Piloform; Bio-Rad) copper slot or mesh grids and dried with a hairdryer. To further increase contrast of cell membranes the sections were stained on drops of lead citrate (Reynolds, 1963). Care was taken to avoid contact with the atmosphere as lead absorbs carbon dioxide and forms a lead carbonate precipitate that contaminates the sections. After washing the grids were examined in a Phillips 201 electron microscope where the morphology of labelled boutons was noted and photographs taken where appropriate.

FIGURE 2:1.

Diagramatic representation of coronal sections through the rostrocaudal extent of the right medulla demonstrating the approximate position of material taken for electron microscope examination. Distance from obex; **A)** -0.7mm **B)** -0.3mm **C)** 0.1mm **D)** 1.0mm.

Orientation bar: D, dorsal; L, lateral. Scale bar = 0.5mm.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8

2:3 RESULTS.

2:3:1 LIGHT MICROSCOPIC OBSERVATIONS.

At the light microscopic level the reaction product of both the TMB and *o*-tolidine method was dense and brown/black in colour although the reaction product of the former method had a granular appearance while that of the latter was amorphous. The degree of sensitivity between the two methods was comparable. With both methods there was some diffuse loss of HRP labelling during the stabilization process. This was due to the solubility of the reaction product in the higher pH of the stabilization solution and occasionally resulted in the almost total loss of the reaction product. In this study this phenomenom appeared to affect the TMB reaction more than the *o*-tolidine reaction. No extraperikaryal reaction product was observed in the dorsal vagal complex after any control experiments and therefore, it was not considered necessary to perform further sham surgical controls. In experiments where HRP was injected into the nodose ganglion vagal afferent labelling tended to be heavier than when the cut end of the cervical vagus was dipped in HRP as SLN afferents were also labelled. Horseradish peroxidase was transported both anterograde to vagal afferent terminals and retrograde to vagal motoneurones (Fig. 2:3A). Labelled cell bodies were detected only on the side of the medulla ipsilateral to the injection site in the DVN and nucleus ambiguus. In addition, a few cells were also observed in the intermediate zone between these two nuclei. Dendrites of labelled cells could be followed for some distance in the DVN but rarely extended further than this nucleus.

The vagal afferent and efferent labelling observed in this study was essentially the same as described by Kalia and Sullivan (1982; Fig. 2:2). Labelled axons, of

mainly small to medium sized calibre, entered the medulla in multiple rootlets, 1.5-2.0mm rostral to obex (Fig. 2:3B & C). Swellings that had the appearance of varocosities could occasionally be observed in labelled fibres running in the vagal afferent tracts. The fibres passed through the spinal trigeminal nucleus and parvicellular reticular formation in a dorsomedial direction before entering the tractus. In the rat efferent fibres are known to be separate from the afferent fibres (Kalia & Sullivan, 1982) and were observed to exit from the medulla at a more ventral level. A few afferent axons terminated in the medial subnucleus, at the level of entry of the afferent tracts. The rest descended through the tract for varying distances before leaving and entering various subnuclei of the nTS. Labelling extended from approximately 2.5mm rostral to 4.0mm caudal to obex and was heaviest just rostral to obex. The medial region throughout the rostrocaudal extent of the nTS was observed to contain the highest density of vagal afferents. More moderate labelling was observed in the intermediate, dorsolateral and ventrolateral regions of the nTS with lighter labelling in the interstitial and ventral subnuclei and subnucleus gelatinosus. Vagal afferent labelling was also detected in the nTS contralateral to the injection site although this labelling was always considerably lighter to that in the ipsilateral aspect. Labelled axons crossed the midline at the level of the commissural subnucleus and either terminated in the contralateral nTS or travelled further rostral before terminating. Vagal afferents were also detected throughout the rostrocaudal extent of the area postrema with some fibres appearing to enter this structure from the commissural subnucleus. Furthermore, the ipsilateral DVN received a small vagal afferent innervation whilst the contralateral side contained only occasional vagal afferent fibres. It was difficult to discriminate the extent of vagal afferent labelling in the ipsilateral DVN from the processes of the labelled vagal motoneurones that were also present. Vagal afferent fibres throughout the dorsomedial medulla varied in calibre from thick to very fine and were either varicose or non-varicose (Fig. 2:3B & C). Axons were observed to

FIGURE 2:2

Series of coronal sections taken through the rostrocaudal extent of the dorsomedial medulla illustrating the central projection of vagal sensory afferents and location of vagal preganglionic motoneurones following unilateral injection of HRP into the left nodose ganglion. Distance from obex; **A)** -0.7mm **B)** -0.3mm **C)** 0.1mm **D)** 1.0mm. Scale bar = 0.5mm. Orientation bar: D, dorsal; L, lateral.

ABBREVIATIONS: AP, area postrema; dlnTS, dorsolateral subnucleus of the nucleus tractus solitarius; mnTS, medial subnucleus of the nucleus tractus solitarius; ncomm, commissural subnucleus; ninTS, interstitial subnucleus of the nucleus tractus solitarius; SG, subnucleus gelatinosus; TS, tractus solitarius; vnTS, ventral subnucleus of the nucleus tractus solitarius; vlnTS ventrolateral subnucleus of the nucleus tractus solitarius; X, dorsal motonucleus of the vagus nerve; XII, hypoglossal motonucleus.

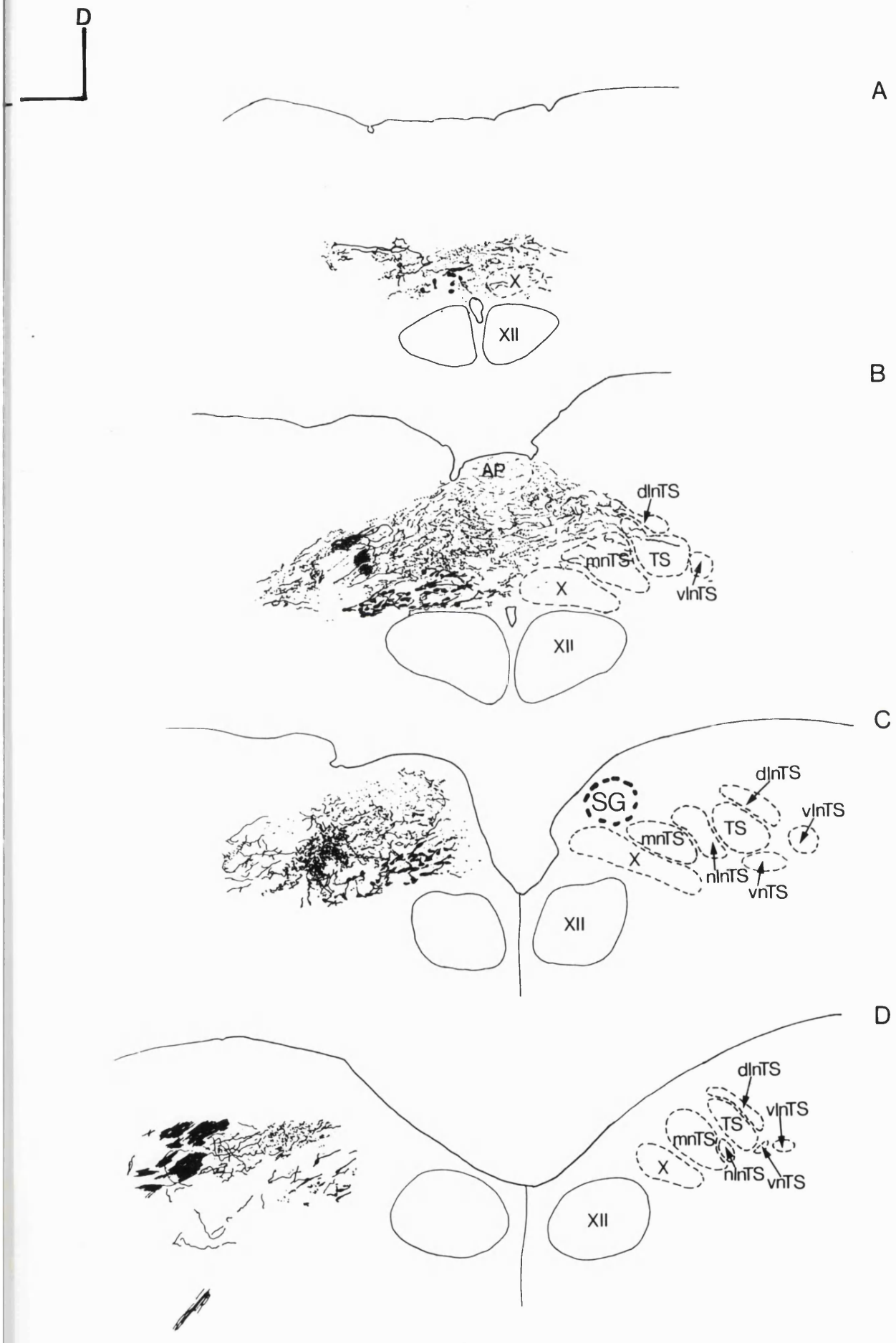
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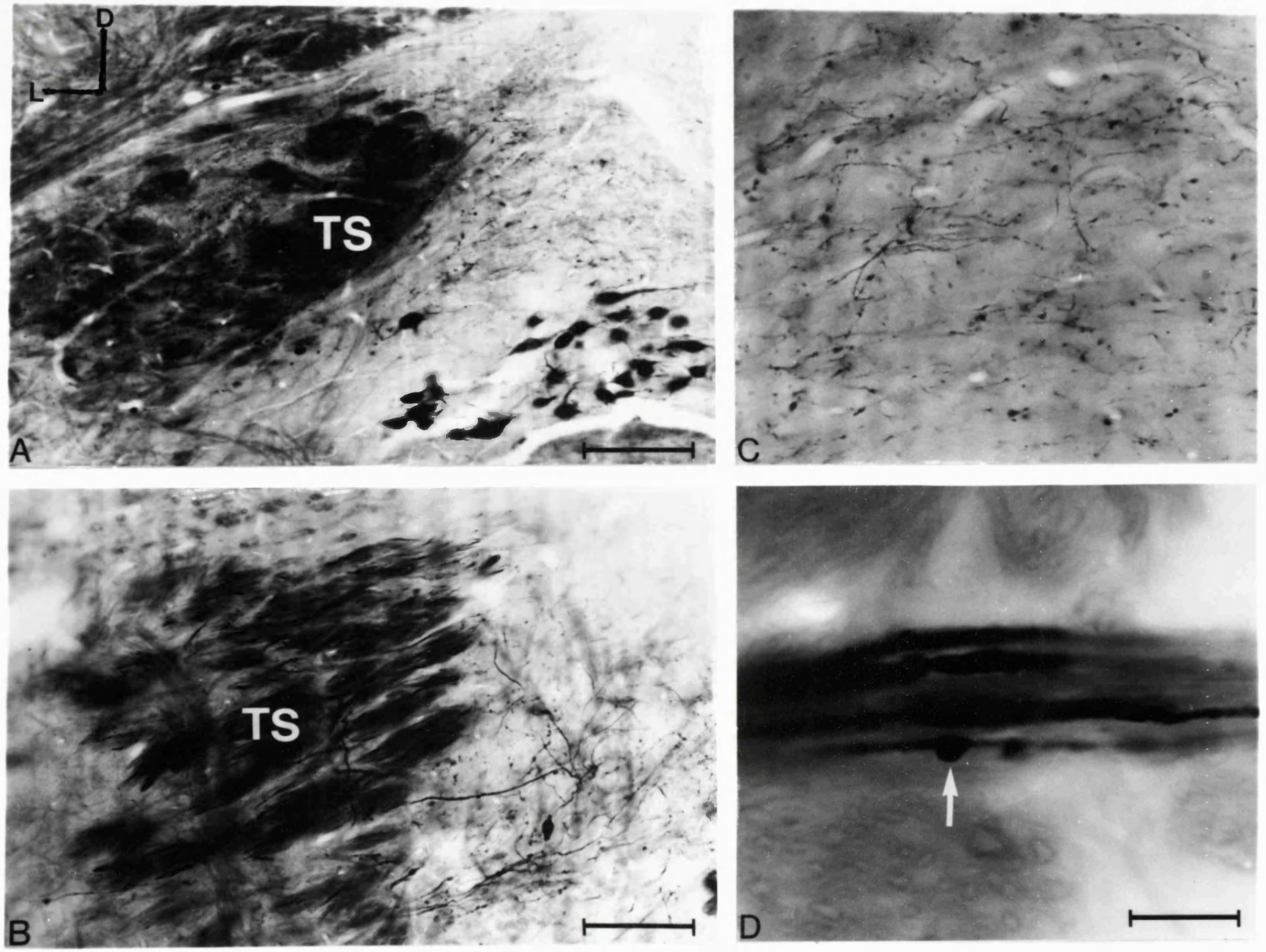
FIGURE 2:3.

Photomicrographs of anterograde and retrograde labelling following injection of HRP into the left nodose ganglion. **A)** a low power photomicrograph showing HRP-labelled vagal sensory afferents and vagal preganglionic motoneurones. Scale bar = 100μm. Orientation bar : D, dorsal; L, lateral. **B)** & **C)** medium power micrographs demonstrating the range of fibre size of vagal sensory afferents and their terminations in the nTS. Scale bar = 50μm. **D)** high power micrograph of the afferent tract containing HRP-labelled fibres with varacosities. Scale bar = 100μm.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subnuclei.

2:3:2 ELECTRON MICROSCOPIC OBSERVATIONS.

The results discussed below were taken from 4 animals that displayed strong and extensive labelling of vagal afferent fibres with HRP combined with good ultrastructural preservation.

The reaction product of the TMB and *o*-tolidine methods had distinctive appearances at the E.M. level. Large amorphous crystals were deposited with the TMB method that were not associated with any particular cell organelles (Fig. 2:4B). In contrast, the reaction product of the *o*-tolidine method filled the terminals with small densely packed granules (Fig. 2:4A). Where the *o*-tolidine reaction product was particulary dense membranes and small cell organelles were obscured and sometimes showed signs of damage. In both TMB and *o*-tolidine reacted material afferent terminals were observed that were not labelled with reaction product but showed signs of degeneration. These afferents were electron dense with loss of synaptic vesicles and disrupted mitochondria but synaptic specializations were maintained (Fig. 2:4C).

Ultrathin sections were taken from the ventrolateral, ventral, dorsolateral, medial, subnucleus gelatinosus and commissural regions of the nTS as well as from the afferent tracts (Fig. 2:3). Although a few, selected boutons were followed in serial section it was not considered necessary to do this routinely due to the amount of material examined (> 400 boutons). It was difficult to examine the area postrema as tissue from this region tended to break up during the processing schedule.

In all fields examined labelled vagal afferents made up only a small proportion

of the total population of afferent terminals. The majority of vagal afferents were unmyelinated although myelinated afferents were also observed. Vagal afferents formed both en passant (Fig. 2:6:D) and more frequently, end terminal boutons that were generally 1-5μm in size. All boutons contained numerous small, round, clear synaptic vesicles approximately 40-60nm in diameter whilst a few boutons also contained 1 or 2 large, clear vesicles. Variable numbers of dense core vesicles were seen in some vagal afferents but rarely numbered more than 5 in the plane of section. Vagal afferents also contained varying numbers of mitochondria. The synaptic contacts observed between labelled vagal afferents and their post-synaptic targets were of the asymmetric type (Gray's type I). This type of synapse was characterized by a dense pre-synaptic density, a widening of the inter-cellular space and synaptic vesicles present near the synaptic cleft. Some of these synaptic specializations were further characterized by the presence of sub-junctional bodies (Fig. 2:6D). Only one possible example of a vagal afferent terminal forming a symmetric contact with another bouton was observed although the synaptic specializations of over 100 labelled boutons were examined (Fig. 2:8). The vagal afferent appeared to be the pre-synaptic structure as there were synaptic vesicles clustered near the synapse of this bouton. Macula adherens were frequently observed and were detected between labelled vagal afferents and a variety of other structures e.g. dendrites, unlabelled afferents, other labelled vagal afferents and soma (Fig. 2:5B). In some instances, both macula adherens and asymmetric contacts were present between labelled afferents and their post-synaptic target. Macula adherens could be distinguished from synapses as they were symmetric and the neurofilaments extended into both structures and were not associated with synaptic vesicles or sub-junctional bodies.

TABLE 2:1. STRUCTURES POST-SYNAPTIC TO VAGAL SENSORY AFFERENTS THROUGHOUT THE ROSTRO-CAUDAL EXTENT OF THE NTS.

	Numbers of afferents	Percentage
Small dendrites 0.17-0.87μm	73	64.6%
Medium dendrites 0.88-1.57μm	27	23.9%
Large dendrites 1.58-2.27μm	11	9.7%
Soma	2	1.8%

terminal boutons in synaptic contact with single small to medium calibre dendrites (88.5%) and less frequently with large calibre dendrites (9.7%). Vagal afferents were most often in contact with dendrites cut in cross-section. Occasionally spiny dendrites were cut in cross-section and labelled vagal afferents were found to make synaptic contact with both the neck of the spine (Fig. 2:7C), the spine itself and the body of the dendrite. One example of a vagal afferent engulfing the spine was also observed (Fig. 2:7C). Some of the small calibre structures that received synaptic input from vagal afferents may also have been spines as they did not contain mitochondria or microtubules. The putative spines were generally smaller than the presynaptic vagal afferent. Occasionally dendrites post-synaptic to vagal afferents were observed containing a few large, round vesicles or multivestibular bodies.

Vagal afferents were sometimes detected making synaptic contact with more than one dendrite in the plane of section (Fig. 2:4C & 5A) whilst some dendrites received multiple afferent input from both labelled and unlabelled structures (Fig.2:7B). The unlabelled structure frequently exhibited a similar morphology to the labelled vagal afferent. Vagal afferents in all subdivisions of the nTS formed "glomerular" structures. These glomeruli consisted of a central vagal afferent terminal that had scalloped edges and were apposed to several small to medium calibre dendrites and other unlabelled axons (Fig.2:5A & B). Labelled afferents were also occasionally observed in apposition with soma (Fig.2:7A). One vagal afferent bouton was observed forming an asymmetric synaptic contact with a dendrite that was further identified by the presence of the *o*-tolidine reaction product as originating from a VPN (Fig. 2:5D). In addition, other labelled dendrites, predominantly in the subnucleus gelatinosus, were detected post-synaptic to boutons morphologically similar to vagal sensory afferents (Fig. 2:7D).

FIGURE 2:4.

Electron micrographs of HRP labelled vagal sensory afferents (asterisk) with peroxidase enzymatic activity revealed with; **A)** the *o*-tolidine method and **B)** the TMB method. **C)** is unlabelled but shows clear signs of degeneration. All the vagal afferents in this figure are forming asymmetric synaptic contacts (arrows) with one or more dendrites of varying calibre. This type of synapse is characterized by a dense post-synaptic density, a widening of the inter-cellular space and synaptic vesicles near the synaptic cleft. Scale bar = 0.5μm.

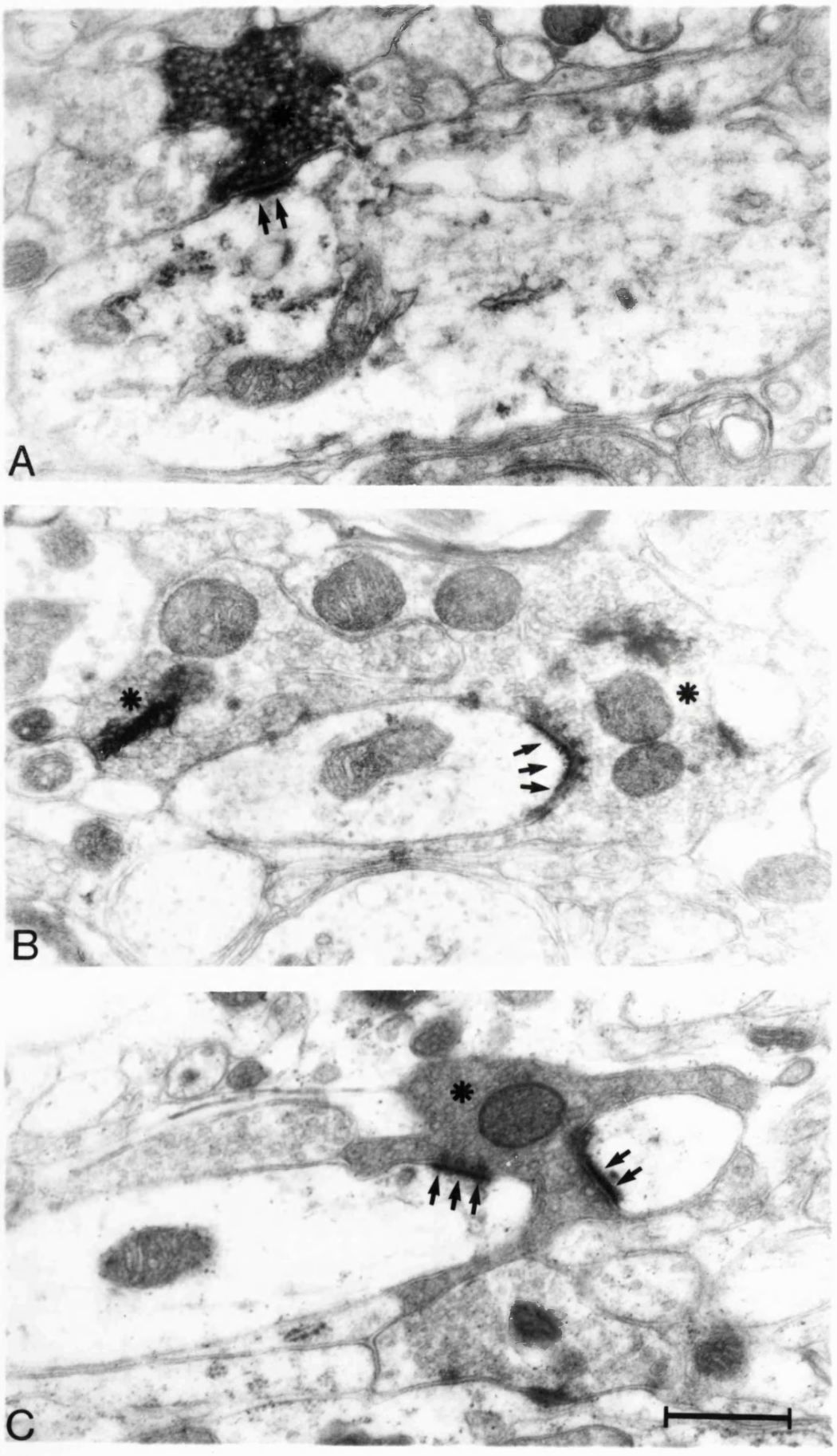
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FIGURE 2:5.

Examples of vagal sensory afferents (asterisk) anterogradely labelled with HRP with peroxidase activity revealed with the *o*-tolidine method. **A)** vagal afferent making asymmetric synaptic contact (arrows) with 2 putative spines. **B)** macula adherens (arrows) between a labelled afferent and dendrite. Note the 'glomerular' arrangement of the vagal afferents in **A** & **B**. **C)** Vagal afferent presynaptic to a possible spine. **D)** HRP-labelled vagal sensory afferent (arrowhead) post-synaptic to a labelled vagal afferent, a presumed vago-vagal contact. Scale bar = 0.5μm.

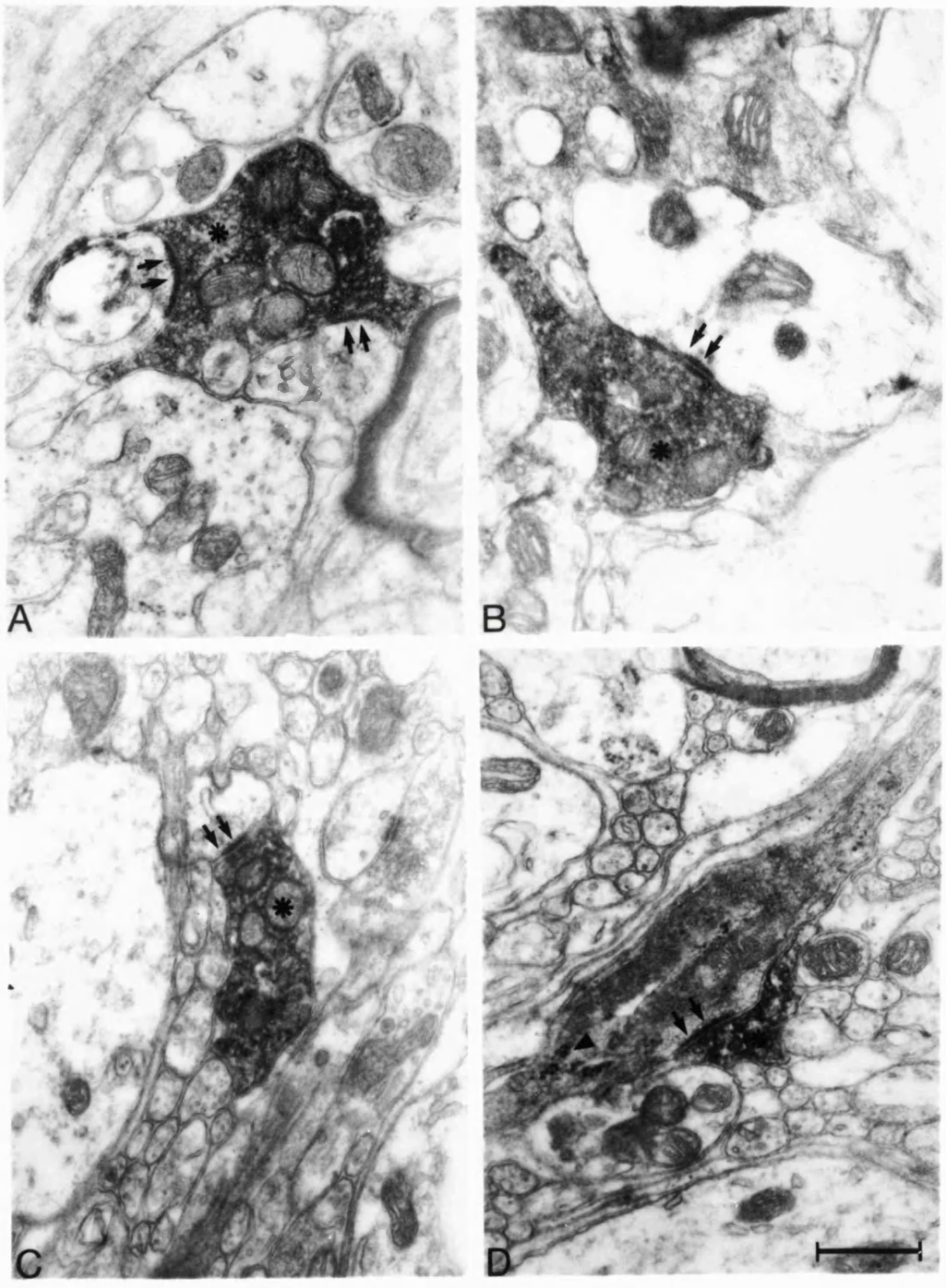
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FIGURE 2:6.

Examples of vagal sensory afferents (asterisk) anterogradely labelled with HRP with the peroxidase activity revealed with the TMB method. **A) & C)** vagal afferents in asymmetric synaptic contact (arrows) with possible spines. **B)** two vagal afferents in apposition to a small calibre dendrite but only one bouton forms a synaptic specialization (arrows). **D)** en passant vagal afferent presynaptic to a dendrite. Scale bar = 1μm.

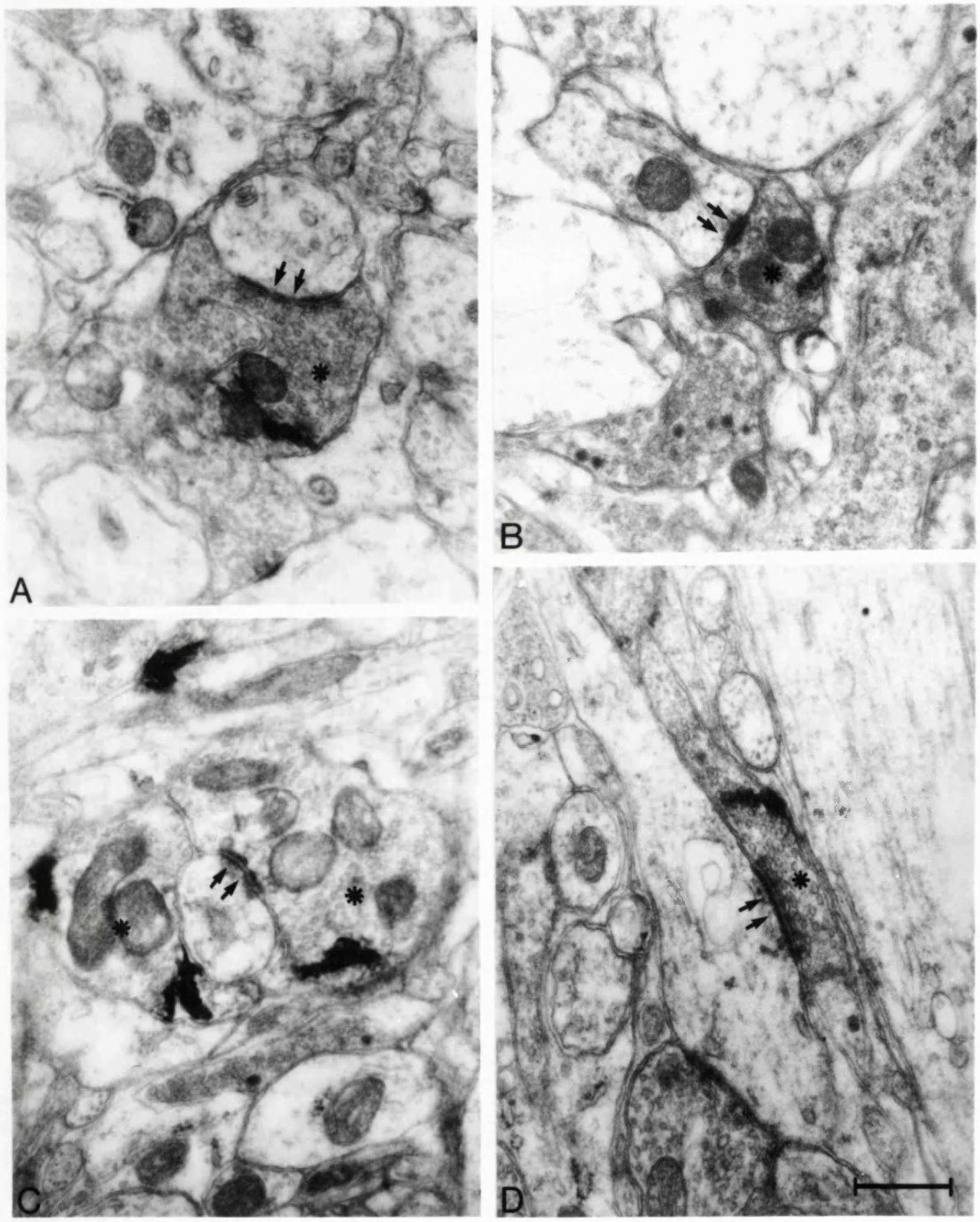
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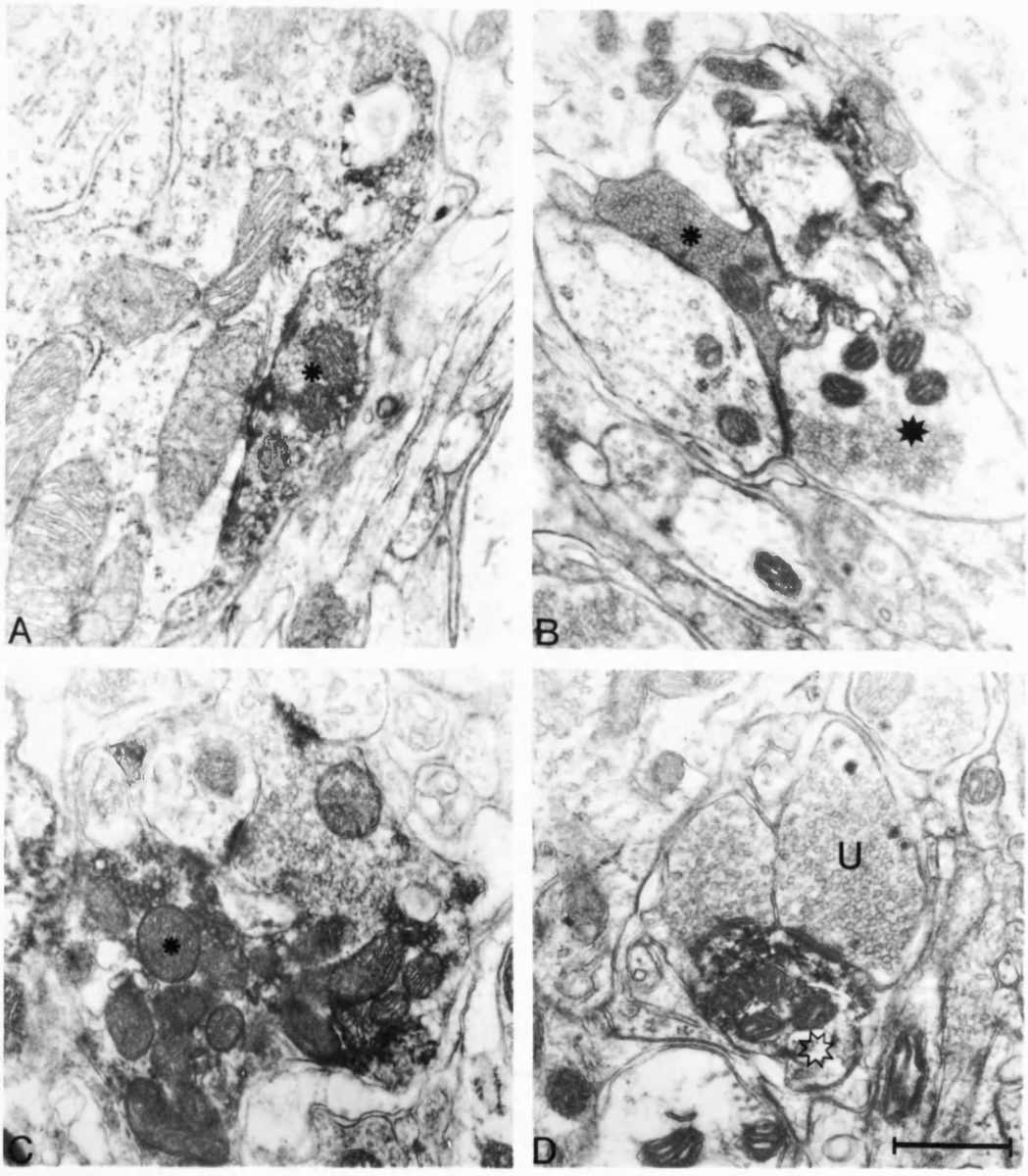
FIGURE 2:7.

Further examples of anterogradely labelled vagal sensory afferents (asterisk). **A)** labelled afferent in synaptic contact with a neurone. **B)** medium calibre dendrite post-synaptic to both a labelled vagal afferent (asterisk) and unlabelled afferent (star). **C)** dendritic spine neck receiving synaptic contact from a vagal afferent. **D)** labelled dendrite (hollow star) in subnucleus gelatinosus post-synaptic to an unlabelled afferent (U). Scale bar = 0.5μm.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2:4 DISCUSSION.

The extent and density of vagal afferent and efferent labelling observed in this study in the dorsomedial medulla agreed with the previous report of Kalia and Sullivan (1982). Vagal afferent fibres of varying calibre and density were detected in all subdivisions of the ipsilateral nTS with lighter labelling in the caudal and intermediate contralateral nTS, the area postrema and DVN. At the ultrastructural level this study demonstrates that vagal sensory afferents had a similar morphology throughout the subdivisions of the rostrocaudal nTS and made synaptic contacts predominantly with dendritic spines and more distal dendrites (88.5%). Vagal sensory afferents formed synaptic contacts of the asymmetric type suggesting that these afferents provide excitatory input. Modulation of vagal afferent input appears to occur mainly at post-synaptic sites as only one possible example of a vagal afferent forming an axo-axonic contact was observed.

2:4:1 TECHNICAL CONSIDERATIONS.

Anterograde neuronal labelling with HRP combined with visualization of peroxidase activity with the *o*-tolidine or TMB histochemical methods is a highly sensitive technique (Mesulam, 1978). However, it is highly unlikely that the total population of vagal sensory afferents were labelled in this study for a number of reasons. Although a number of pressure injections were made into the nodose ganglion the number of perikarya taking up the tracer is unknown. In addition, there was some loss of staining due to the solubility of the reaction product and the variability of the stabilization procedure. Another consideration is the specificity of the neuronal tracer. As there are no reports that HRP is taken up preferentially by certain classes of fibre it

is assumed that all classes were labelled. This is partially confirmed by the large range of calibre of vagal afferent fibres observed at the light microscope level and the detection of both myelinated and unmyelinated labelled fibres at the electron microscope level.

Although the injections in this study were aimed at the nodose ganglion on some occasions hypoglossal motoneurones were also lightly labelled. It is possible that the hypoglossal nerve was inadvertently injected with tracer as it runs close to the nodose ganglion at this level. As previous reports (Nazruddin et al., 1989; Neuhuber & Mysicka, 1980) have stated that hypoglossal nerve afferents project to regions outside the nTS it is assumed that the labelled boutons observed in this study were purely of vagal origin. This was further confirmed by the complete lack of HRP labelled vagal afferents observed in the control experiments. Unlabelled axons were observed that show signs of degeneration. These axons were assumed to be vagal afferents whose parent cell body was damaged during the injection process as degenerating structures were not detected in control material.

2:4:2 MORPHOLOGICAL PROFILE OF VAGAL AFFERENTS.

All the vagal afferents observed in this study were generally of a similar size and contained round, clear synaptic vesicles indicating that they formed a homogeneous group as regards synaptic vesicles. A proportion of vagal afferents contained low numbers of dense core vesicles but due to the numbers of labelled boutons examined in this study (> 400) it was not possible to follow all of them in serial section. It is possible that the central terminations of vagal sensory afferents contain neuropeptides (see chapter 4) as this type of subcellular organelle has been assumed to contain these neurochemicals (see Thureson & Klein, 1990). This suggestion has recently been

further verified by the ultrastructural visualization of neuropeptide immunoreactivity in dense core vesicles (De Biasi & Rustioni, 1988; Merighi et al.,1989, 1991).

2:4:3 SYNAPTIC CONTACTS OF VAGAL AFFERENTS.

Vagal afferents in this study were observed making predominantly asymmetric synapses with their post-synaptic targets. If the assumption is correct that this type of synapse is providing excitatory input (Andersen et al., 1963; Andersen & Eccles, 1965; Eccles, 1964; Gray, 1962) it is possible that these boutons may ultilize excitatory amino acid/s as a neurotransmitter (see chapter 3). However, it should be noted that there is indirect evidence for GABAergic boutons forming asymmetric synapses in various CNS regions (see Ribak & Roberts, 1990). If vagal afferents transmit solely excitatory input then the inhibitory responses recorded from nTS neurones following sensory afferent stimulation (Felder & Mifflin, 1988; Mifflin et al., 1988a, 1988b) are probably due to the presence of inhibitory interneurones. Neurones immunoreactive for the inhibitory amino acid, GABA, have been detected in the nTS (Izzo et al., 1992; Maley & Newton, 1985) and may receive direct synaptic input from vagal afferents (Maqbool et al., 1991). The GABA-mediated inhibition of neurones synaptically activated by vagal afferent stimulation (Bennett et al., 1987) further supports this suggestion.

Slowly adapting and superior largyngeal nerve afferents appear to be amenable to presynaptic inhibition (Barillot, 1970; Richter et al., 1986; Rudomin, 1967). However, only one possible example of a vagal afferent making an axo-axonic contact (the assumed substrate of presynaptic inhibition) was observed in this study following the examination of over a 100 vagal afferent synaptic specializations (Fig. 2:8). There are difficulties in identifying symmetric synapses in HRP reacted material due to the

reaction product obscuring the membranes. This problem was faced by other workers who have reported vagal sensory afferents making axo-axonic contacts (Chiba & Doba, 1976; Richter et al., 1985b, 1988b; Sumal et al., 1983). If these contacts do exist they appear to be very rare and were missed entirely by several workers examining vagal afferent synaptology (Chazal et al., 1991; Gwyn et al., 1982; Leslie et al., 1982). Furthermore, Izzo and co-workers did not observe any GABAergic boutons in synaptic contact with other boutons in the cat nTS whilst a similar study (Maqbool et al., 1991) found only one indistinct example (see Fig. 3E). Although GABAergic axons were observed in the cat ventrolateral medulla in contact with other axons (Lipski et al., 1990) this material had been stained both for immunocytochemistry and to reveal intracellular-labelled neurones making interpretation difficult. Therefore, the lack of definitive neuroanatomical evidence for the presence of vagal afferents forming synaptic contacts with other axons raises the possibility that these primary afferents may be modulated presynaptically by other mechanisms. For example, Richter and co-workers (1986) suggested that vagal afferents terminating in the region of the dorsal respiratory neurones may be depolarized by the high levels of extracelluar potassium found in the proximity of these cells (Richter et al., 1978).

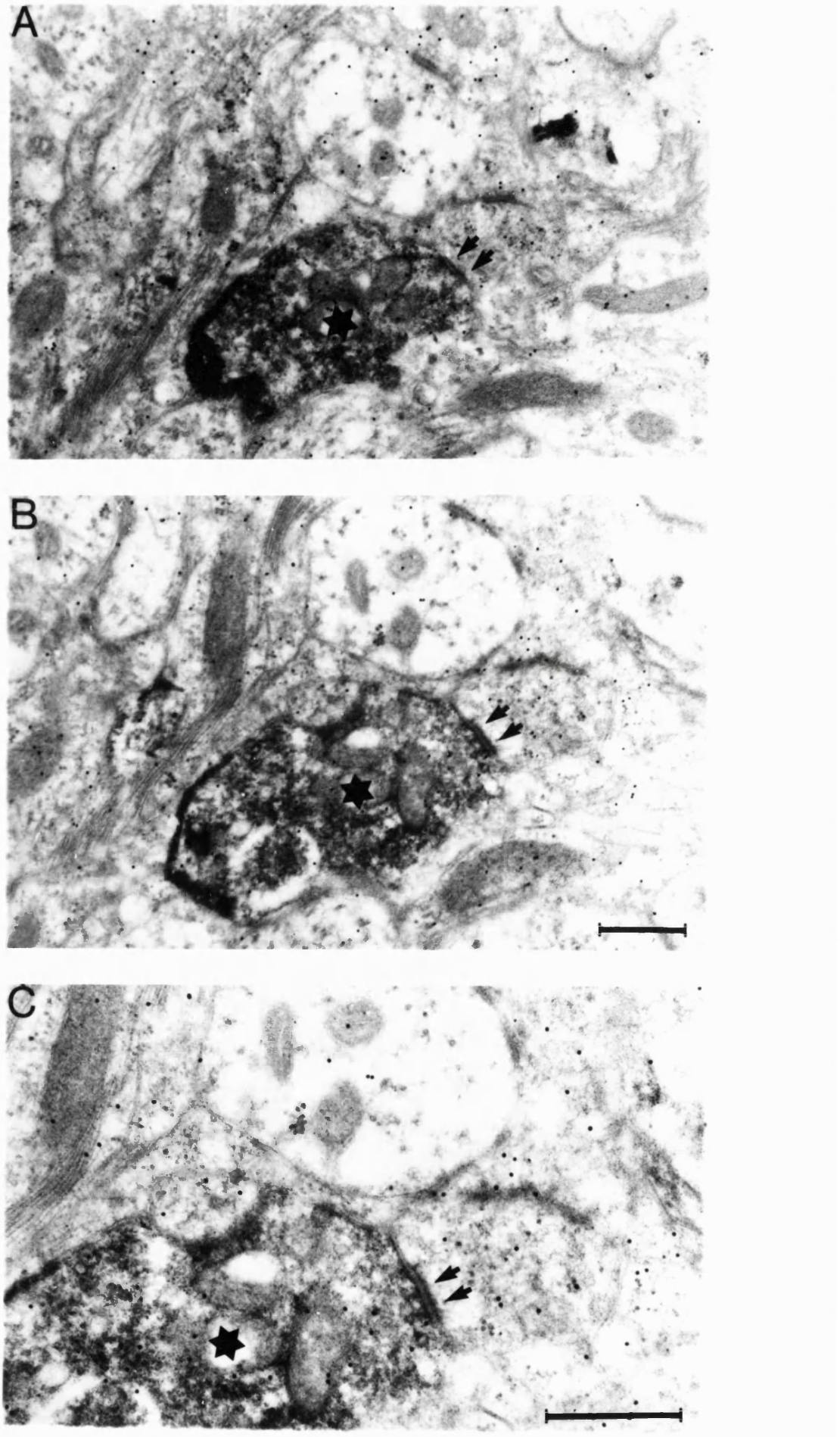
2:4:4 POST-SYNAPTIC TARGETS OF VAGAL AFFERENTS.

This study confirms and extends the results of other studies (Chazal et al., 1991; Chiba & Doba, 1976; Gwyn et al., 1982; Leslie et al., 1982; Sumal et al., 1982), that vagal afferents make synaptic contact predominantly with distal dendrites and dendritic spines (88.5%) and more rarely proximal dendrites (9.7%) and perikarya (1.8%). However, it should be noted that not all dendrites would be cut in vertical cross-section and oblique or tangential sections can give a misleading impression of size and shape. Input onto spines and more distal regions of the neurone are more

FIGURE 2:8.

Electron micrograph of serial sections (**A** & **B**) taken from the nTS demonstrating a labelled vagal afferent (star) forming a possible symmetric type synapse (arrows). Scale bar = 0.5μm. **C**) is a higher power micrograph of the synapse shown in **B**). Scale bar = 0.5μm.

(Immunogold can be observed in these micrographs as the material was taken from the study in chapter 3B)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susceptible to modulation than more proximal synaptic inputs. At these locations postsynaptic responses elicited by primary afferent inputs can be modulated by inputs located more proximally on the cell. Vagal afferents also formed the central terminal of glomerular structures. These complex arrangements are assumed to form divergent and diffuse synaptic systems capable of influencing several neurones simultaneously.

Only a single example of a labelled vagal afferent was observed making synaptic contact with a labelled dendrite, a presumed vago-vagal contact. Previous studies have reported abundant vago-vagal contacts in the rat subnucleus gelatinosus although not elsewhere in the nTS (Rinaman et al., 1989). However, Rinaman and co-workers studies utilized HRP conjugated to cholera toxin as a neuronal tracer, a compound that fills the dendritic tree better than the HRP, which may explain the lack of vago-vagal contacts detected in this study.

Although no attempt was made in this study to identify the neurochemical content of the post-synaptic targets previous studies have suggested that vagal sensory afferents provide synaptic input to neurones containing catecholamines (Sumal et al., 1983) met-enkephalin (Velley et al., 1991), substance P (Kawai et al., 1989) and possibly GABA immunoreactivity (Maqbool et al., 1991). As sensory vagal afferents have a widespread distribution throughout this region and subserve many functions it is probable that further studies will reveal many different post-synaptic targets.

2:4:5 CONCLUSIONS.

In summary, this study has demonstrated that the central projections of vagal sensory neurones have a similar morphology throughout the different subnuclei of the rostrocaudal extent of the nTS, despite their diverse functional properties.

Furthermore, the results from this study suggests that vagal afferent input is open to modulation which probably occurs principally at post-synaptic sites.

CHAPTER 3A

A light microscopic study of glutamate-immunoreactive neurones and axon terminals in the dorsomedial medulla.

CONTENTS

3A:1 INTRODUCTION.

For many years it was known that the brain contained high concentrations of certain amino acids e.g. glutamate and aspartate and that these substances had powerful excitatory effects on neuronal activity (Curtis & Watkins, 1961). However, confirming that glutamate and aspartate were neurotransmitters in the CNS proved to be more difficult. This was partly due to the difficulty in dissociating their other roles e.g. in intermediary metabolism, as a precursor of the inhibitory amino acid GABA and as important building blocks in proteins and peptide synthesis, from their possible neurotransmitter role. The advent of specific agonists and antagonists did much to aid the elucidation of their excitatory effects and today it is agreed that glutamate (and aspartate) largely satisfy the four main criteria for classification as a neurotransmitter (for review see Curtis & Johnson, 1974; Fonnum, 1981).

A number of studies support the role of glutamate as a neurotransmitter in the nTS. Biochemical assay suggests that the nTS, particulary the medial region, contains relatively high levels of glutamate (Dietrich et al., 1982) where neurotransmitter-like release has been demonstrated both *in vivo* (Granata & Reis, 1983; Lawrence & Jarrott, 1994) and *in vitro* (Kihara et al., 1989; Meeley et al., 1989). A high affinity uptake system for glutamate has been shown (Perrone, 1981; Siemers et al., 1982; Simon et al., 1985; Sved, 1986) that appears to be heterogeneously distributed (Simon et al., 1985). The nTS also has a higher concentration of glutamate binding sites than is generally observed in other brainstem regions (Monaghan & Cotman, 1985). In addition, there is evidence that glutamate plays a role in the nTS in a number of physiological functions. Microinjection of glutamate or glutamate agonists into the nTS evokes swallowing (Hashim & Berger, 1989; Jean et al., 1986; Kessler et al., 1990), changes in cardiovascular parameters (Kubo & Kihara, 1988; Le Galloudec et al.,

1989; Reis et al., 1981; Talman et al., 1980) and the Breuer-Hering response (McCrimmon et al., 1993) whilst these responses, elicited by activation of peripheral afferents, are suppressed by prior application of glutamate antagonists. Further evidence for glutamate having a central role in cardiorespiratory regulation comes from studies showing blockade of the arterial chemoreceptor reflex with a broad spectrum EAA antagonist (Zhang & Mifflin, 1993) and excitation of functionally identified respiratory neurones and presumed reflex interneurones with glutamate in the nTS (Henry & Sessle, 1985).

The present study was undertaken to provide a detailed description of glutamate immunoreactive neurones and axon terminals throughout the nTS with regard to their topographical organization. To achieve these aims antisera raised against a haemocyanin-glutaraldehyde conjugate of glutamate was utilized in conjunction with post-embedding immunocytochemical techniques.

3A:2 METHODS.

3A:2:1 TISSUE PREPARATION.

Twelve male Sprague-Dawley rats (275-325g) were perfusion fixed through the ascending aorta with 2% parformaldehyde and 2% glutaraldehyde, the brains removed and left overnight in the same fixative. Coronal sections were cut from each medulla at 80μm on a vibrating microtome. The tissue was washed well in 0.1M PO_4 buffer and then post-fixed for 1 hour in 1% osmium tetroxide, dehydrated and embedded in resin. For full details of these procedures see chapter 2.

These animals formed part of a larger study and some the resultant material was also used in chapter 3B.

3A:2:2 PRE-EMBEDDING IMMUNOCYTOCHEMISTRY.

Pre-embedding immunocytochemical techniques were performed on sections of medulla taken from 5 animals. The sections were incubated for 1 hour in non-immune goat serum diluted 1:10 in PBS containing 0.1% Triton X-100. The sections were subsequently incubated in rabbit anti-glutamate (Arnel) diluted 1:1000-1:5000 in PBS, overnight at 4°c. The sections were then washed in PBS and incubated for 5-6 hours in biotinylated goat anti-rabbit (Vector Laboratories) at a 1:50 dilution in PBS. After further washes in PBS the sections were incubated with a streptavidin biotinylated complex (Vectastain Elite ABC Kit; Vector Laboratories) diluted 1:50 in PBS. Finally the peroxidase activity was revealed by incubating the sections in a VIP peroxidase substrate kit (Vector Laboratories) for 5-10 minutes. After washing the sections were mounted on subbed slides, dehydrated and coverslipped.

3A:2:3 POST-EMBEDDING GLUTAMATE IMMUNOCYTOCHEMISTRY ON SEMI-THIN SECTIONS.

Regions of the medulla containing the entire nTS, DVN and a portion of the hypoglossal nuclei at 5 different levels were identified at the light microscopic level and re-embedded prior to re-sectioning. Semi-thin 1μm sections were cut with a glass knife on an ultramicrotome, collected from the water-filled boat, placed on chrome alum-gelatin coated slides and dried in an incubator at 60°c.

To allow the primary antibody access to the tissue the resin was first etched for 30-60 minutes in ethanoic sodium hydroxide (12g sodium hydroxide in 100mls of

100% ethanol, left overnight), washed well in 100% ethanol and then rinsed several times in several dist. H_2O. The osmium tetroxide was bleached from the sections by treating with freshly made 1% periodic acid for 10 minutes, rinsing in dist. H_2O followed by 0.5% sodium borohydride for 15 minutes. The sections were washed in several changes of dist. H_2O and reionized in 0.2M Tris/0.1M phosphate buffered saline buffer (TPBS; pH7.4). The following steps were performed in a humidity chamber to prevent evaporation of reagents. Sections were incubated in 2% normal goat serum (NGS) diluted with TPBS (pH7.4) for 30 minutes and without washing were transferred to rabbit anti-glutamate diluted 1:1000 overnight at room temperature. The primary antibody was detected using either A) the avidin and biotinylated horseradish peroxidase complex (ABC method) or B) the immunogold silver intensification (IGS) method.

A) ABC method: Sections were washed well in buffer and then incubated in biotinylated goat anti-rabbit IgG diluted 1:100 for 2-4 hours, washed in 0.2M TPBS and transferred to streptavidin biotinylated HRP complex (Vectastain Elite ABC kit; Vector Laboratories). To reveal the peroxidase enzymatic activity sections were either reacted using DAB (see section 2:2:3a) or the VIP peroxidase substrate kit (Vector Laboratories).

B) IGS: Following the primary antisera incubation the sections were washed and then incubated in goat anti-rabbit IgG conjugated to 15nm colloidal gold (Biocell) diluted 1:100 for 2-4 hours. After washing thoroughly in dist. H_2O the immunogold was visualized with a silver enhancing kit (Vector Laboratories).

All reactions were monitored with the light microscope and stopped with dist. H_2O. The sections were dehydrated, coverslipped with XAM (Merck) and examined

on an Olympus Vannox microscope to determine the distribution of the glutamate-immunoreactive structures.

3A:2:4 CONTROLS FOR IMMUNOCYTOCHEMICAL DETECTION OF GLUTAMATE.

Controls verifying the specificity of the glutamate antisera have been described in detail by Hepler and co-workers (1988). In addition, some sections were treated as described above but the primary antibody was omitted and replaced with non-immune serum or were incubated in primary antibody previously absorbed with glutamate alone or glutamate conjugated to haemocyanin with glutaraldehyde. The amino acid was conjugated to haemocyanin according to the procedure of Hepler et al. (1988). Briefly, 100μm of L-glutamate (Sigma) was dissolved in 1ml of dist. H_2O and mixed with an eqal volume of 0.1M PO_4 buffer (pH7.4) containing 12mg haemocyanin (Sigma). To this solution 40μl of 25% glutaraldehyde was added. After incubation at room temperature for 24 hours the mixture was dialysed against dist. H_2O for 3 days. One mililitre of the diluted antisera was then absorbed with the conjugateat different concentrations (undiluted, 1:10, 1:100 or 1:1000 in PBS).

3A:3: RESULTS.

All the data used in this study were obtained using post-embedding immunocytochemistry as no specific immunolabelling was detected with pre-embedding techniques. Glutamate immunostaining was performed on semi-thin sections taken from the dorsomedial medulla. The results discussed below are taken from material obtained from 4 rats that exhibited high levels of glutamate

immunoreactivity. No glutamate immmunostaining was observed in 3 rats whilst only relatively weak glutamate immunoreactivity was observed in a further 3 animals. The brains from 2 rats were not processed due to poor fixation. Examination of material revealed immunolabelling for glutamate that was associated with both neuronal perikarya and punctate structures, presumably boutons, that was easily distinguishable from background levels of staining. Glutamate-immunoreactive neurones were observed only when the primary antisera was localized using either gold-labelled secondary antibodies followed by silver intensification (Fig. 3A:2) or using the ABC method in combination with the VIP substrate kit (Fig. 3A:3). In material in which the immunoperoxidase complex was detected using DAB only axonal labelling was observed.

In order to examine the specificity of the glutamate antisera sections were incubated with primary antisera preabsorbed with glutamate or glutamate conjugated to glutaraldehyde. Preabsorption with the glutamate conjugate at dilutions of 1:100 and above abolished the immunostaining although some weak immunostaining was observed following absorption of glutamate alone. In addition, replacement of the primary antisera with the non-immune sera resulted in a complete lack of immunostaining.

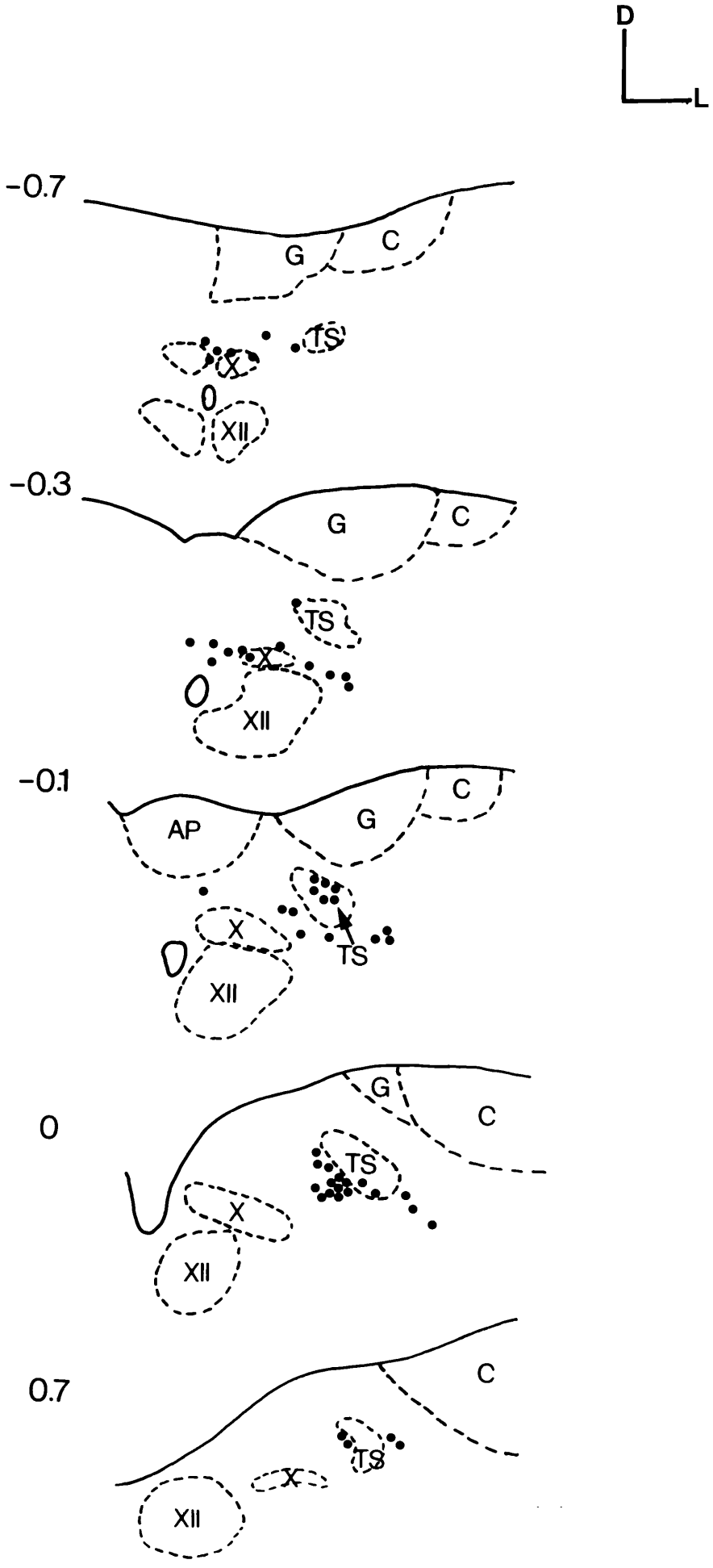
3A:3:1 GLUTAMATE-IMMUNOREACTIVE NEURONES.

Glutamate-immunoreactive neurones were observed throughout the rostrocaudal extent of the nTS. At the most caudal levels of the nTS the majority of glutamate-immunoreactive cells were detected above the central canal extending over the dorsal edge of the DVN. Occasional immunoreactive cells were also observed in the medial nTS. Further rostral, at the most caudal level of the area postrema, immunolabelled neurones were still observed above the central canal but at this level only 1 or 2 cells

FIGURE 3A:1.

Line drawing of a series of sections through the right dorsomedial medulla showing the distribution of glutamate-immunoreactive perikarya. Each dot indicates a single immunoreactive neurone on a representative 1μm section. Numbers on left side indicate the distance in millimeters from obex. Orientation bar: D, dorsal; L, lateral. Scale bar = 0.5mm.

Abbreviations: AP, area postrema; C, cuneate complex; G, gracilis complex; TS, tractus solitarius; X, dorsal motonucleus of the vagus nerve; XII, hypoglossal motonucleus.


D
L
-0.7
G
C
TS
X
0
XII
-0.3
G
C
TS
X
0
XII
-0.1
AP
G
C
X
TS
0
XII
0
G
C
TS
X
XII
0.7
C
TS
X
XII

extended into the DVN with a few glutamate-immunoreactive neurones in the medial portion of the nTS. At the level of the area postrema two distinct groups of immunolabelled cells were present. The larger group was located within the tractus with a smaller group ventrolateral to the tractus. Infrequent glutamate-immunoreactive cells were also seen in the medial and ventral regions of the nTS and in the commissural subnucleus. A large group of immunolabelled neurones was observed medial to the tractus at the level of obex. Other immunoreactive cells were also located in the tractus and ventrolateral to the tractus. At more rostral levels glutamate-immunoreactive cells were sparse and were only situated medial and lateral to the tractus. Labelled neuronal perikarya were also observed in the adjacent gracile nucleus (Fig. 3A:2A). No immunoreactive neurones were seen in the DVN at more rostral levels or within the hypoglossal motonucleus at any level (Fig. 3A:2).

The morphological characteristics of the glutamate-immunoreactive neurones were examined in serial sections. Immunoreactive perikarya were either ovoid or round in shape, approximately 10μm in diameter and generally smaller than the glutamate-immunoreactive cells observed in the adjacent gracile nuclei. From an examination of the semi-thin sections the majority of glutamate-immunoreactive neurones were seen to have a smooth, unindented nuclei (Fig. 3A:3C). As only the perikarya and occasionally the proximal dendrites of neurones were immunostained with this antisera it was not possible to examine the dendritic morphology of these cells.

3A:3:2 GLUTAMATE-IMMUNOREACTIVE BOUTONS.

The nTS was densely innervated with immunoreactive boutons that were not associated with any particular structure or subnuclei within the full rostrocaudal extent of the nTS. The DVN and hypoglossal motonuclei also contained immunoreactive boutons that had a homogeneous distribution. Comparison of the three areas examined

FIGURE 3A:2

Low power light micrographs of 1μm sections showing the distribution of glutamate-immunoreactive neurones (arrows) at two levels of the right dorsomedial medulla. **A)** 0.1mm caudal to obex **B)** level of obex. Orientation bar: D, dorsal; L, lateral. Scale bar = 200μm.

ABBREVIATIONS: AP, area postrema; TS, tractus solitarius.

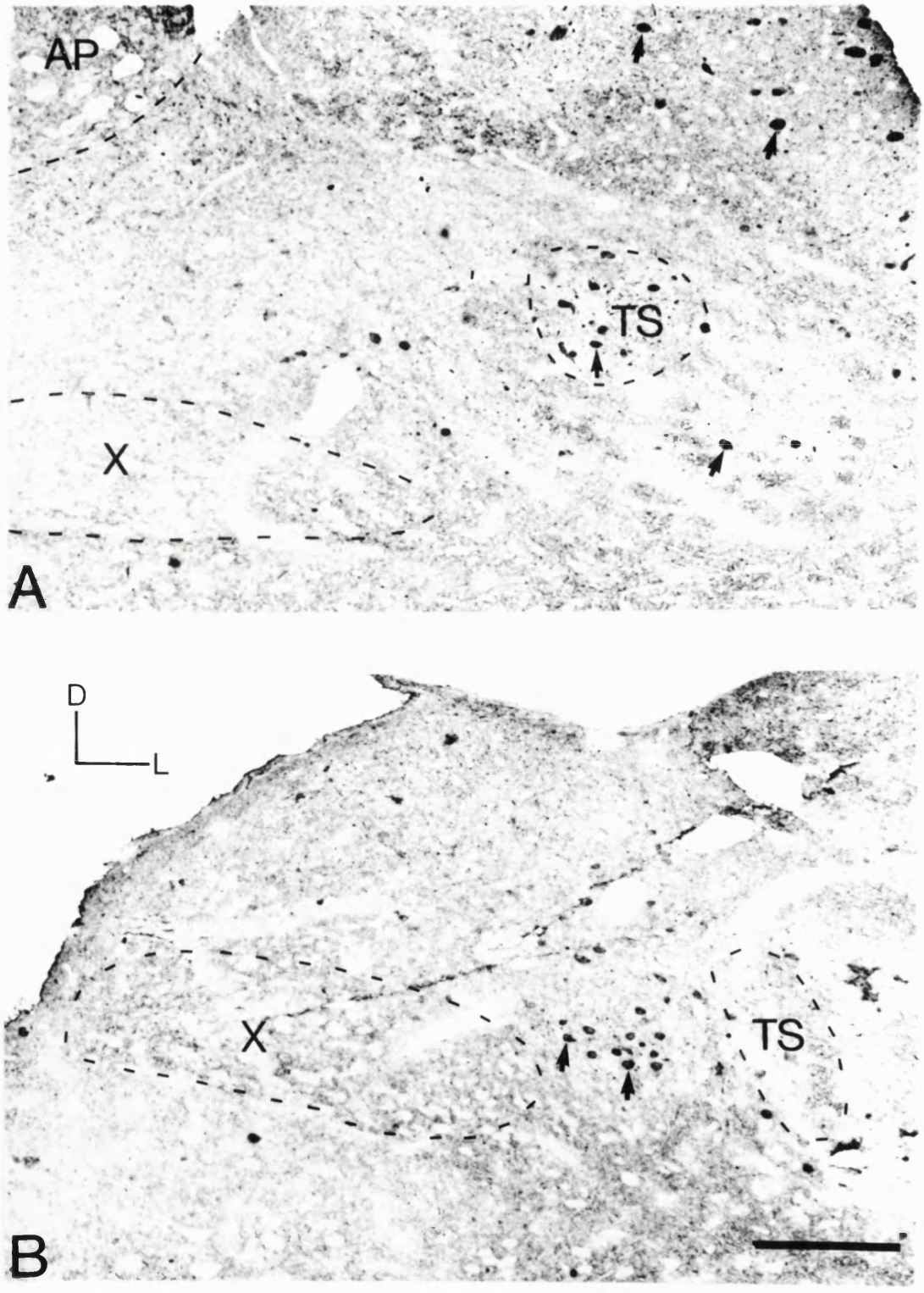

AP
TS
X
A
D
L
X
TS
B

FIGURE 3A:3.

Light micrographs demonstrating glutamate-immunoreactive boutons and perikarya in the right nTS. A) Medium power light micrograph of the rostral nTS (0.7mm rostral to obex) showing glutamate-immunoreactive neurones medial to the tractus (TS). Orientation bar: D, dorsal; M, medial. Scale bar = 50μm. B) High power light micrograph of one of the immunoreactive neurone shown in A). C) High power micrograph of immunoreactive (arrows) neurones and boutons (hollow arrows) in the ventrolateral subnuclei of the nTS at a level just caudal to obex (-0.1mm). Non-immunoreactive neurones (stars) can also be seen in the surrounding neuropil. Scale bar B & C =20μm.

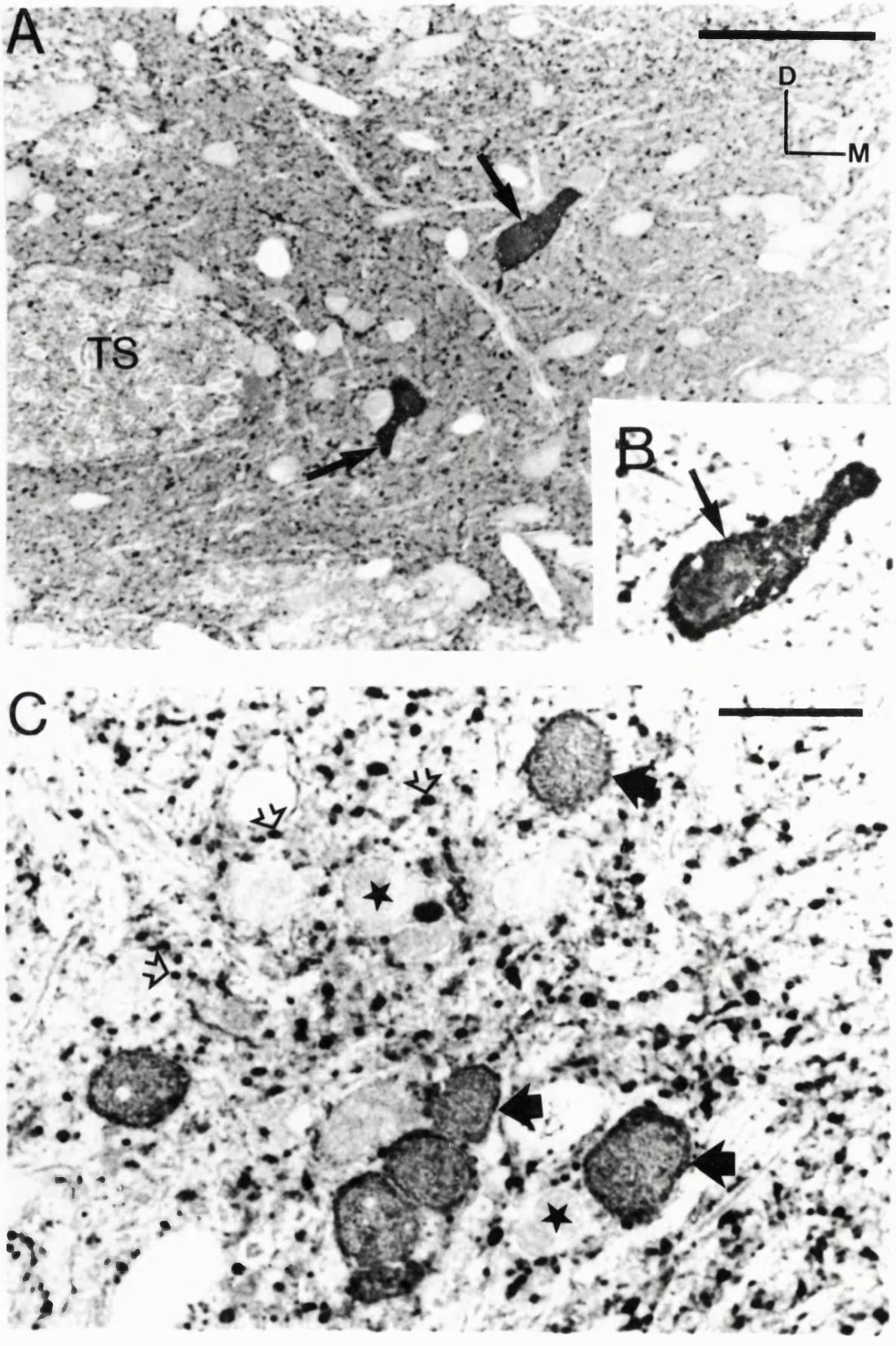
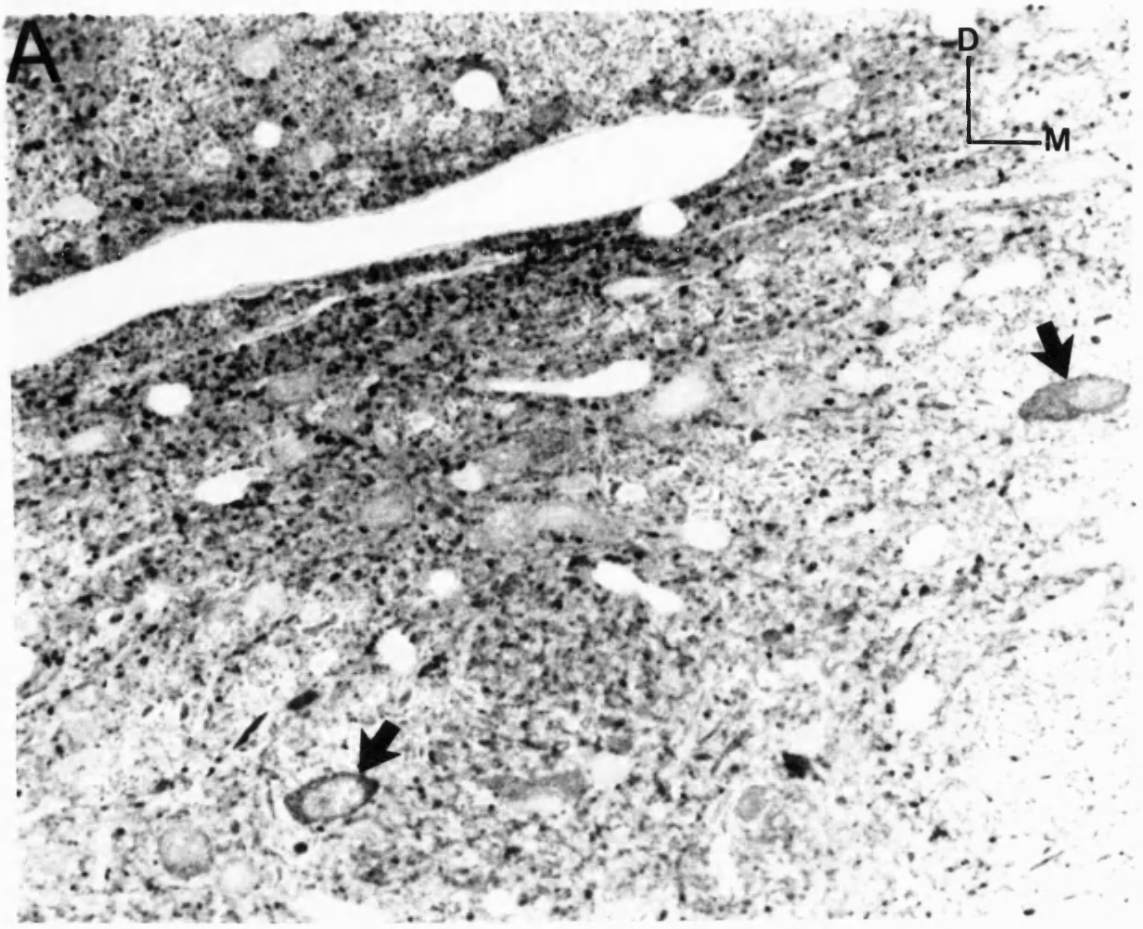
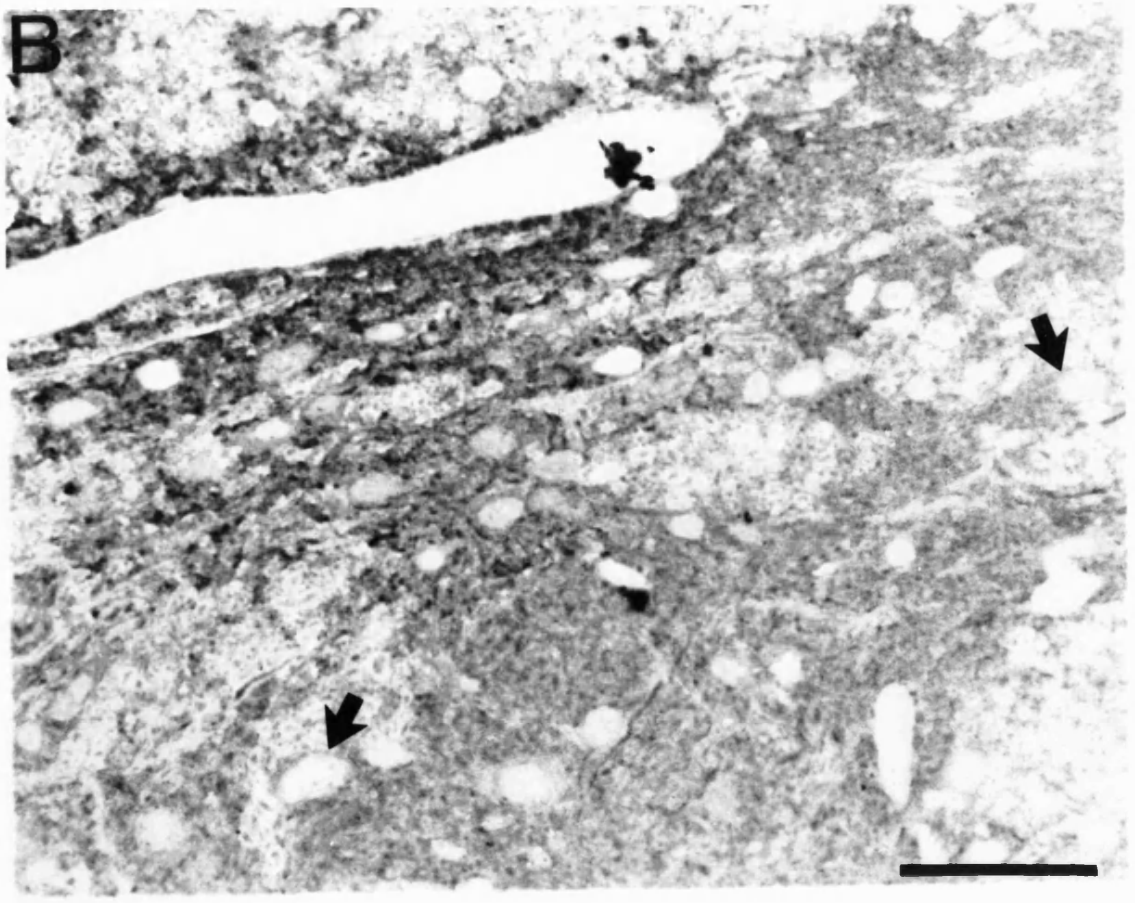

A
D
M
TS
B
C

FIGURE 3A:4.

Medium power micrograph of serial sections (**A** & **B**) in the left nTS immunostained with **A**) glutamate antisera and **B**) glutamate antisera following absorption with glutamate conjugated to haemocyanin. Note that absorption of the antisera abolishes neuronal (arrows) and presumptive bouton immunolabelling. Orientation bar: D, dorsal; M, medial. Scale bar= 50μm.


A
D
M


B

revealed a slight variation in the extent of innervation of the immunoreactive boutons with the greatest density in the hypoglossal motonucleus. The majority of cells in the nTS were in close apposition with immunoreactive boutons although the density of these contacts appeared greater on DVN and hypoglossal neurones.

3A:4 DISCUSSION.

In this study the presence of glutamate-immunoreactive perikarya and presumptive axonal structures have been demonstrated in the dorsomedial medulla and their distribution described using a post-embedding technique in combination with highly sensitive detection systems.

3A:4:1 TECHNICAL CONSIDERATIONS.

No immunostaining was observed when pre-embedding techniques were employed. The reason for this lack of immunostaining is unclear. However one possible explanation may relate to the the fact that the primary antisera is raised against a glutaraldehyde conjugate. The various steps involved in preparing the material for examination in the electron microscope may subtly alter the glutamate in the tissue so it more closely mimics the antigen that the antisera is raised against.

Using post-embedding methods variations in the degree of immunostaining between animals were observed. This variation occurred despite the fact that all the material was carefully processed using the same schedule. It is known from pilot studies and from other groups (Battaglia & Rustioni, 1988; Tracey et al., 1991) that the pattern of glutamate immunostaining is critically dependent on the fixation of the material. It is possible that minor differences in the perfusion technique led to

variations in the speed and the degree of fixation and therefore, the subsequent immunostaining.

Sections immunostained with the ABC technique combined with peroxidase visualization with the DAB reaction revealed only lightly stained glutamate-immunoreactive axonal structures. The signal to noise ratio was considerably improved by the use of more sensitive techniques e.g. the IGS and ABC technique combined with the VIP substrate kit which resulted in neurones and presumptive boutons immunostained well above background. The organization of glutamate-containing boutons and perikarya was identical with both these immunocytochemical techniques demonstrating that the increased level of immunostaining observed was due to increased sensitivity and was not an artefact of the detection system.

The antisera employed in this study recognizes glutamate in glutaraldehyde fixed material and has been extensively characterized (Hepler et al., 1988). No cross-reactivity was detected when the antisera was tested against a series of different amino acid conjugates including aspartate when used at optimum dilutions. The specificity of the antisera in the material used in this study was confirmed by the lack of immunostaining obtained when the antisera was replaced with non-immune serum or was absorbed with glutamate conjugated to haemocyanin with glutaraldehyde (Fig. 3A:4) whilst only very weak immunostaining was observed after absorption with glutamate alone.

Although it is difficult to prove conclusively that the immunolabelling achieved with the glutamate antisera is related to neurotransmitter glutamate rather than glutamate in metabolic pools there is some indirect evidence that the immunolabelling observed in this study may be associated with glutamate involved in neurotransmission. The distribution of glutamate-immunoreactive neurones in the dorsomedial medulla did not

correspond with areas with high metabolic activity (Torrealba & Munoz, 1992). For example, neurones in the DVN which have high levels of the metabolic marker, cytochrome oxidase (Torealba & Munoz, 1992), were not immunoreactive. This is in contrast to a previous study that demonstrated many glutamate-immunoreactive neurones in this nuclei (see Fig. 10, Ruggierio, 1994). In addition, previous studies using the same antisera have demonstrated glutamate-immunoreactive pyramidal neurones in the cortex (Conti et al., 1987, 1989), a population of neurones that had been suggested from other evidence to be glutamatergic (for review see Fonnum, 1981). Therefore, the presence of higher levels of glutamate in putative boutons and only a specific population of neurones detected with highly selective antisera together with previous studies suggesting a functional role for glutamate in this area (see Introduction for refs.) is strongly suggestive that these immunoreactive-structures utilize glutamate as a neurotransmitter

3A:4:2 GLUTAMATE-IMMUNOREACTIVE NEURONES.

This study identifies a population of glutamate-immunoreactive neurones in the nTS which are of a similar size and morphology. From the appearance of the cell bodies it appears that the majority of these immunoreactive perikarya may be orientated in a rostrocaudal direction. Although not extensively studied these neurones have smooth nuclei in contrast to the indented nuclei of neurones commonly observed containing GABA in the cat nTS (Izzo et al., 1992). The numbers of immunoreactive neurones in this study are likely to be an underestimation as the levels of glutamate in neuronal perikarya were generally near the limits of detection, unlike the axonal boutons which were observed even when less sensitive detection methods were used. Neuronal immunolabelling was observed in the intermediate nTS with a compact collection of immunoreactive neurones observed within and just medial to the tractus, in a region that closely corresponds to the subnucleus centralis (see Altshculer et al.,

1989). There is some indirect evidence that the group of immunoreactive neurones observed in this area may project to the rostral ventrolateral medulla (RVLM). A discrete projection has been shown from this area to the retrofacial nucleus and nucleus ambiguus (Cunningham & Sawchenko, 1989; Ross et al., 1985) which is suggested to be involved in the tonic and reflex control of blood pressure and oesophageal motility (see Cunningham & Sawchenko, 1990 for review). Further evidence comes from the demonstration of an [^{3}H]aspartate uptake mechanism on nTS neurones that project to the RVLM (Somogyi et al., 1989). A somatostatin, enkephalin and SP-containing pathway has also been shown from the nTS to the RVLM (Cunningham et al., 1991; Cunningham & Sawchenko, 1989; Morilak et al., 1989) raising the possibility that glutamate is co-localized with these neuropeptides.

3A:4:3 PRESUMPTIVE GLUTAMATE-IMMUNOREACTIVE BOUTONS.

Presumptive glutamate-immunoreactive boutons were numerous throughout the nTS as well as adjacent motonuclei. There was no apparent difference in the density of immunoreactive boutons in the various subnuclei of the nTS although there was some variation in density between the different nuclei of the dorsomedial medulla. Previous studies have provided evidence that glutamate in the nTS is functionally involved in baro- and chemoreceptor reflexes, respiration and deglutition (see Introduction for refs). The density and distribution of putative glutamate-immunoreactive boutons in the nTS support a role for glutamate in these physiological functions. Furthermore, the homogeneous distribution of glutamate-immunoreactivity throughout the nTS combined with the possibly that all neurones in this region are in synaptic contact with immunoreactive boutons suggests that this excitatory amino acid may be involved in the whole range of nTS functions.

3A:4:4 CONCLUSIONS.

In summary, this study demonstrates that the nTS is densely innervated with glutamate-immunoreactive presumptive boutons implying an important role for this excitatory amino acid in the functions of this area. The presence of a discrete group of immunoreactive neurones in the medial region of the intermediate nTS raises the possibility that these neurones may project to the RVLM and may be involved in tonic and reflex cardiovascular and oesophageal control.

CHAPTER 3B

Demonstration of glutamate in vagal sensory afferents: a combined anterograde tracing and immunocytochemical electron microscopic study.

CONTENTS

3B:1 INTRODUCTION.

Although it has been established that glutamate is contained within dorsal root perikarya and primary afferent terminals in the spinal cord (Battaglia & Rustioni, 1988; DeBiasi & Rustioni, 1988; Merighi et al., 1991; Wanaka et al., 1987) the presence of this excititatory amino acid in vagal sensory afferents is still controversial. Nodose ganglionectomy or intracranial section of the vagus and glossopharyngeal nerves was reported to cause an ipsilateral decrease in glutamate (Dietrich et al., 1982; Meeley et al., 1989; Perrone, 1981; Reis et al., 1981) but results from other studies also employing indirect approaches have been conflicting. A difference in the high affinity uptake of glutamate after nodose ganglionectomy between ipsi- and contralateral sides and control animals was reported by Perrone (1981) but these results were not confirmed by other studies using comparable methods (Simon et al., 1985; Sved et al., 1986). Some studies have utilized the fact that the metabolically stable compound D-aspartate is taken up by the same uptake system as L-glutamate (Streit, 1980). Following injection of [^{3}H] D-aspartate into the nTS Schaffer and co-workers (1990) detected significant numbers of labelled cells in the nodose ganglia but only occasional labelled cells were detected using the same technique in another study (Backes & Sved, 1990). Another approach has been to measure the release of glutamate in the nTS following electrical or natural stimulation of vagal afferents (and carotid sinus nerve afferents) with push-pull cannulae (Granata & Reis, 1983) or *in vivo* microdialysis (Allchin et al., 1992; Lawrence & Jarrott, 1994; Sved & Salter, 1990). Again the indirect nature of the techniques used resulted in disparaties in the results obtained from these studies.

In the present study post-embedding immunocytochemical techniques at the electron microscope level were used to confirm the presence of glutamate in synaptic terminals in the nTS and identify their morphological characteristics. In addition, this study investigates glutamate-immunoreactivity in sensory afferents of the vagus nerve.

3B:2 METHODS.

3B:2:1 ANTEROGRADE LABELLING.

Vagal afferents were labelled in 12 male Sprague-Dawley rats (275-325g) following the unilateral injection of HRP into the nodose ganglia as described in section 2:2:1. Animals were allowed to survive for 48 hours before they were perfusion fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1M PB. The brains were removed, post-fixed overnight and coronal sections of the medulla cut at 80μm on a vibrating microtome.

3B:2:2 HRP HISTOCHEMISTRY.

The transported HRP was revealed using the TMB or DAB histochemical reaction (see section 2:2:3).

3B:2:3 TISSUE PROCESSING

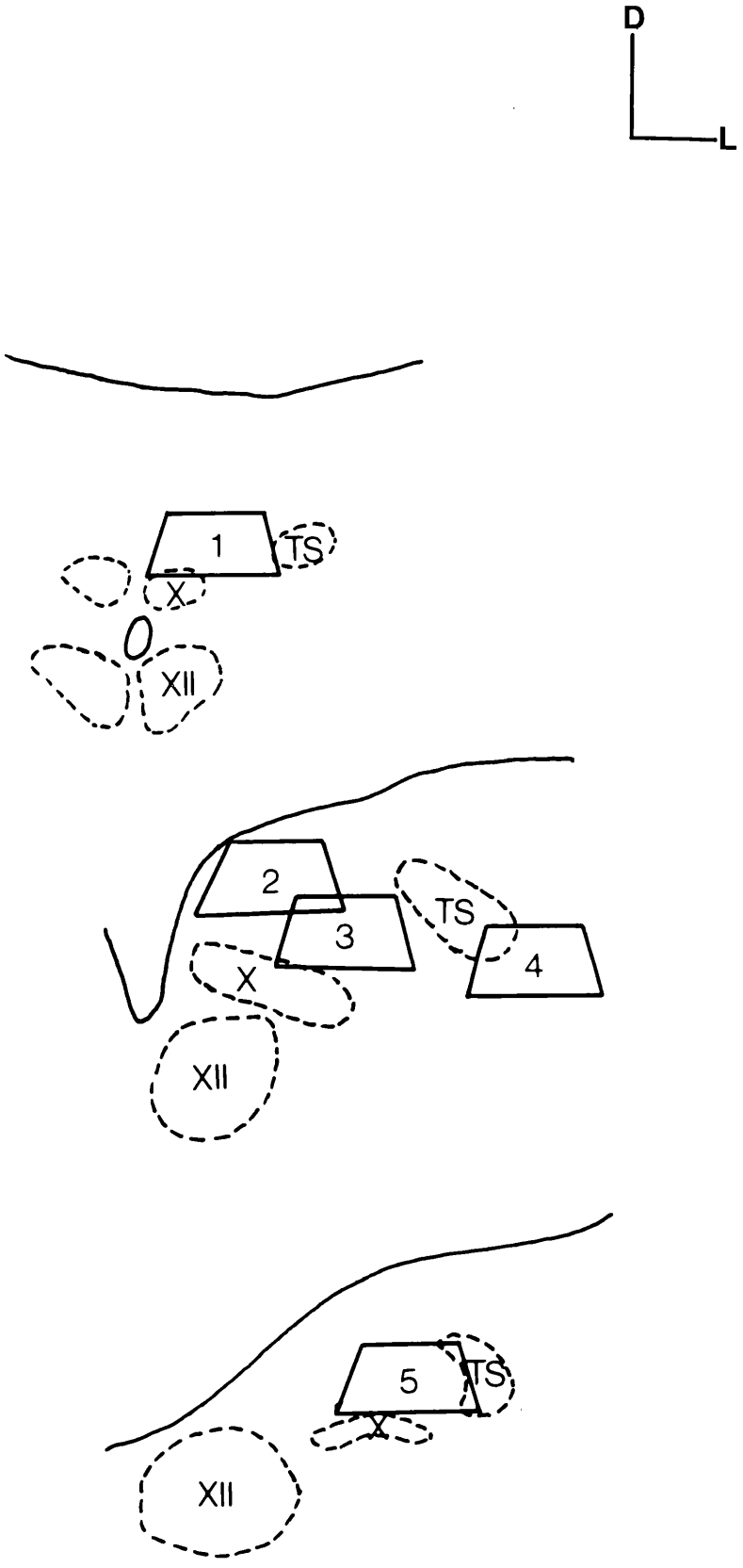
The tissue was washed well in buffer and then post-fixed for 1 hour in 1% osmium tetroxide, dehydrated and embedded in resin as described in section 2:2:4.

3B:2:4 POST-EMBEDDING GLUTAMATE IMMUNOCYTOCHEMISTRY ON ULTRATHIN SECTIONS .

Different regions of the nTS (see Fig. 3B:1) from 4 animals, containing anterogradely labelled sensory vagal afferents, were identified at the light microscope level and re-embedded prior to sectioning. These animals had good ultrastructural preservation and prior immunostaining on 1μm resin sections had revealed high levels of glutamate immunoreactivity. Gold/silver sections were cut on an ultramicrotome and collected on plastic-coated nickel slot grids. These sections were immunostained for glutamate immunocytochemistry following the procedures described by de Zeeuw et al., (1988). All the incubations described below were carried out at room temperature by floating the grids section side down on drops of reagents that had been filtered (Millipore; 0.22μm pore size) in a humidity chamber. Sections were first washed in 0.2M TPBS pH7.4 containing 0.02% Triton X-100 (TPBS-Tx) and 1% NGS before incubation overnight in rabbit anti-glutamate diluted 1:1000-1:10 000 in the same buffer. After several washes in TPBS-Tx pH7.4 and followed by TPBS-TX pH8.2 the grids were incubated in goat anti-rabbit IgG conjugated to 15nm colloidal gold diluted 1:10 with TPBS-Tx pH8.2 for 2-3 hours. The grids were washed in TPBS-Tx pH8.2 and then in several changes of distilled H_2O. To increase contrast of the material the sections were stained with 1% aqueous uranyl acetate for 30 minutes followed by lead citrate. The grids were examined in an electron microscope (Phillips 201) where the morphology and type of synapse of all boutons was documented and photographs taken where appropriate.

FIGURE 3B:1.

Diagramatic representation of coronal sections through extent of the right dorsomedial medulla demonstrating the approximate position of material taken for electron microscope examination. Orientation bsr: D, dorsal; L, lateral. Scale bar = 0.5mm.


D
L
1
TS
X
O
XII
2
3
TS
4
X
XII
5
TS
X
XII

3B:2:5 QUANTITATION AND ANALYSIS.

Forty seven boutons, identified as vagal afferents by the presence of HRP reaction product, were assessed for glutamate immunoreactivity by counting gold particles on electron micrographs. Photomicrographs were printed to a final magnification of x60 000. The areas of the axonal profiles were computed and the gold particles counted. Particles over mitochondria were excluded from the counts since the anti-glutamate anti-sera is known to cross-react with intermediates in the Krebbs Cycle (Fonnum et al., 1988). The value for the area of each profile was used to calculate the number of gold particles/μm^2. Gold particle densities were also determined over at least 3 dendrites in each photomicrograph to assess background labelling. A vagal afferent was judged to be glutamate-immunoreactive if the concentration of gold particles over the profile exceeded the 99% confidence limit (mean + 2.576% standard deviations) of the concentration of gold particles over dendrites in the same micrographs. In addition, 13 boutons forming asymmetric type synapses and 12 boutons forming symmetric type synapses, but not containing HRP reaction product, were analysed as described above.

3B:2:6 CONTROLS FOR IMMUNOCYTOCHEMICAL DETECTION OF GLUTAMATE.

The specificity of the primary antisera in these experiments was confirmed by replacing the glutamate with non-immune serum. Sections were also incubated with primary antisera previously absorbed with glutamate conjugated to haemocyanin with glutaraldehyde or glutamate alone. Furthermore, in one experiment immunostaining was compared, in serial section, to immunostaining using antisera to GABA (Arnel).

3B:3 RESULTS.

Examination of ultrathin sections revealed that colloidal gold particles indicative of glutamate immunoreactivity covered a proportion of boutons and mitochondria generally. Terminals containing vesicles that were labelled with many gold particles sometimes lay next to or near terminals containing vesicles that were labelled with very few gold particles. Other neuronal elements e.g. dendrites and glial processes had a low gold particle density that was never above background levels (Fig. 3B:2,3,4 & 5).

3B:3:1 MORPHOLOGY OF GLUTAMATE-IMMUNOREACTIVE BOUTONS.

Gold level density over axonal profiles was compared with the density over surrounding dendrites. Boutons were considered to be glutamatergic if they had a gold particle density equal or greater than 42.4μm^2 (mean+ 2.576 X S.D.; 99% confidence levels). Of the 13 boutons forming asymmetric type synapses 12 (92.3%) were labelled with 51 or more gold particles. The highest labelling over an axonal profile was 130.1 gold particles/μm^2 whist the mean level of labelling in the positive profiles was 111.9 +19.2. Immunogold labelled boutons were detected throughout the caudal, intermediate and rostral levels of the nTS. These boutons had common morphological characteristics e.g. they were of a similar size and contained round, clear synaptic vesicles (approximately 40-60μm in diameter) whilst some also contained dense core vesicles. Post-synaptic targets were most frequently small to medium-sized calibre dendrites although immunoreactive boutons in apposition to large calibre dendrites and perikarya including retrogradely labelled VPNs were also detected. Dendrites were often seen to receive multiple synaptic inputs, from both labelled boutons and unlabelled boutons (Fig. 3B:2D). Examination of alternate, serial sections

FIGURE 3B:2.

Electron micrograph of serial sections (**A** & **B**) in the nTS immunostained with glutamate (**A**) or GABA (**B**) antisera, respectively. Note that the glutamate and GABA antisera immunolabel different boutons and that these boutons form synaptic contacts with the same dendritic structure. **C**) and **D**) Examples of glutamate-immunogold labelled boutons in the nTS forming asymmetric synapses (arrows) with unlabelled dendrites. Unlabelled boutons (star) with background levels of gold particles are easily distinguished from the immunoreactive boutons. These boutons form symmetric type synapses with the dendrite. This type of synapse is characterized by a widening of the inter-cellular space, thickening of both the pre- and post-synaptic membranes and an acculmulation of synaptic vesicles near the synaptic cleft. Scale bar = 0.5μm.

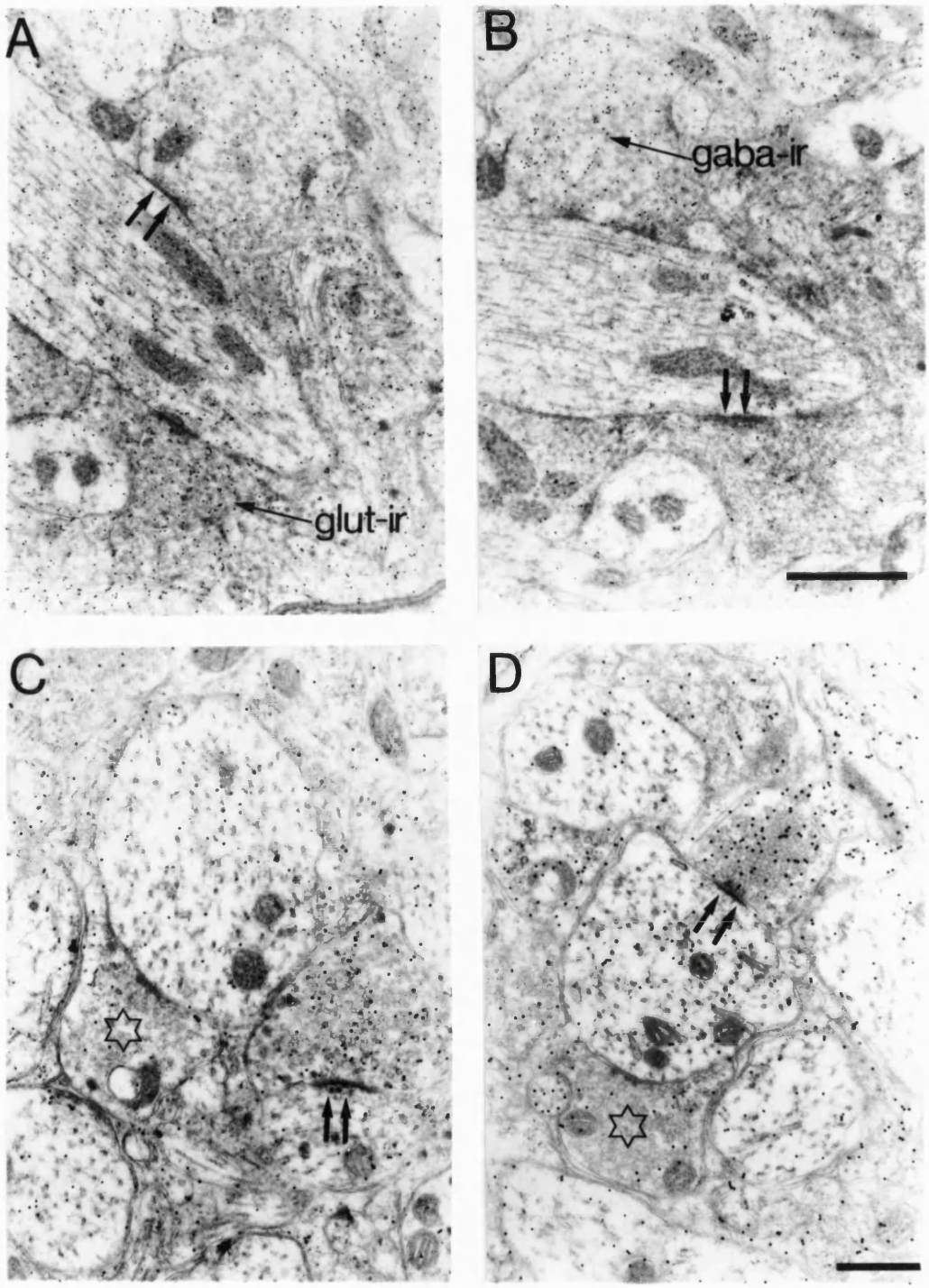

A
glut-ir
B
gaba-ir
C
D

FIGURE 3B:3.

Serial section of vagal afferent bouton, identified by TMB reaction product (hollow arrows), immunogold labelling with **A**) glutamate antisera and **B**) glutamate antisera following preadsorption of the primary antisera with glutamate conjugated to haemocyanin. As the sections were mounted on different grids the orientation of each micrograph is slightly different (see dendrites D1 and D2 for orientation). Scale bar = 0.5μm.

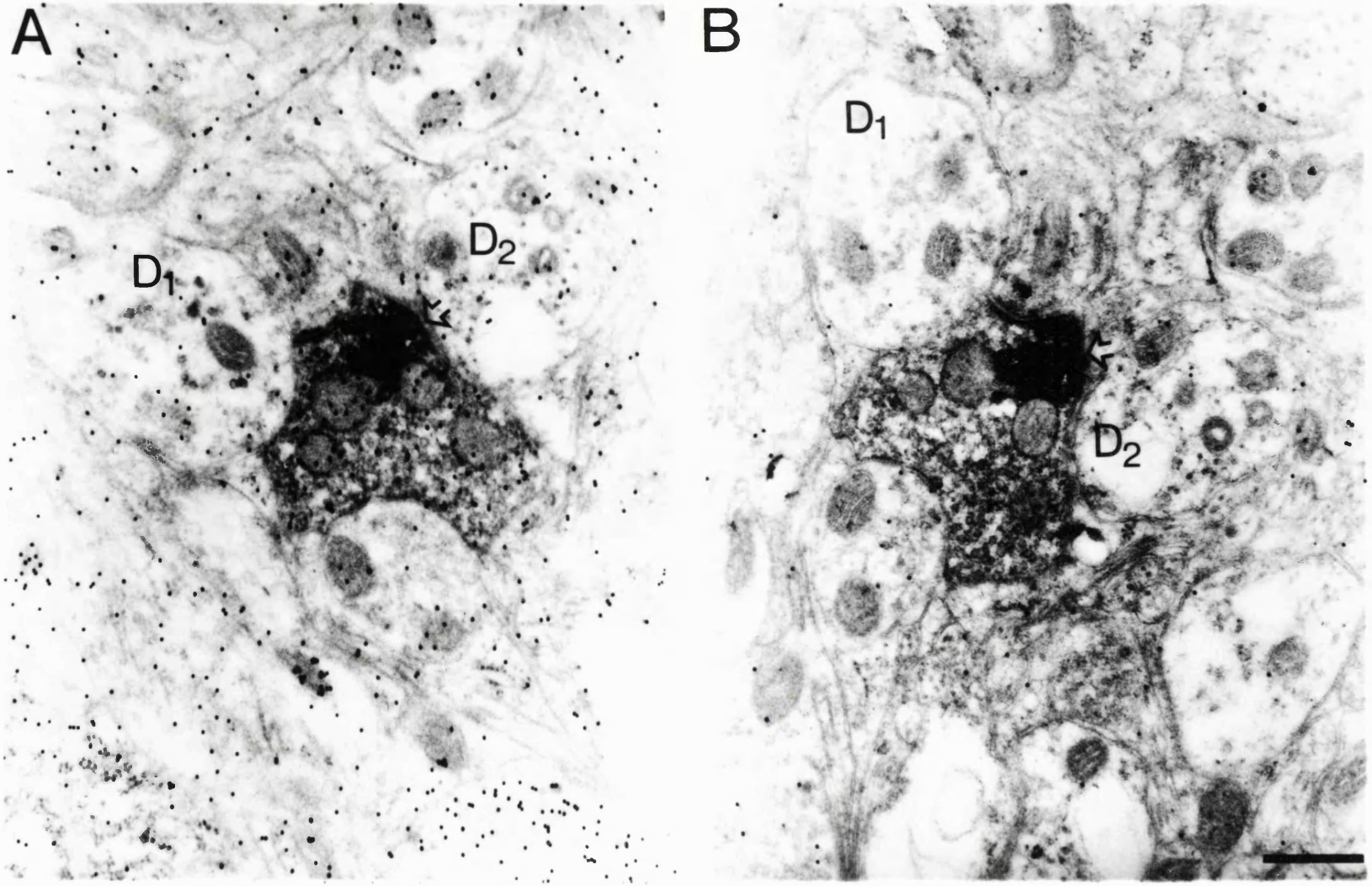

A
D1
D2
B
D1
D2

FIGURE 3B:4

Serial section of glutamate immunogold labelled vagal afferent (hollow arrow). Notice that the number of gold particles in both sections is higher than background (for comparison see bouton labelled with star). Scale bar = 0.5μm.

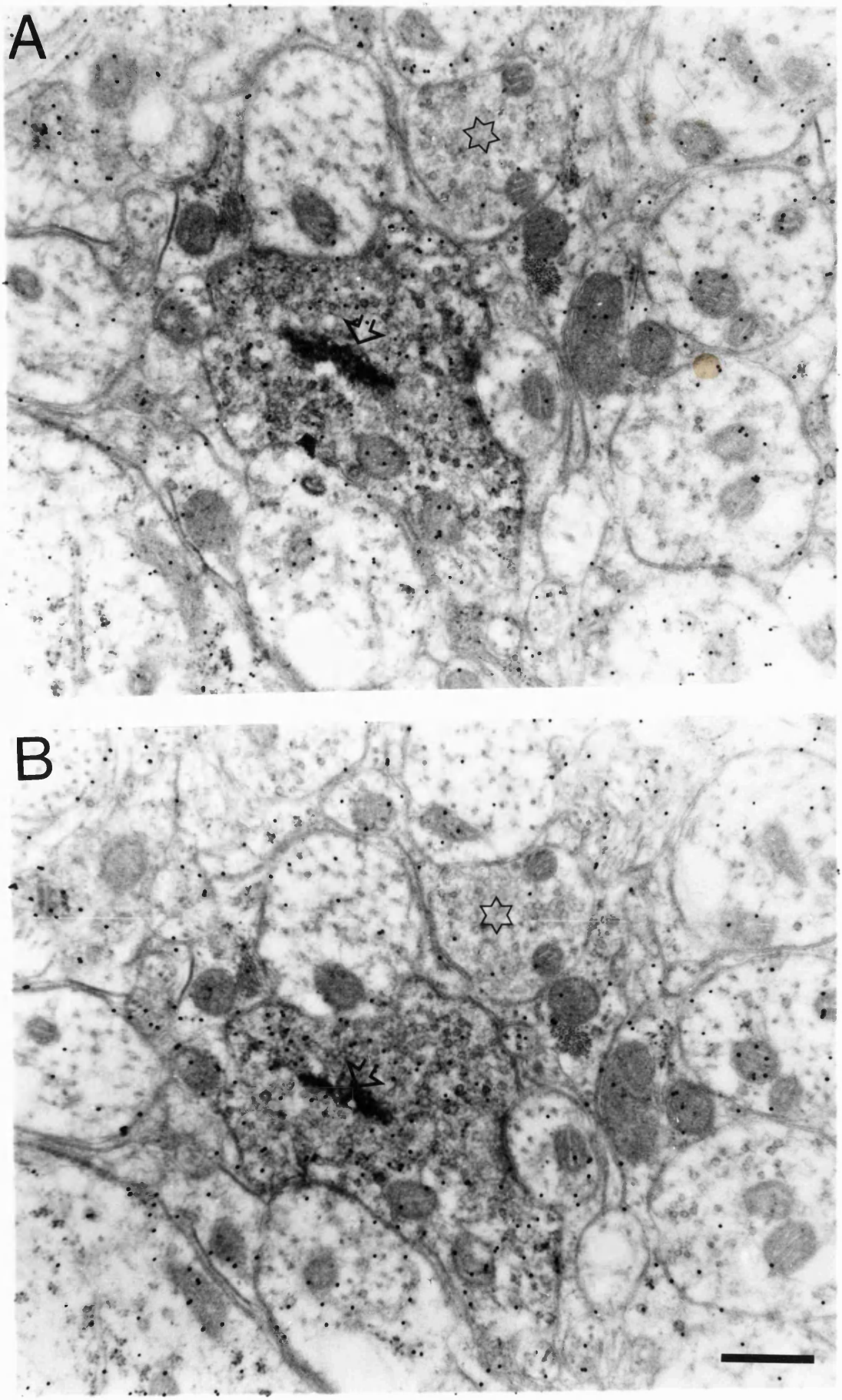
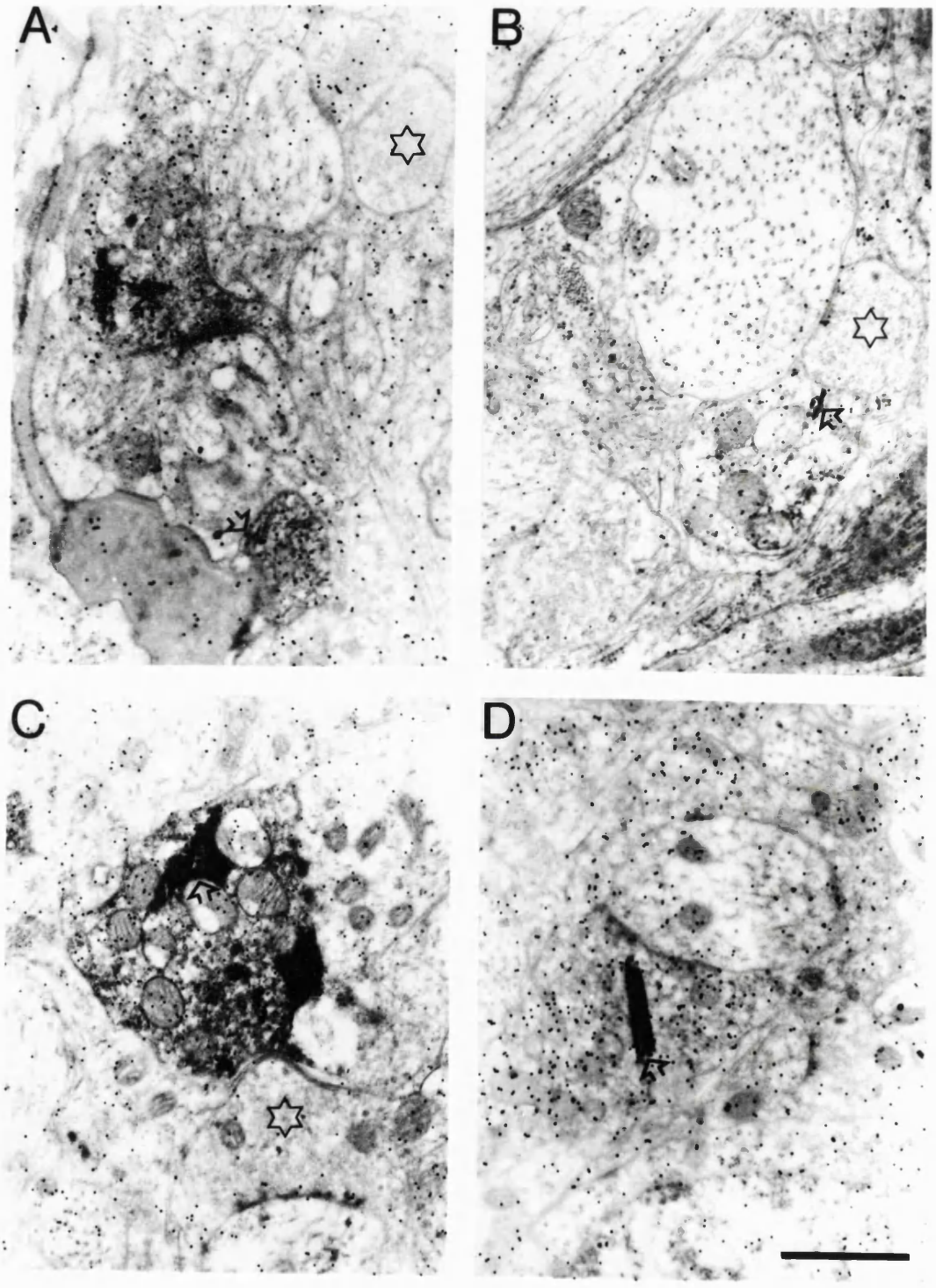

A
B

FIGURE 3B:5.

Further examples of labelled vagal afferents identified by TMB reaction product (hollow arrows) that have immunogold particle densities greater than surrounding structures. Unlabelled boutons (stars) contain only background levels of gold. Scale bar = 1μm.


A
B
C
D

immunostained to GABA and glutamate revealed that some dendrites receive multiple synaptic input from boutons immunoreactive for both these amino acids and that the synaptic contacts formed by boutons containing these two amino acids were distinguishable, with GABA-immunoreactive boutons forming exclusively symmetric synapses and glutamate-immunoreactive boutons forming asymmetric synapses (Fig. 3BA & B).

A small group of boutons with flattened or pleomorphic synaptic vesicles and making asymmetric synapses were observed that had only background levels of gold particles and were presumably immunonegative, as were all boutons forming symmetric synaptic contacts. Immunonegative boutons had a mean gold particle density of $17.8 \pm 8.6 \mu m^2$.

3B:3:2 GLUTAMATE-IMMUNOREACTIVE VAGAL AFFERENT TERMINALS.

Examination of material in this study concentrated primarily on material where the transported HRP was visualized with the TMB method as the reaction product from the DAB reacted material was hard to detect following the post-embedding procedures. Analysis of 47 boutons, identified as vagal afferents by the presence of HRP reaction product, revealed that 43 (91.5%) had levels of gold particles significantly greater than surrounding dendrites (mean + 2.576 X S.D.; 99% confidence limit). Labelled vagal afferents were detected throughout the rostrocaudal extent of the nTS (Fig. 3B:3A,4 & 5). However, the levels of immunogold labelling observed over these labelled afferents were never as high as that seen over the most intensely immunogold labelled boutons, none of which contained HRP reaction product. The mean level of gold particles over glutamatergic vagal afferents was $62.3 \mu m^2$ as compared to a mean of 111.9 gold particles/μm^2 for boutons forming asymmetric synapses but not containing HRP

reaction product.

3B:3:3 CONTROLS.

To evaluate the specificity of the glutamate antisera, in this material, ultrathin sections were incubated in the primary antisera preabsorbed with glutamate alone or glutamate conjugated to glutaraldehyde. Immunostaining was abolished following absorption with the conjugate (Fig. 3B:3). A few gold particles were observed over the sections but these were probably due to the secondary antisera. Very weak immunostaining was observed after absorption of glutamate alone. No immunostaining was detected when non-immune serum replaced the primary antisera. Furthermore, examination of alternate serial sections immunostained for either GABA or glutamate that terminals immunoreactive for GABA were never immunoreactive for glutamate and *vice versa* (Fig. 3B:2A & B).

3B:4 DISCUSSION.

The present study provides evidence that a population of boutons in the nTS utilizes glutamate as a neurotransmitter. These immunoreactive boutons have a similar morphological profile to each other and were identified as containing round, clear synaptic vesicles and occasionally dense core vesicles and forming exclusively asymmetric synapses. A proportion of the glutamate-immunoreactive boutons were further identified as originating from vagal sensory neurones.

3B:4:1 TECHNICAL CONSIDERATIONS.

As discussed previously the antisera used in this study recognizes both metabolic and neurotransmitter glutamate making the identification of structures that utilize glutamate as a neurotransmitter difficult. However, there was some evidence that the structures identified as immunolabelled in this study are glutamatergic. High densities of immunogold were localized over axonal terminals, particularly synaptic vesicles and synaptic contacts. Although mitochondria were also generally immunolabelled this was discounted as the labelling probably reflects glutamate synthesis in the mitochondrial matrix (Fonnum et al., 1988). Furthermore, examination of alternate sections immunostained for GABA or glutamate revealed that terminals were only immunoreactive with one of the antisera. Only background levels of immunogold were observed following immunostaining for glutamate over boutons identified as GABAergic on consecutive sections although glutamate is known to be involved in GABA synthesis and breakdown.

The major advantage of the post-embedding immunocytochemical technique used to detect glutamate in the nTS in this study, as compared to the indirect techniques used in other studies (see Introduction for refs.), is that it confirmed the presence of glutamate in synaptic structures and allowed the morphological features of these boutons to be identified. In addition, combining the immunocytochemical technique with anterograde labelling from nodose ganglion sensory neurones permitted the positive identification of vagal afferents in the same material. However, the possibility that the TMB histochemical reaction used to reveal the transported HRP interferred with the immunostaining had to be considered. Examination of material at the light and electron microscope level revealed that the TMB reaction appeared to cause a slight loss of immunostaining. In addition, the TMB reaction appeared to have a further effect on the immunostaining as the density of gold particles over boutons containing reaction

product rarely matched the density of the most intensely immunolabelled boutons.

3B:4:2 MORPHOLOGY OF GLUTAMATE-IMMUNOREACTIVE BOUTONS.

Examination of ultrathin sections immunostained for glutamate revealed a population of synaptic boutons that were presumed to be equivalent to the punctate structures observed at the light microscope level. All immunoreactive boutons contained round, clear synaptic vesicles whilst a proportion also contained low numbers of dense core vesicles. The glutamate-immunoreactive boutons observed in this study formed exclusively asymmetric synapses with their post-synaptic targets. This type of synaptic contact has been associated with excitatory transmission although it should be noted that indirect studies for GABA using antisera raised against glutamic acid decarboxylase (GAD), the synthetic enzyme for GABA, have reported immunoreactive boutons forming asymmetric synapses (for refs. see Ribak & Roberts, 1990). Boutons containing round, clear vesicles contained gold particle levels that varied from background to heavily labelled and therefore, it not possible to determine if this entire population of morphologically similar boutons are glutamate-containing.

Comparison of the glutamate-immunoreactive boutons observed in this study reveal similarities with those described in other regions of the CNS e.g. the hippocampus, spinal trigeminal nucleus (Clements et al., 1990), spinal cord (Clements et al., 1990, De Biasi & Rustioni, 1988), lateral geniculate nucleus (Montero & Wenthold, 1989) and cerebellum (Somogyi et al., 1986). Therefore, it is suggested that the presence of round, clear synaptic vesicles and the formation of asymmetric synapses with predominantly small to medium sized dendrites are common characteristics of glutamatergic terminals throughout the CNS.

A small population of boutons making asymmetric synapses but containing

flattened or pleomorphic synaptic vesicles were observed containing only background levels of immunogold and presumably do not utilize glutamate as a neurotransmitter. As many studies have demonstrated a close association between the presence of asymmetric synapses and excitatory synaptic input it is possible that these boutons utilize some other excitatory neurotransmitter.

3B:4:3 GLUTAMATE-IMMUNOREACTIVE VAGAL SENSORY AFFERENTS.

The results from this study demonstrate that vagal afferents contain elevated levels of glutamate. Previous studies have indicated that glutamate release in the nTS is enhanced following stimulation of the vagus nerve (Granata & Reis, 1983) supporting a possible role for glutamate in vagal afferents. This hypothesis is further supported by lesion studies that have demonstrated a decrease in glutamate following removal of the nodose ganglion (Meeley et al., 1989; Perrone, 1981; Reis et al., 1981). However, the findings in these studies have been challenged by other workers who failed to detect any changes in glutamate release following vagal nerve stimulation (Allchin et al., 1992; Sved & Salter, 1990) or in the high affinity glutamate uptake system following nodose ganglionectomy (Simon et al., 1985; Sved et al., 1986). Given the high density of glutamate-containing structures in the nTS it is therefore, not surprising that some studies failed to detect the relatively small proportion of glutamate originating from the nodose ganglia with the use of indirect techniques. The direct visualization of glutamate in vagal afferents strongly supports the role of this or a closely related amino acid in the vagal sensory afferent system.

Analysis of HRP labelled vagal afferents in the nTS revealed that a high proportion (91.5%) contained significantly higher levels of immunogold particles than surrounding dendrites. However, the results from the analysis of this material must be assessed cautiously. As discussed above the histochemical reaction had a detrimental

effect on the immunostaining. There was variability in the level of immunolabelling between experiments which might reflect a variation in levels of glutamate or differences in the fixation and processing procedures of individual animals. Finally, there is also the possibility that vagal afferents were damaged and their neurotransmitter content was affected during the injection procedure. Vagal afferents containing elevated levels of glutamate were detected throughout all regions of the nTS examined implying that this excitatory amino acid may be involved in a wide variety of functions.

3B:4:4 ORIGIN OF GLUTAMATE-IMMUNOREACTIVE TERMINALS IN THE NTS.

In order to determine the function of glutamate in the nTS it is important to establish the origin of this innervation. This study has demonstrated that the nodose ganglia supplies a proportion of the glutamate-immunoreactive boutons to this nuclei. It is possible that some of the HRP labelled boutons, identified as being glutamatergic in this study, originated from cell bodies located in the jugular or petrosal ganglia as these ganglia together with the nodose form a fused mass in the rat (Altschuler et al., 1989). The lack of terminal labelling observed in the dorsal vagal complex following spillage of HRP around the nodose ganglia strongly suggests that the labelled boutons do not originate from surrounding tissues. The contribution of the vagal afferent system to the total number of glutamate-immunoreactive boutons in the nTS is probably fairly small as reflected by the the very high density of the immunoreactive boutons and their homogeneous distribution, which did not reflect the organization of vagal afferents in this region (see chapter 2). Thus, glutamate terminals in the nTS must have additional sources. There is evidence that the afferent component of other cranial nerves e.g. the glossopharyngeal and carotid sinus nerves also contain glutamate (Lawrence & Jarrott, 1994; Okada & Miura, 1992). A number of studies have suggested that baroreceptor afferents that terminate in the nTS utilize glutamate as a

neurotransmitter (see Lawrence & Jarrott, 1994) whilst glutamate-immunoreactive neurones were detected in the petrosal ganglia, a proportion of which innervated the carotid sinus nerve (Okada & Miura, 1992). Further possible sources include the intrinsic glutamate-containing neurones identified in this study, other cranial nerve ganglia as well as the known excitatory projections to the nTS from other CNS regions (see Spyer, 1993).

3B:4:5 CONCLUSIONS.

In summary, this study suggests that glutamate is an important excitatory neurotransmitter not only of vagal sensory afferents but of nTS afferents generally. These immunoreactive boutons probably originate from multiple sources. Glutamate-immunoreactive boutons have similar morphological features and can be distinguished from GABA-immunoreactive boutons in the nTS.

CHAPTER 4

The central distribution of SP, CGRP and 5-HT in vagal sensory afferents in the dorsal medulla.

CONTENTS

4:1 INTRODUCTION.

The nature of the chemical transmitters and neuromodulators utilized by sensory neurones of the vagus nerve has been the subject of a number of studies (see Dockray & Sharkey, 1986). The majority of these have provided evidence to support the presence of SP, CGRP and 5-HT, all of which have been immunocytochemically localized in neuronal perikarya in the nodose ganglion (Gamse et al., 1979: Gaudin-Chazal et al., 1982; Helke & Hill, 1988; Katz & Karten, 1980; Ling et al., 1992; Lundberg et al., 1978; Nosjean et al., 1990; Thor et al., 1988). More recently, *in situ* hybridization studies have lent further support for the utilization of SP and CGRP by these neurones (Czyzyk-Krzeska et al., 1991).

Whilst there is some direct evidence to support the presence of these putative neurotransmitters/neuromodulators at the peripheral ends of vagal sensory afferents (Chery-Croze et al., 1988; Dey et al., 1990; Green & Dockray, 1987; Lindh et al., 1983; Springall et al., 1987) evidence of these substances at the central terminals has come mainly from indirect studies. For example, the loss of SP (Baude et al., 1989; Gillis et al., 1980; Helke et al., 1980; Lorez et al., 1983) and CGRP (Torrealba, 1992) from the nTS following lesions of the vagus nerve have suggested the presence of these peptides at the central ends of this nerve. Evidence that 5-HT is in the central ends of vagal nerve afferent neurones has been suggested by the demonstration of [^{3}H]5-HT uptake from the nTS and its subsequent transport to neurones in the nodose ganglia (Gaudin-Chazal et al., 1982b; Nosjean et al., 1990).

The aim of the present study was to establish whether SP, CGRP and 5-HT are present in the central terminals of vagal sensory neurones of the rat and, if so, to determine whether these neurochemicals are restricted to any particular afferent system

by comparing their pattern of termination with the previously described viscerotopic organization of these afferents. To achieve these aims anterograde labelling from the nodose ganglion was combined with immunocytochemistry to detect either SP, CGRP or 5-HT. Double-labelled structures were detected using a laser scanning confocal microscope and computer aided image capture system to increase both the ease and reliability of analysis.

4:2 METHODS.

4:2:1 SURGICAL PROCEDURES.

Male Sprague-Dawley rats (220-375g) were used throughout this study. The anaesthesia, surgical and perfusion procedures are described in detail in chapter 2.

4:2:2 ANTEROGRADE TRACERS.

The first part of this study involved ultilising a number of anterograde tracers to find a technique which would successfully and reliably label vagal afferents.

a. Horseradish peroxidase combined with histochemistry.

Multiple pressure injections of 20% HRP (w/v) were made into the right nodose ganglion of 6 rats (220-250g). After a survival period of 48 hours the animals were perfusion fixed with 4% paraformaldehyde and 0.1% glutaraldehyde. A low concentration of glutaraldehyde was used as this fixative has an adverse effect on antigenicity of the putative neurotransmitters. Vibratome sections (60μm) were reacted

using the *o*-tolidine method (see section 2:2:3b). The nodose ganglion of one animal was injected with HRP and intensification with cobalt chloride was omitted from the stabilization procedure of the subsequent *o*-tolidine reaction. In a further animal the immunofluorescent procedure was performed prior to the sections being reacted with the *o*-tolidine method. For comparison, a few sections were first reacted with the o-tolidine method and then incubated sequentially in the primary and secondary antisera.

b. Detection of horseradish peroxidase using anti-peroxidase antisera.

The right nodose ganglia of 2 rats were injected with 20% HRP (w/v) and following a 48 hour survival period the animals were perfusion fixed with 4% paraformaldehyde. To aid penetration of the antibody, frozen sections (60μm) were prepared and Triton 100-X added to the primary antibody. A few sections were reacted with the *o*-tolidine method to ensure that good anterograde labelling had been achieved. The remainder were incubated in goat anti-peroxidase (1:50-1:100; Jackson ImmunoResearch Laboratories Inc. USA) with or without 0.1% Triton 100-X for 5 hours. After washing in phosphate buffered saline (PBS) the sections were incubated for 2 hours in donkey anti-goat conjugated to Texas Red (1:50; Jackson ImmunoResearch Laboratories Inc. USA).

c. Biocytin.

A solution of 5% biocytin (Sigma Chemicals Co.) in 2M sodium methyl sulphate was injected into the right nodose ganglion of 3 animals. Following a 24 hour survival period the rats were perfusion fixed with 2% paraformaldehyde and 1% glutaraldehyde. Higher concentrations of glutaraldehyde do not appear to affect the detection of biocytin. Sections, cut on a vibrating microtome, were incubated in biotinylated-strepavidin conjugated to HRP (1:5; Amersham International) for 2 hours

and peroxidase activity was revealed using p-phenylenediamine and pyrocatechol (Hanker et al., 1977) with nickel and cobalt intensification.

d. Biotinylated-spacer-peroxidase.

An aqueous solution of 2.5% biotin-spacer-peroxidase (Amersham International) was injected into the right nodose ganglion of 2 rats. After 24 hours the animals were perfusion fixed with 3% paraformaladehyde and 1% glutaraldehyde. The success of the injection was tested by reacting a few sections with the *o*-tolidine method whilst the remainder were incubated in biotinylated-streptavidin conjugated to fluorescein (1:50; Amersham International) with 0.1-0.5% Triton 100-X for 2 hours.

e. *Phaseolus vulgaris* leuco-agglutinin.

Multiple pressure injections of *Phaseolus vulgaris* leuco-agglutinin (PHA-L) were made into the right nodose ganglion (n=2). Due to the slow transport of the tracer the animals were allowed to survive for 13 days post-injection. The tissue was perfusion fixed with 4% paraformaldehyde and 50-80μm sections cut on a freezing microtome. To detect the lectin sections were incubated in goat anti-PHA-L (1:1000; Vector) for 18-40 hours at 4°c followed by rabbit anti-goat conjugated HRP (1:50; Dakopatts) for 2 hours. Peroxidase activity was revealed using DAB (50mg/ml in 0.05M Tris-HCl pH7.2, containing 0.01% H_2O_2) for 5-10 minutes.

f. Cholera toxin subunit-B.

Multiple pressure injections of cholera toxin subunit-B (CT-B; 0.5mg/30μl dist. H_2O; List Laboratories) were made slowly into various sites in the ganglion

(approximately 20-30µl in total) of 14 rats (250-320g). After a 48 hour survival period the animals were re-anaesthetized and perfusion fixed with 4% paraformaldehyde with or without 0.05% glutaraldehyde in 0.01M PB (pH7.4). Coronal sections of the medulla were cut (30-40µm) on a vibrating microtome.

4:2:3 ANTEROGRADE LABELLING COMBINED WITH IMMUNOFLUORESCENT DETECTION OF SP, CGRP AND 5-HT.

Only HRP and CT-B produced dense labelling of vagal afferent axons in the medulla. Therefore, only these tracers were combined with immunofluorescent detection of putative neurotransmitters in further experiments.

a. Horseradish peroxidase.

Sections were incubated in either rabbit anti-SP (1:5000; Eugene Tech) or rat anti-5-HT (1:6000; SeroTech) and 0.2% Triton 100-X. The tissue was incubated in the primary antisera for 12-36 hours on a rocker table at 4°c and the antigens were visualized with a biotinylated-streptavidin-fluorescein detection system (1:50). In one experiment the immunofluorescent procedure was performed prior to the sections being reacted with the *o*-tolidine method.

b. Cholera toxin subunit-B.

To reveal the transported CT-B sections were incubated in goat anti CT-B (1:5000-1: 10 000; List Laboratories) for 48-72 hours at 4°c with 0.1-0.2% Triton 100-X. Rabbit anti-SP (1:1000; Eugene Tech), rabbit anti-CGRP (1:1000; Affinity) or rat anti-5-HT (1:2000; SeroTech) were added to the sections and incubated for a further 24 hours. After washing in PBS fluorescein isothiocyanate (FITC)-labelled donkey anti-

goat IgG and Texas red-labelled donkey anti-rabbit IgG (for the detection of the SP or CGRP) or donkey anti-rat conjugated to Texas red (for the detection of 5-HT) were added for 2 hours. All secondary antibodies were diluted with PBS to give a final dilution of 1:50. Sections were subsequently washed in PBS, mounted onto slides and coverslipped with an aqueous mountant (Citifluor; National Diagnostics). All the fluorescent-labelled secondary antisera were obtained from Jackson ImmunoResearch Laboratories Inc., USA.

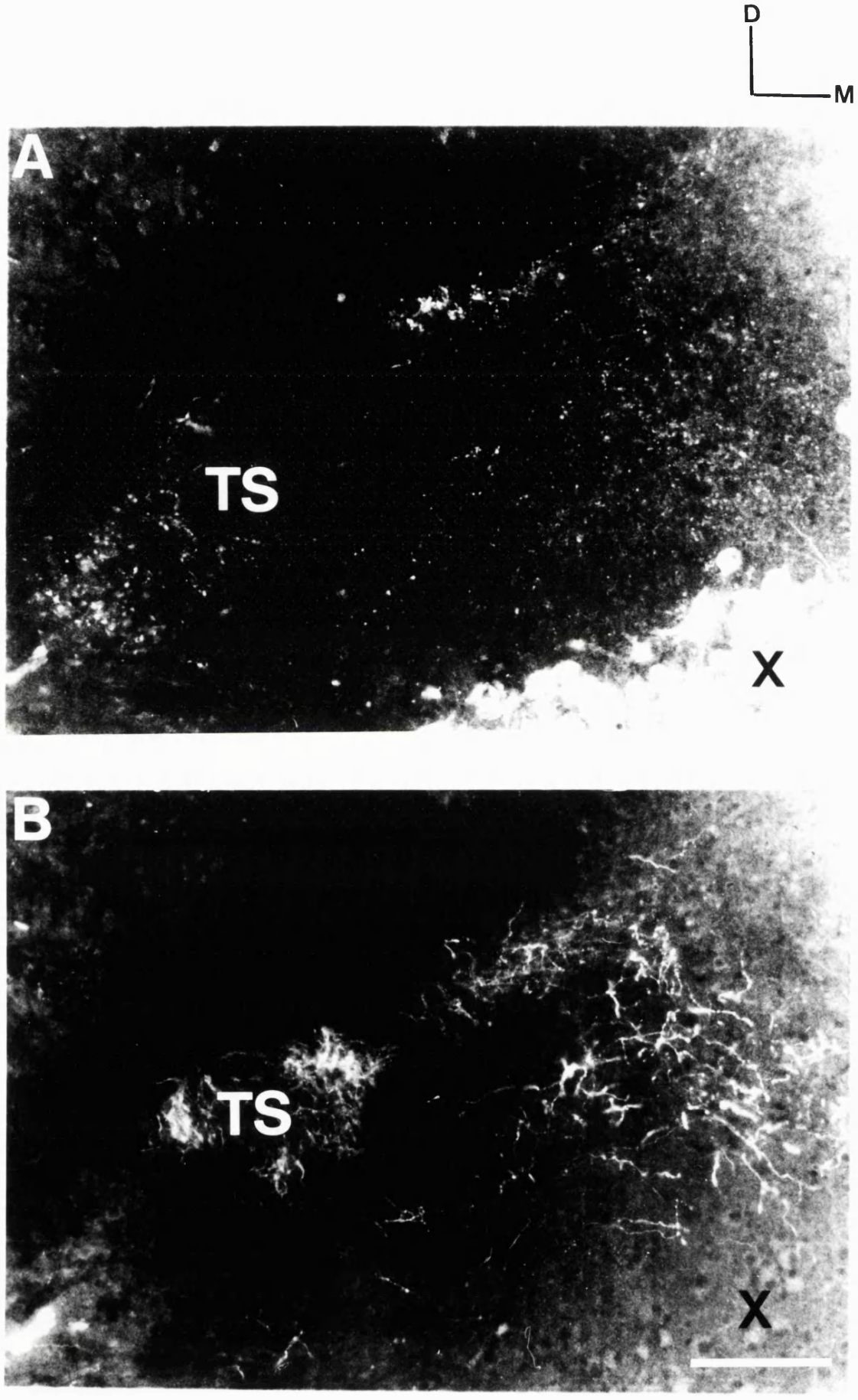
4:2:4 ANALYSIS OF SECTIONS.

As only CT-B could be combined successfully with the immunofluorescent detection of putative neurotransmitters all sections for analysis were taken from this set of experiments. Sections were examined initially on an Olympus Vanox fluorescent microscope equipped with filters having excitation wavelengths 450-490nm and 510-560nm. Only experiments that exhibited dense and entensive afferent labelling and strong 5-HT, SP or CGRP immunofluorescence were analyzed on the laser scanning confocal microscope.

Sections were examined on a laser scanning confocal microscope using a Krypton/Argon laser and equipped with an image capture system (Bio-Rad MRC-600). The microscope was operated in the double channel mode so that Texas red-labelled structures were recorded in one channel and FITC-labelled structures in the other channel. The tissue was examined with a x40 oil immersion objective as this gave the brightest image and collected a suitable amount of data from each field (110 x 80μm). Optical sections were collected at 2μm intervals from each field using the ZSERIES command. The number of optical sections taken from each position varied but averaged about 12. The total projection through the section was displayed on a high

FIGURE 4:1.

Low power fluorescence micrographs of an individual section of the left nTS to show concurrently both anterogradely labelled vagal afferents and CGRP immunoreactivity. A) Vagal afferent axons and preganglionic motoneurones labelled following the injection of CT-B into theipsilateral nodose ganglion. B) Micrograph of the same section showing CGRP-immunoreactive axonal labelling in and around the tractus solitarius (TS). Orientation bar: D, dorsal; M, medial. Scale bar= 100μm.


D
M
A
TS
X
B
TS
X

resolution monochrome monitor with each channel displayed individually (see Fig. 4:3A & B). Photographs of each field were taken on Kodak Technical Pan 2415 film and high contrast prints obtained. Images were also dispalyed on a colour monitor with the two channels represented by different colours (CT-B in green and the putative neurotransmitter in red) and the two images then superimposed. All digitised images were stored on optical disc (Magdisc). Any structures that were detected on both the channels were seen in yellow (see Fig. 4:3C & D). All double-labelled axonal structures were recorded and their location within the dorsomedial medulla mapped.

For each of the three putative neurotransmitters studied 5 levels through the rostrocaudal extent of the nTS of at least 9 animals were examined and fields from each of the major subnuclei scanned (>120 fields per putative neurotransmitter). The area postrema and the DVN were also examined, although it was not possible to analyse the ipsilateral DVN as retrograde labelling of vagal preganglionic motoneurones by the CT-B hindered analysis of afferent fibre labelling in this region.

4:2:5 IMMUNOCYTOCHEMICAL CONTROLS.

Controls for the specificity of antibodies to CGRP and 5-HT are described in detail elsewhere (Consolazione et al., 1981; Gulbenkian et al., 1986). The specificity of the antibodies to SP has been determined using a number of different techniques. First, the pattern of immunostaining was identical to that described previously in a number of regions of the CNS. Furthermore, preabsorption with synthetic SP (10μM) resulted in a total loss of staining. Absorption with other tachykinins was not examined and, therefore, it is possible that this antiserum may cross-react with these related compounds. The specificity of the secondary antibodies in the double-labelling procedure were also examined; 1) sections were incubated with CT-B antiserum and reacted with either Texas red-labelled donkey anti-rat or donkey anti-rabbit IgG

(detection systems for the putative transmitters), 2) sections incubated with either SP, CGRP or 5-HT antisera were reacted with FITC-labelled donkey anti-goat IgG (detection system for the CT-B). No immunostaining was observed following these incubations.

4:2:6 CONTROLS FOR LASER SCANNING CONFOCAL MICROSCOPIC ANALYSIS.

Controls were performed to examine the possible overlap of excitability and/or emission wavelengths with the filters used on the microscope. Analysis of sections that had been incubated to detect CT-B alone (using FITC-labelled secondary antibodies) produced labelling only on the appropriate channel. Similarly, sections incubated to detect a single putative neurotransmitter (using Texas red-conjugated secondary antibodies) produced labelling exclusively on the other channel. 'Bleed-through' from one channel to the other was only encountered at very high laser intensities.

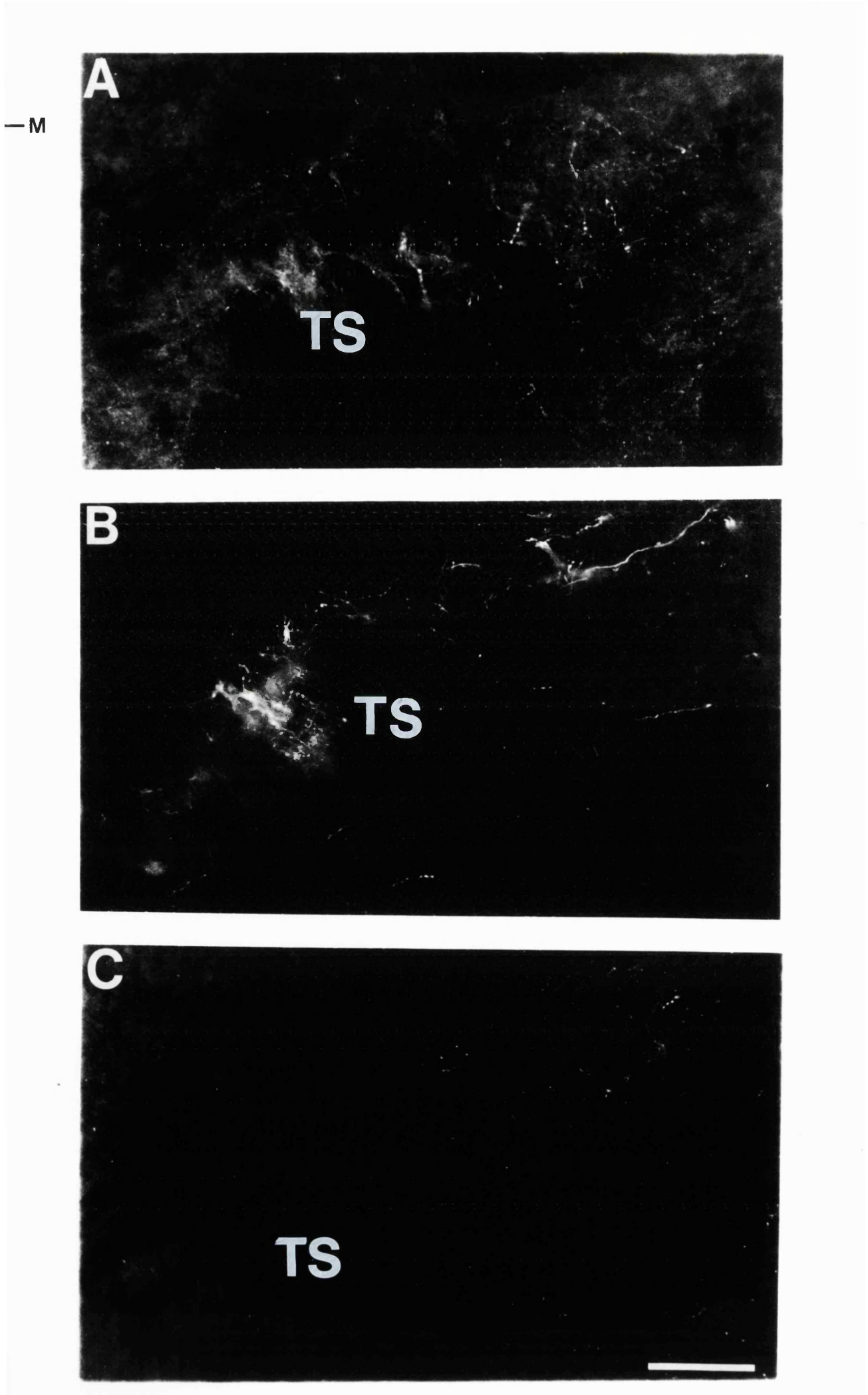
4:3 RESULTS.

4:3:1 ANTEROGRADE TRACERS.

Limited attempts were made with a number of anterograde tracers to label vagal afferents. No labelling was achieved with either biocytin or PHA-L and it was not possible to detect transported HRP with an anti-peroxidase antibody in this study. Sections reacted with the *o*-tolidine method exhibited very lightly stained vagal afferents terminals and efferent cell bodies confirming that biotinylated-peroxidase was transported anterogradely but attempts to detect this tracer with biotinylated-streptavidin

FIGURE 4:2.

Low power fluorescent micrographs of sections taken from similiar levels of the left nTS (approximately 0.1mm caudal to obex) demonstrating; A) SP immunoreactivity. B) CGRP immunoreactivity. C) 5-HT immunoreactivity. Orientation bar: D, dorsal; M, medial. Scale bar= 100μm.


A
M
TS
B
TS
C
TS

conjugated to fluorescein were unsuccessful.

Good vagal afferent axonal and terminal and efferent cell body labelling was obtained using either CT-B or HRP (combined with histochemical detection as a neuronal tracer). Although vagal afferent labelling was as extensive when CT-B was used as the anterograde tracer it did not seem to be as dense as the labelling achieved with HRP combined with peroxidase histochemistry. 'Punctate' labelling was obtained with CT-B that lacked the intra-varocosity labelling that was observed with HRP (Fig. 4:1A).

4:3:2 ANTEROGRADE TRACING COMBINED WITH IMMUNOFLUORESCENT DETECTION OF SP, CGRP AND 5-HT.

a. Horseradish peroxidase combined with histochemistry.

Strong immunofluorescent staining for SP and 5-HT was achieved on sections that had been reacted previously with *o*-tolidine. However, no vagal afferents containing immunoreactivity for SP, CGRP or 5-HT were observed and in areas of dense vagal afferent labelling immunofluorescent labelling appeared diminished. A possible explanation was that the cobalt chloride used in the stabilization process quenched the immunofluorescence but when this compound was omitted all the HRP reaction product was lost. The attempt to overcome these problems by reversing the order of the HRP histochemistry and immunofluorescence resulted in considerable reduction in both the intensity of the vagal afferent labelling and the immunostaining for SP and 5-HT. Again, no double-labelled structures were detected.

b. Cholera toxin subunit-B.

Following the injection of CT-B into the nodose ganglion labelled axonal structures were observed throughout the nTS, DVN and area postrema, with an ipsilateral predominance. The pattern of this labelling was similar to that reported previously in the rat using HRP (Kalia & Sullivan, 1982). Labelled axons were seen as punctate structures, presumably boutons (Fig. 4:1A), with no apparent labelling between these structures. Lighter labelling of axons was detected ipsilateral to the injection in both the vagus nerve efferent and afferent tracts, as well as in the tractus solitarius. The injection of CT-B also resulted in the labelling of neuronal perikarya in the ipsilateral DVN and nucleus ambiguus. As CT-B could be successfully detected with an immunofluorescent antibody system and could therefore, be combined readily with the immunofluorescent detection of putative neurotransmitters this tracer was used in all subsequent experiments.

4:3:3 IMMUNOFLUORESCENT DETECTION OF SP, CGRP AND 5-HT.

a. Substance P.

Immunoreactivity was observed in axonal fibres and bouton-like swellings throughout the medulla of the rat with a distribution similar to that described previously by other groups in this species (Kalia et al., 1984; Newton et al., 1985; Yamazoe et al., 1984). No immunoreactive perikarya were observed in the medulla with this antibody. Moderate densities of SP were observed in caudal nTS increasing in density rostral to obex. The pattern of SP immunoreactivity was relatively homogeneous throughout the nTS and DVN, with the exception of the tractus, which contained only light labelling and a region in the medial subnucleus which exhibited a distinct paucity of SP immunoreactivity. Similarly the area postrema contained few SP-

immunoreactive axonal structures (Newton et al., 1985). Within the medulla immunoreactive fibres were also observed in the vagal afferent tract.

b. Calcitonin gene-related peptide.

Immunoreactivity for this peptide in the region of the nTS has been previously described in detail in the cat (Torrealba, 1992) but descriptions in the rat have been relatively brief (Kawai et al., 1985; Morishima et al., 1985; Rosenfeld et al., 1983). Therefore, although not the purpose of this study a more detailed description of the CGRP-immunoreactive labelling in the nTS, DVN and area postrema will be given here.

At caudal levels of the nTS a band of CGRP-immunoreactive axonal fibres were observed running across the commissural subnucleus and appeared to form a dense

plexus on the midline that also extended into the DVN. Further rostral this plexus became less obvious and CGRP-immunoreactive axons and bouton-like swellings filled the medial and commissural subnuclei. However, at this level the heaviest CGRP-immunoreactive axonal labelling was observed in the dorsolateral and interstitial subnuclei and the tractus was also filled with CGRP-immunoreactive fibres and boutons (Fig. 4:2B). The area postrema contained few immunoreactive fibres, the majority of which were located on the dorsal and ventral edges of this structure. Moderate densites of CGRP-immunoreactive axonal profiles were observed at the level of obex with the greatest concentration found in the lateral subnuclei, the subnucleus gelatinosus and the tractus. Rostral to obex CGRP immunostaining was markedly reduced and mainly confined to the tractus and the lateral subnuclei. Only infrequently were fibres observed in the DVN at this level. Many CGRP-immunoreactive fibres were detected in the vagal afferent tracts. Neuronal perikarya were also labelled with

this antibody in the medulla but only in the compact region of the nucleus ambiguus and in the facial nucleus.

c. 5-Hydroxytryptamine.

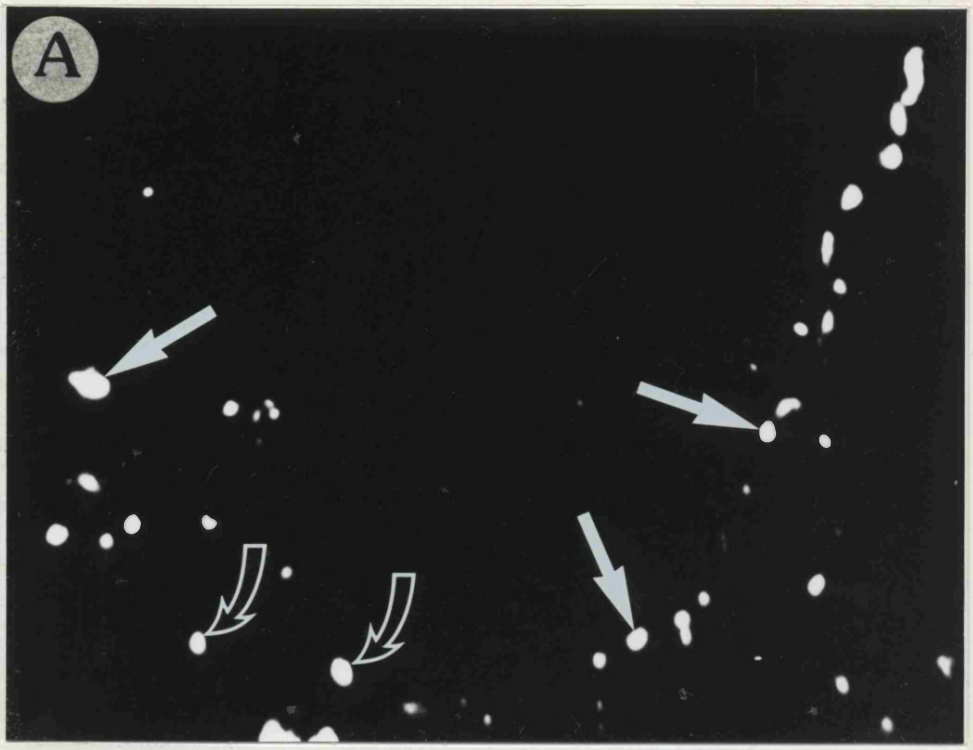
The distribution of axons and perikarya labelled for 5-HT in the medulla was similar to that previously described in the rat (Newton et al., 1985; Palkovits et al., 1985; Steinbush, 1981). In this study 5-HT-immunoreactive axonal profiles in the dorsomedial medulla were generally less numerous than those observed following SP or CGRP immunocytochemistry (Fig. 4:2C). Briefly, moderate densities of 5-HT-immunoreactive axonal fibres and bouton-like swellings were observed throughout the nTS with a slightly higher density in the DVN. At levels rostral to obex increased densities of 5-HT immunoreactivity were observed in the ventrolateral subnucleus. In the area postrema the pattern of immunostaining agreed with that described by Newton and co-workers (1985) with most 5-HT-immunoreactive fibres detected along the ventral and ventrolateral regions and with fewer fibres in the main body of this structure.

4:3:4 DISTRIBUTION OF CHEMICALLY IDENTIFIED VAGAL AFFERENTS.

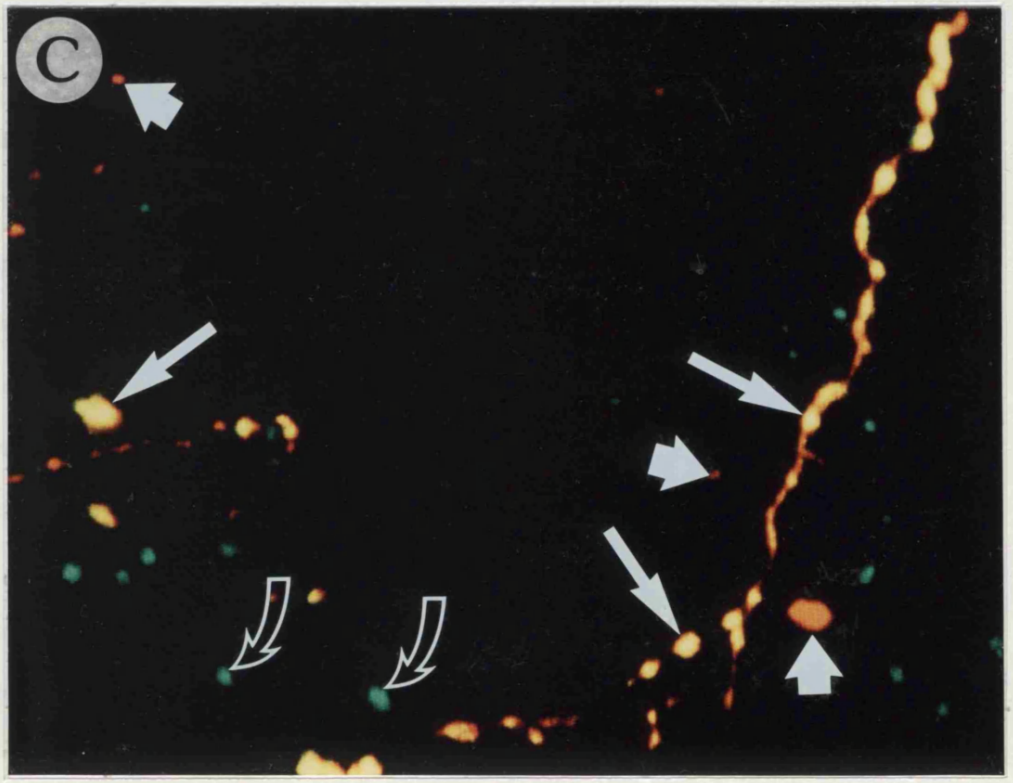
Due to the variability of both the anterograde labelling achieved with CT-B and the labelling obtained with antibodies to the putative transmitters from experiment to experiment, analysis was only carried out on sections from experiments in which dense labelling of both vagal afferents and the putative neurotransmitter was obtained. Identified vagal afferents in the medulla were found to contain each of the putative neurotransmitters studied, the number of which varied from experiment to experiment. Therefore, little attempt was made to quantify the results. Nevertheless, in all these experiments double-labelled axonal structures constituted a minor proportion of both

FIGURE 4:3.

Images of fibres in the dorsomedial medulla labelled by the transport of CT-B injected into the nodose ganglion and/or immunoreactive for CGRP. These images were captured using a confocal microscope and represent a Z-series of optical sections through a 40μm section. **A)** Image of CT-B labelled vagal afferents. **B)** Image taken from the same region but showing CGRP- immunoreactive axonal structures. Some structures are clearly labelled in both images (solid arrows). These structures are more easily identified in **C)** in which the two preceeding images have been represented by different colours (CT-B in green and CGRP-immunoreactive in red) and then merged. Thus structures CGRP-immunoreactive but not labelled from the nodose ganglion are seen in red (short hollow arrows), labelled vagal afferents not immunoreactive for CGRP seen in green (curved hollow arrows) and CGRP-immunoreactive vagal afferents appear yellow (solid arrows). Images are taken from the commissural subnucleus. Scale bar= 20μm.


A


B


C

the CT-B-labelled afferents and of the neurochemically identified axons within the nTS and surrounding regions. Consequently the data are compiled from a large number of fields within defined subnuclei and taken from a number of different experiments.

a. Substance P-immunoreactive vagal afferents.

Analysis throughout the rostrocaudal extent of the nTS, area postrema and contralateral DVN failed to reveal more than a few examples of vagal afferents that contained SP immunoreactivity. At caudal levels SP-vagal afferent terminals were detected only in the medial subnucleus (Fig. 4:5A). The distribution of SP-immunoreactive vagal afferent terminals was most extensive at the level of the area postrema (Fig. 4:5B) where they were observed in most nTS subnuclei and in the area postrema (Fig. 4:5B). The contralateral DVN, in its medial aspect, also contained double-labelled terminals (Fig. 4:5B). The majority of vagal afferents containing SP immunoreactivity were observed in the medial portion of the rostral nTS (Fig. 4:4A & B), but in only one experiment (RS140) were more than a few double-labelled terminals detected in this area. At this level occasional double-labelled terminals were

also situated in the tractus and the ventrolateral subnucleus (Fig. 4:5C). At the most rostral extremes of the nTS double-labelled structures were only observed in the tractus (Fig. 4:5D).

b. Calcitonin gene-related peptide-immunoreactive vagal afferents.

Vagal afferents containing CGRP were more numerous than those containing SP althought the density of SP-immunoreactive axonal labelling in the dorsomedial medulla was always greater in these experiments. The caudal nTS, from the level of the decussation of the pyramids to the level of obex, contained the greatest number of

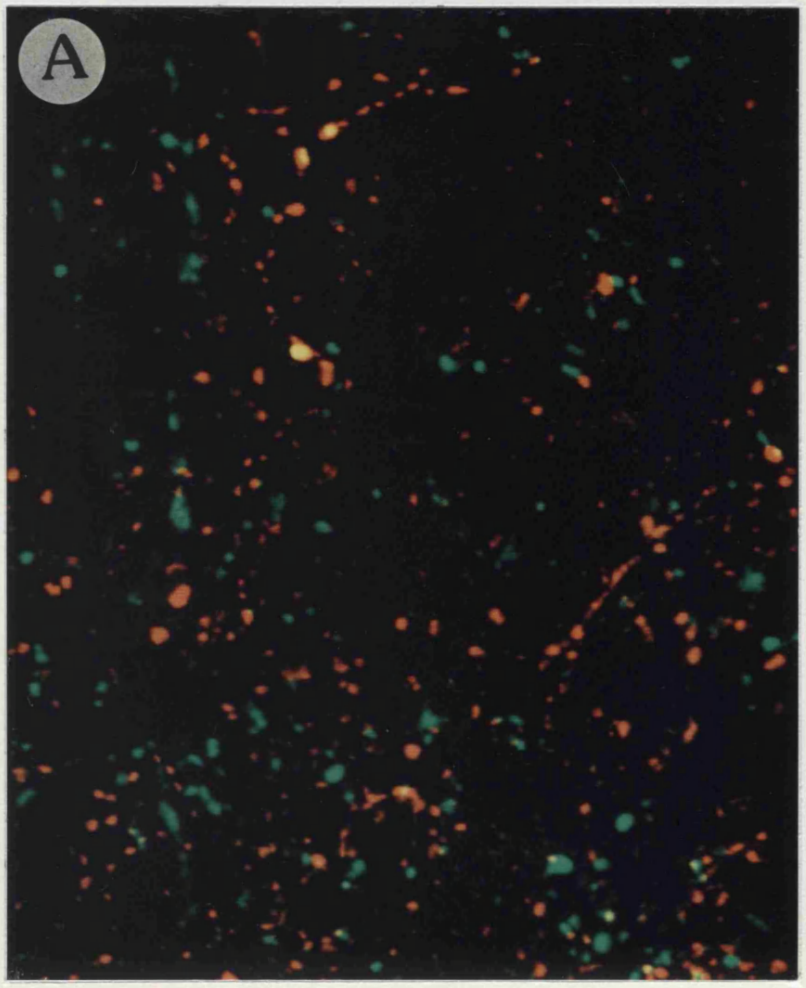
vagal afferents immunoreactive for CGRP. At this level double-labelled structures were detected throughout the different subnuclei with a predominance in the medial and commisural subnuclei (Fig. 4:6A & B). At the level of the AP, particulary at more caudal levels, considerable numbers of double-labelled structures were observed in the medial and commissural regions (Fig. 4:6A & B). Few double-labelled fibres were found in the medial . aspect of the contralateral DVN (Fig. 4:6B). At the level of obex CGRP-immunoreactive vagal afferents were primarily detected in the medial subnuclei of the nTS, but some double-labelled axons were also found in the dorsolateral and ventral subnuclei and in the tractus (Fig. 4:6C). At more rostral levels of the medulla CGRP-containing vagal afferents were observed in the tractus (Fig. 4:6D).

c. 5-Hydroxytryptamine.

The distribution of 5-HT-immunoreactive vagal afferents in the dorsomedial medulla was more distinctive than that seen with either SP or CGRP (Fig.4:6). No vagal afferents containing 5-HT immunoreactivity were observed at the most caudal levels of the nTS (Fig. 4:6A). The majority of serotonergic vagal afferents were detected at the level of the area postrema where they were seen throughout this structure. These afferents tended to be most numerous on around, the ventral edge of the area postrema (Fig. 4:6B). In the majority of fields examined in this area the greater proportion of 5-HT-immunoreactive structures were also identified as vagal afferents, but the majority of vagal afferents were not serotonergic (Fig. 4:4D & E). At this level 5-HT containing vagal afferents were also observed in the tractus and in the contralateral DVN (Fig. 4:6B). At more rostral levels serotonergic vagal afferents were only detected in the ventral region of the nTS (Fig. 4:6C & D).

FIGURE 4:4.

Examples of merged confocal microscope images of labelled vagal afferents (green) and immunoreactivity for SP (**A&B**), CGRP (**C**) and 5-HT (**D&E**; red). (**A&B**) SP-immunoreactive vagal afferents in the medial nTS (yellow). (**C**) CGRP-immunoreactive vagal afferents in the medial nTS. (**D&E**) 5-HT-immunoreactive vagal afferents in **D**) the area postrema and **E**) the ventral nTS. Scale bar=20μm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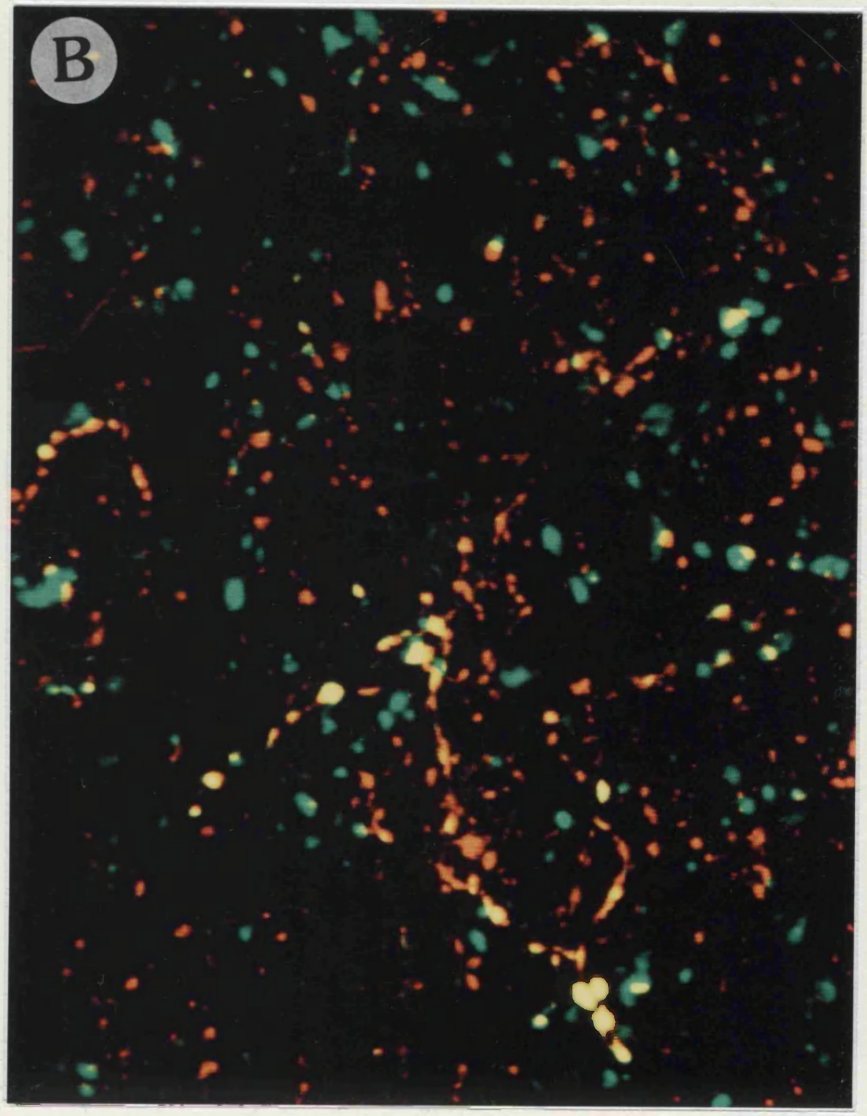
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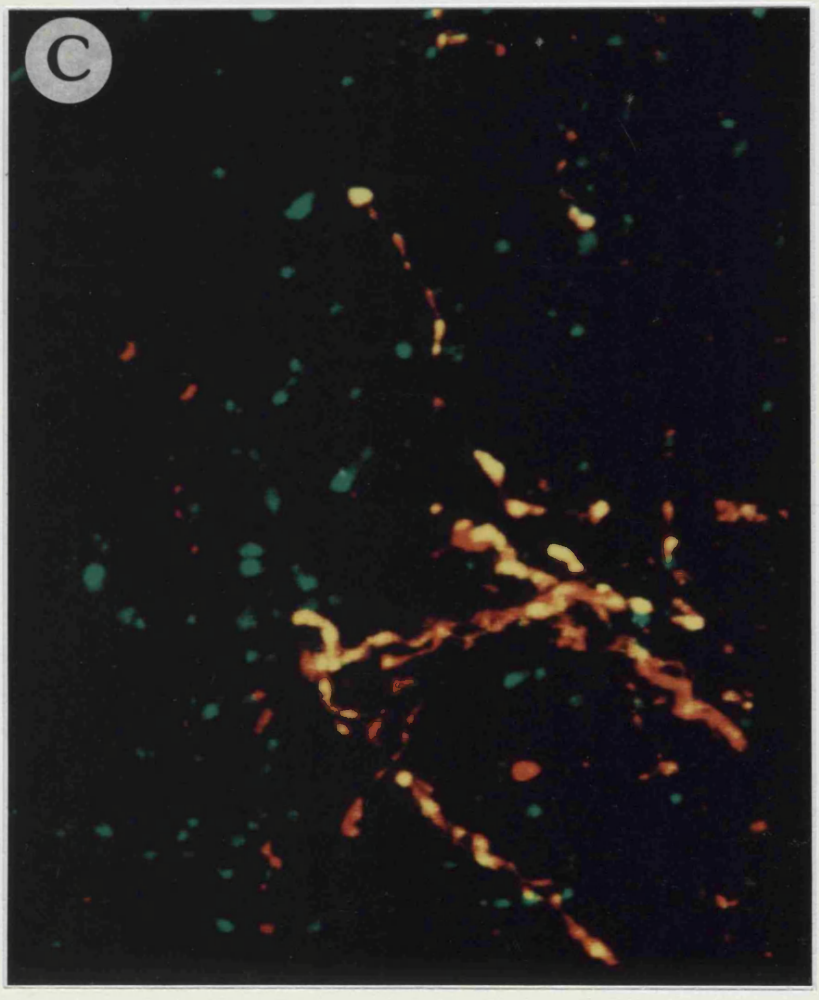
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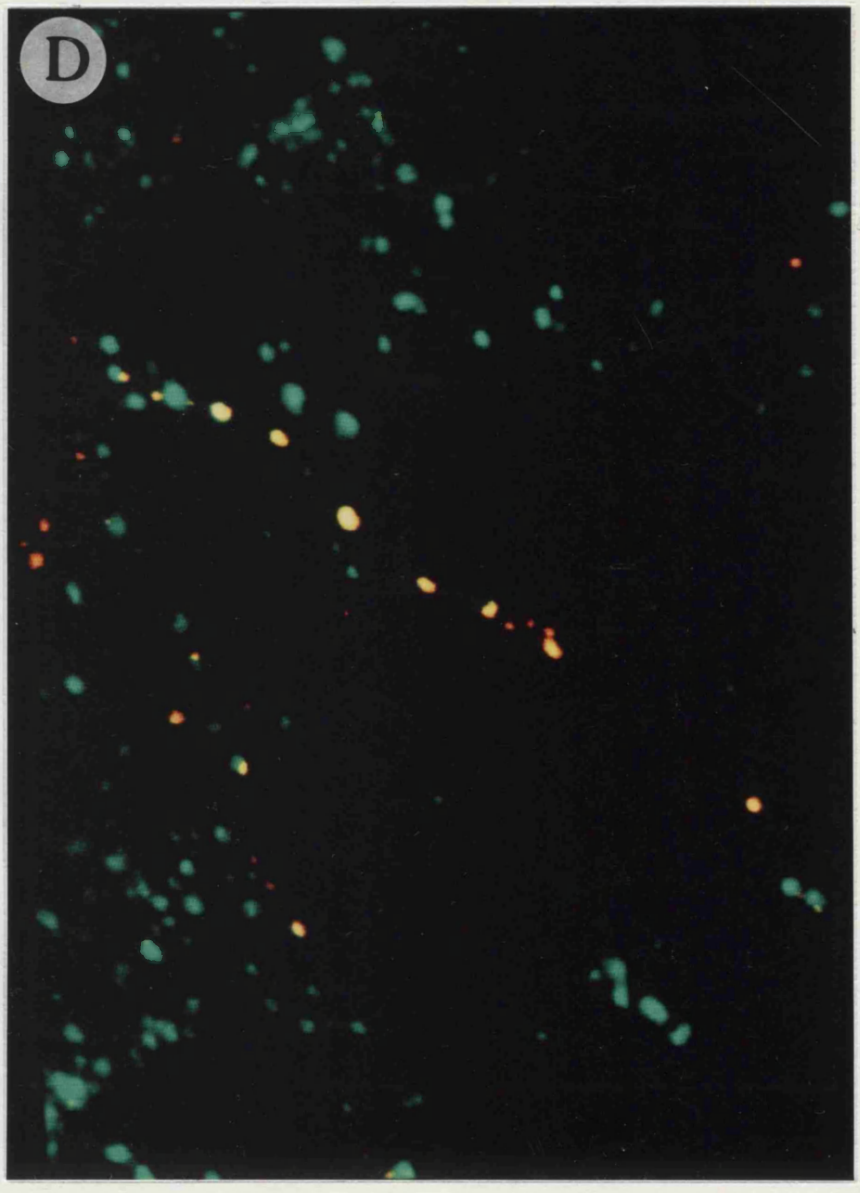
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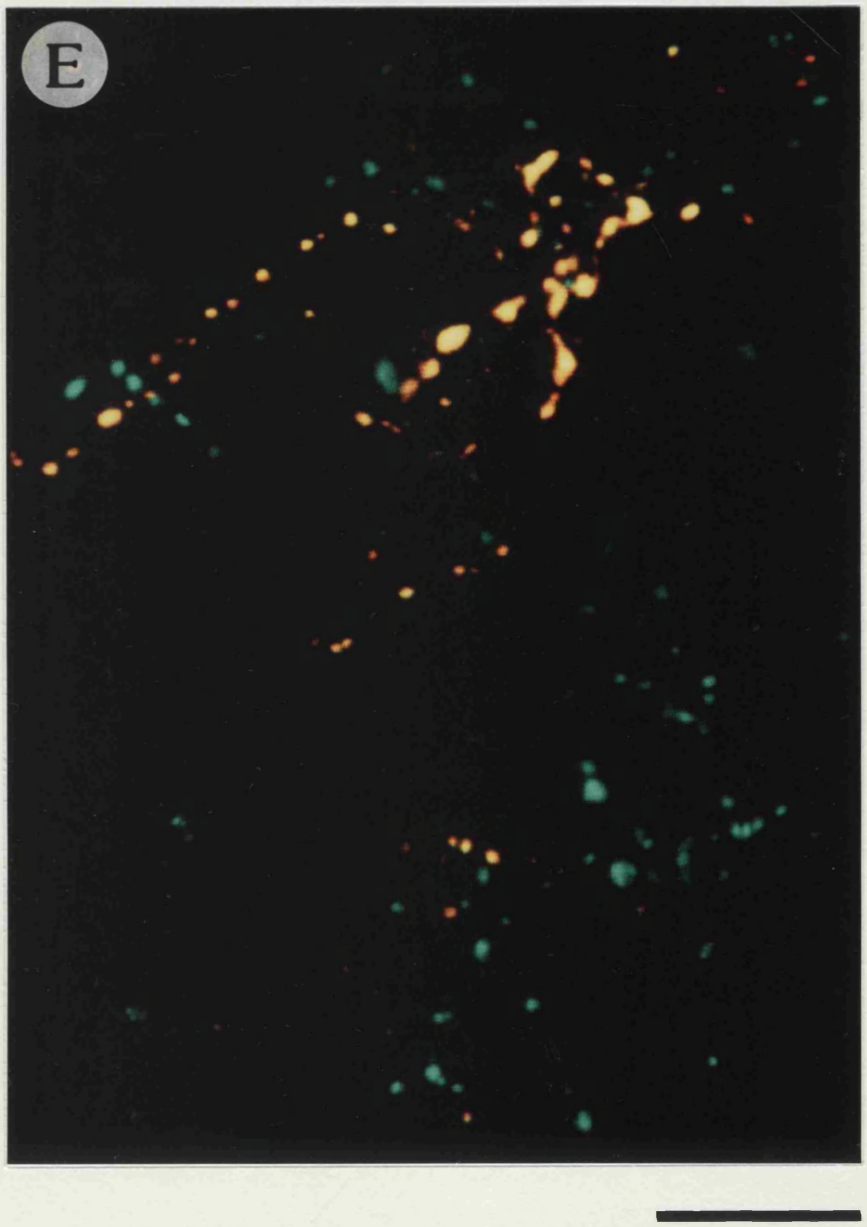
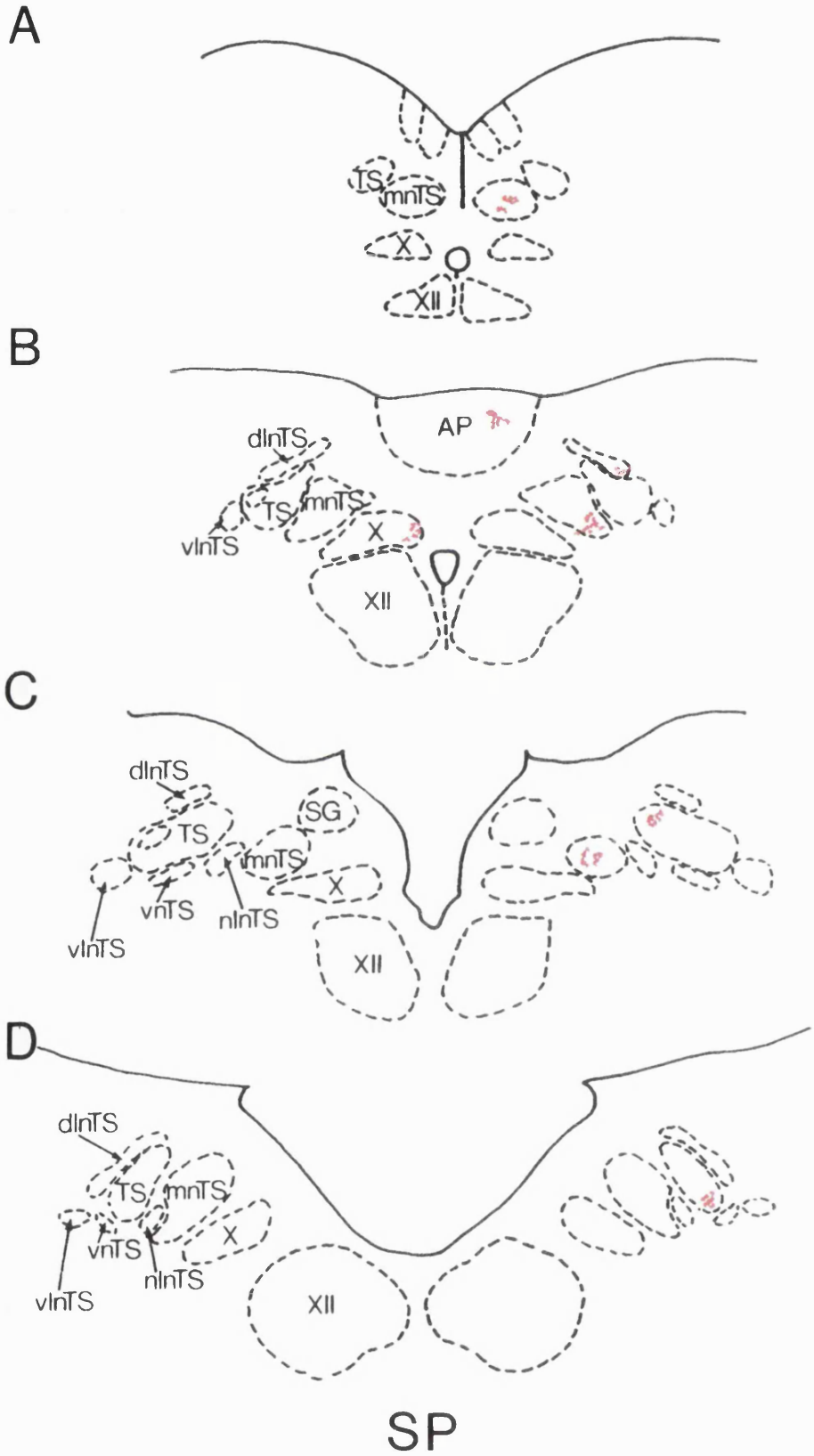

E

FIGURES 4:5, 6 & 7.

Diagramatic representation of the distribution of vagal afferents identified as containing **5**) SP, **6**) CGRP and **7**) 5-HT at four different levels through the medulla. Results are taken from at least 9 experiments. **A**) -0.7 to -0.31mm caudal to obex, **B**) -0.3 to -0.01mm caudal to obex, **C**) obex to +0.29mm rostral, **D**) +0.3 to 2.0mm rostral to obex.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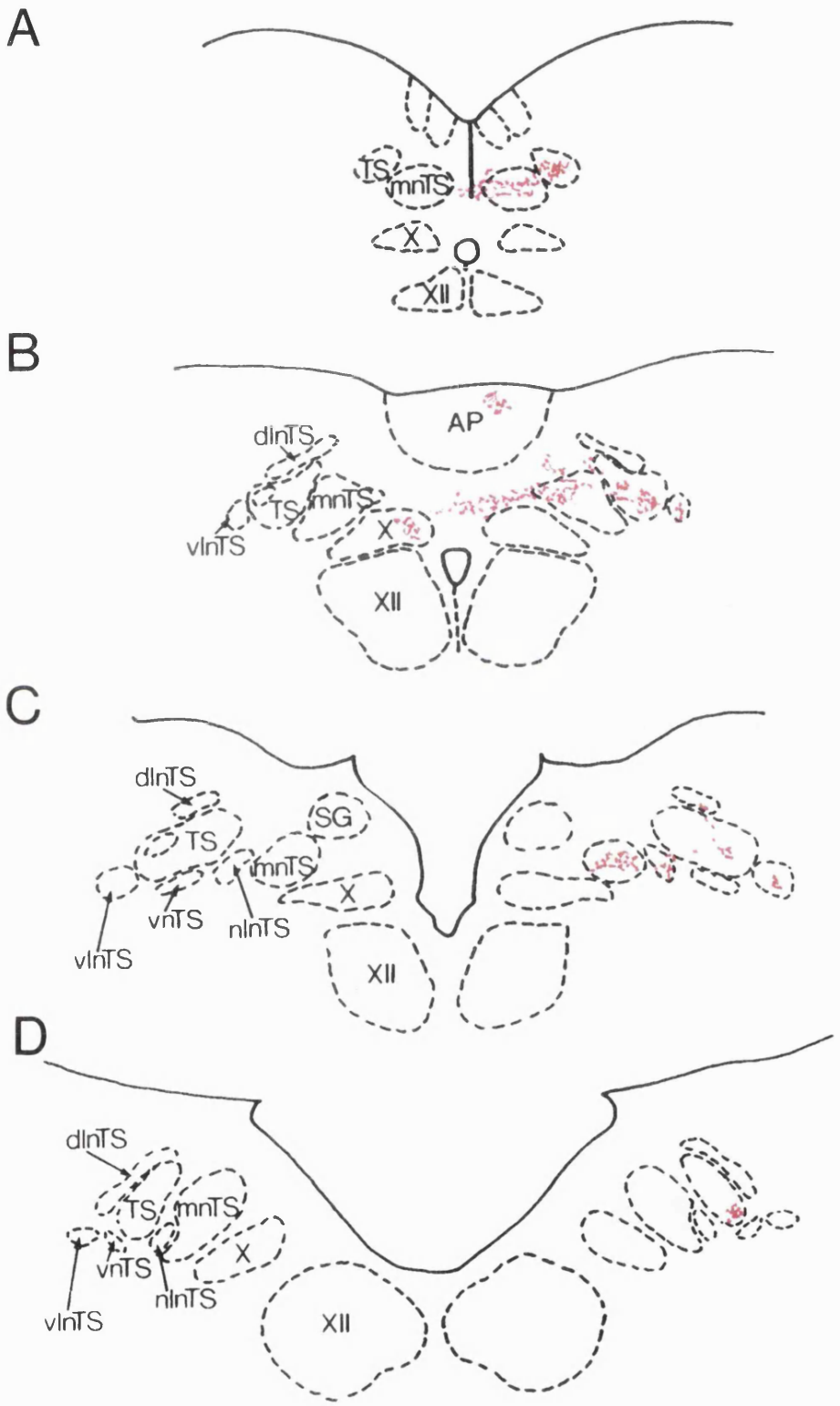
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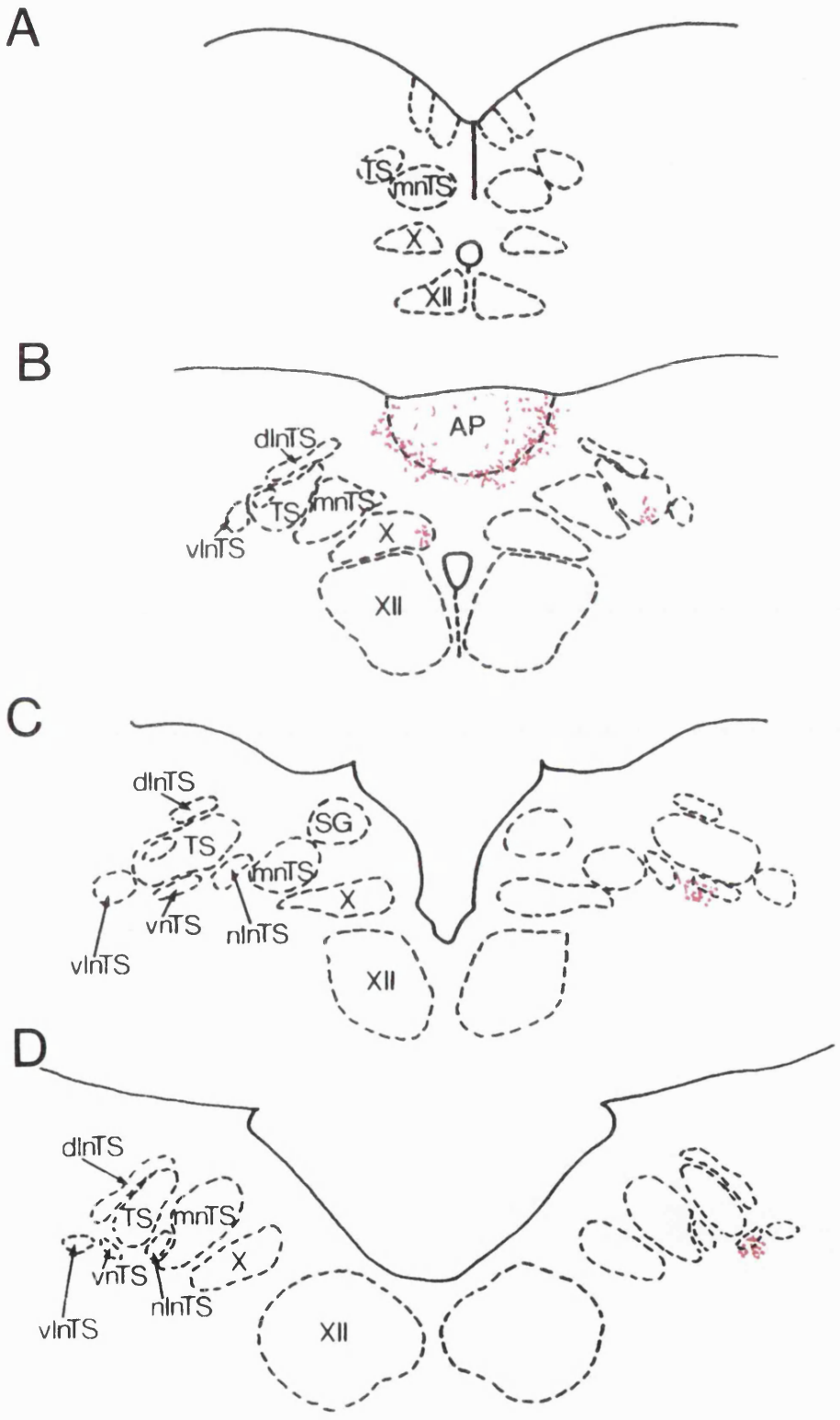
CGRP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5-HT

4:4 DISCUSSION.

The results of the present study provide direct evidence for the presence of 5-HT, SP and CGRP immunoreactivity in the central terminations of vagal sensory afferents and reveal a distinctive distribution of some of these putative transmitter substances within these afferents.

4:4:1 TECHNICAL CONSIDERATIONS.

Little success was achieved in labelling central vagal afferents with the limited attempts made with a number of neuronal tracers. Horseradish peroxidase or its conjugates combined with HRP histochemistry (Mesulam, 1978) are very sensitive anterograde tracers but these techniques are less suitable for combining with the immunocytochemical detection of putative neurotransmitters and neuromodulators as the electron dense reaction product appears to quench the immunofluorescence. To avoid the use of HRP histochemistry an attempt was made to detect the transported HRP with an anti-peroxidase antibody. Although HRP was transported to the dorsal medulla the enzyme was not detected with this antiserum in these experiments. As other groups (Lindh et al., 1989) have used this method with success with similar schedules, it is possible that the antisera used in this study was less sensitive that that used in other studies.

Other anterograde tracers, that have not previously been applied to peripheral nerves were also investigated. Biocytin, a soluble complex of biotin and lysine, has recently been described as an anterograde tracer in the CNS (Izzo, 1992; King et al., 1991). When biocytin was injected into the nodose ganglion no vagal afferent labelling

was detected. The plant lectin, PHA-L, is also a sensitive anterograde tracer in the CNS (Gerfen & Sawchenko, 1984) but no vagal afferent labelling was achieved with this tracer in these studies. Although only limited attempts were made with these neuronal tracers, it is possible that the central vagal afferents were not labelled as sensory vagal neurones may lack the requisite uptake mechanisms. Biotinylated-spacer-peroxidase had not previously been reported as an anterograde tracer but this substance was only weakly transported (as detected using peroxidase) and did not appear sensitive enough to warrant further investigation.

The vagal afferent labelling achieved with CT-B appeared as extensive as that obtained with HRP or its conjugates, but in general the labelling was less dense. This may be explained partly by the lack of intra-axonal labelling between the bouton-like swellings that resulted following labelling with CT-B (Fig. 4:1). The major advantage of using CT-B was that it could be detected with an immunofluorescent technique and could be combined readily with the immunofluorescent detection of putative neurotransmitters using different fluorochromes. Studies in the peripheral nervous system have suggested that not all sensory neurones possess the GM1, monoganglioside plasma membrane receptor for CT-B (Robertson & Arvidsson, 1985; Robertson & Grant, 1985). In dorsal root ganglion CT-B preferentially labelled large neurones and, therefore, failed to label the majority of unmyelinated and small myelinated primary afferent fibres (Robertson & Grant, 1985). However, similar studies in the nodose ganglion have demonstrated that all visceral primary ganglion neurones have the GM1 receptor (Robertson et al., 1992). Nevertheless, the possibility that vagal sensory neurones bind and transport CT-B to different degrees cannot be excluded.

Although the injections in this study were placed in the nodose ganglion possible labelling of afferents originating from ganglia of the glossopharyngeal nerve

must also be considered, as these ganglia form a fused mass interconnected by groups of cells in the rat (Altschuler et al., 1989). The vagal afferent labelling appeared to be considerable but it is accepted that not all the sensory neurones of the vagus nerve were labelled as some of these lie outside the area of injection e.g. in the jugular ganglion. An additional source of unlabelled vagal afferent terminals originate from the contralateral side as these experiments employed only unilateral injections into the nodose ganglion.

4:4:2 SUBSTANCE P.

The results from this study suggest that only a few SP-immunoreactive axons in the rat dorsomedial medulla originates from neurones in the nodose ganglia. Due to the small number of double-labelled axons observed in these studies it was not possible to determine any pattern to the distribution of SP-containing vagal afferents.

These results contrast sharply with findings from lesion studies which have suggested that a significant proportion of the SP-immunoreactive terminals in the nTS originate from the nodose ganglia (Baude et al., 1989; Gillis et al., 1980; Helke et al., 1980; Lorez et al., 1983). This discrepancy may be due to a number of factors. First, two of the studies were performed in cat (Baude et al., 1989; Gillis et al., 1980) a species in which radioimmunoassay has indicated that the SP-content is higher than in the rat (Dockray & Sharkey, 1986). However, other factors must also contribute to the apparent differences between the results of the present study and those of the lesion studies as some were performed in the same species (Helke et al., 1980; Lorez et al., 1983). One important consideration is the specificity of the lesions. For example, the study of Gillis and co-workers (1980) involved deafferentation of both the vagus and glossopharygeal nerves and therefore, some of the decrease in SP content in the medulla may have been due to degeneration of SP-containing glossopharyngeal

afferents. Similarly treatment of neonatal rats with capsaicin in the study of Lorez and co-workers (1983) would have also destroyed other unmyelinated and some thinly myelinated afferents in the glossopharyngeal nerve and possibly the trigeminal nerve, which has projections terminating in the nTS (Contreras et al., 1982; Hamilton & Norgren, 1984; Jacquin et al., 1982; Jacquin et al., 1983; Torvik, 1956). Interestingly, it has been shown that capsaicin treatment of rats results in a loss of SP-immunoreactive fibres in and around the nTS that is similar to that seen following transection of the trigeminal nerve (South & Ritter, 1986). In addition, these workers observed no changes in SP immunoreactivity following unilateral removal of the nodose ganglion.

A further consideration when assessing changes following lesions is that the changes observed may be due not only to the loss of the primary neurones but also to secondary degenerative changes of their postsynaptic targets, as suggested by a number of studies (Gobel, 1984; Gobel & Binck, 1977; Kapadia & LaMotte, 1987; Pinching & Powell, 1971; Sugimoto & Gobel, 1984). Such changes might result in the loss of SP-containing neurones that have been shown to be in the nTS (Yamazoe et al., 1984).

Assuming that very few central terminals of vagal afferents contain SP then it would be expected that this peptide is present in a small number of cell bodies in the nodose ganglion. Although a number of studies have demonstrated SP in perikarya in the nodose ganglion limited information is available regarding the total numbers of these neurones (Helke & Hill, 1988; Helke & Niederer, 1990; Helke & Rabchevsky; 1991). Ligation studies on the cervical vagus nerve have demonstrated an accumulation, central to the ligation, of SP immunoreactivity in a very high proportion of these axons (Lundberg et al.,1978). Furthermore, with the application of multiple ligatures Brimijoin and co-workers (1980) demonstrated that over 95% of the SP in the vagus nerve is transported peripherally. Assuming that the SP in the vagus nerve is

restricted to sensory fibres only (as yet there is no evidence to suggest that SP is present in motor fibres of this nerve) these ligation studies, together with the findings of the present study suggest that SP is mainly associated with the peripheral axons of vagal sensory neurones where it may have a function in local effector reflexes (for review see Holzer, 1988).

As central vagal afferents containing SP were so sparse it was difficult to discern any pattern of distribution and so comparisons with the known viscerotopic organization of the vagal afferent system were not possible. Hence, no indication of which peripheral organ or function these SP-containing afferents were associated with could be inferred from the results. However, vagal afferent fibres immunoreactive for SP have been detected in a variety of end organs that includes the airways (Dey et al., 1990; Lundberg et al., 1984), oesophagus (Green & Dockray, 1987, Lundberg et al., 1982), lungs (Lundberg et al., 1982) stomach and pancreas (Sharkey et al., 1984). Substance P immunoreactivity has been detected in discrete fibres in the tunica adventitia of the aortic arch (Helke et al., 1980) and a few nodose ganglion perikarya that project in the aortic depressor nerve were also observed to be SP-immunoreactive (Helke et al., 1980). Therefore, it is possible that the vagal afferents identified chemically in this study innervate a number of end organs.

The dorsomedial medulla is densely innervated by SP-immunoreactive fibres, the vast majority of which apparently do not originate from the nodose ganglia. Other possible sources include SP-immunoreactive primary afferents originating from the jugular, glossopharyngeal and trigeminal ganglia (Cuello & Kanazawa, 1978; Helke & Hill, 1988; South & Ritter, 1986) and/or SP projections from other CNS regions i.e. medullary raphe nuclei (Thor & Helke, 1987). Substance P interneurones, situated mainly in the commissural and medial subnuclei of the nTS may also contribute to this innervation (Yamazoe et al., 1984).

4:4:3 CALCITONIN GENE-RELATED PEPTIDE.

Although previous studies have demonstrated the presence of CGRP in the nodose ganglion (Czyzyk-Krzeska et al., 1991; Helke & Hill, 1988; Torrealba, 1992) this study provides direct evidence that this peptide is contained within the central terminals of vagal afferents, primarily those found in the caudal and intermediate nTS.

The presence of CGRP in the central ends of vagal sensory afferents has been implied by marked reductions in CGRP immunoreactivity in the cat nTS and area postrema following unilateral nodose ganglionectomy (Torrealba, 1992). The results of this study do not indicate such a large proportion of CGRP-containing vagal sensory afferents in the dorsal medulla. This discrepancy may reflect a species difference as a far greater proportion of cat nodose ganglion perikarya are reported to be CGRP-immunoreactive (Torrealba, 1992), or as discussed previously, a difference in methodology (see above).

In the present study vagal afferents containing CGRP were far more numerous than those containing SP. This may reflect higher numbers of nodose ganglion perikarya that are reported to contain CGRP than SP in the rat (Czyzyk-Krzeska et al., 1991; Helke & Niederer, 1990) or, alternatively, CGRP may be transported centrally to a greater degree than SP. However, like SP it has been suggested that CGRP is transported principally towards the periphery (see Dockray & Sharkey, 1986).

A distinguishable pattern of CGRP-containing vagal afferents was observed in the dorsomedial medulla in this study with the greatest density of fibres found in the medial and commissural subnuclei of the nTS, a region receiving vagal afferent innervation from most visceral organs (Kalia & Mesulam, 1980b). This suggests that

CGRP-containing vagal afferents are not restricted to the innervation of any particular end organ. In the periphery CGRP-containing vagal afferents have been detected in the airways and oesophagus, with a smaller innervation of the heart (Cadieux et al., 1986; Lundberg et al., 1985; Springall et al., 1987) and gastrointestinal tract (Su et al., 1987).

From the results of the present study it appears that the nodose ganglion contributes a relatively small proportion of the total CGRP-immunoreactive axons observed in the dorsomedial medulla. A number of other sources of this peptide in the dorsomedial medulla have previously been identified. These include the petrosal, trigeminal and jugular ganglia (Helke & Hill, 1988) and possibly from local neurones, as CGRP-immunoreactive perikarya have been reported in the rat nTS (Kawai et al., 1985; Morishima et al., 1985; Rosenfeld et al., 1983).

4:4:4 5-HYDROXYTRYPTAMINE.

Previous studies have demonstrated the presence of serotonergic-immunoreactivity in the perikarya of vagal sensory neurones in the nodose ganglion (Gaudin-Chazal et al., 1982a; Nosjean et al., 1990; Thor et al., 1988). The present study extends these findings by demonstrating that this neurotransmitter substance is also present in the central terminals of these sensory afferents. Analysis of the organization of these chemically identified afferents reveals that they are mainly restricted to the area postrema and the adjacent regions of the nTS, suggesting that they are related to afferents originating from particular end organs or associated with particular functions.

The rat area postrema and the surrounding region of the nTS receives afferent innervation primarily from the accessory coeliac, coeliac and hepatic branch of the

subdiaphragmatic vagus (Altschuler et al., 1991; Norgren & Smith, 1988, Rogers & Hermann, 1983). Therefore, the 5-HT-containing vagal afferents observed in this study may be the central portions of neurones whose peripheral fibres innervate the coecum, liver and/or hepatic portal vein.

Recent studies have suggested that serotonin in the area postrema is associated with gastric chemoreception and the emetic reflex although its exact role is far from clear (Leslie & Reynolds, 1991). Antagonists of the 5-HT_3 receptor have proved very effective anti-emetic drugs in patients receiving chemo- and radiotherapy. Furthermore in experimental animals it has been shown that 5-HT_3 antagonists injected into the area postrema produce a transient but marked inhibition of emesis (Higgins et al.,1989). Binding studies have demonstrated the highest density of 5-HT_3 receptors to be in the dorsomedial medulla in all the species examined (Barnes et al., 1990; Higgins et al., 1989; Hoyer et al., 1989; Kilpatrick et al., 1989; Leslie et al., 1990; Pratt & Bowery, 1989; Reynolds et al., 1989; Waeber et al., 1989). Nodose ganglionectomy in both the rat (Pratt & Bowery, 1989) and ferret (Leslie et al., 1990) results in a reduction in binding to these receptors in this region of the medulla, suggesting that these receptors are located on vagal sensory afferents. This is further supported by the visualization of mRNA for 5-HT_3 receptors in the nodose ganglion and vagus nerve using *in situ* hybridization (Palacios et al., 1990). However, it should be noted that the distribution of these receptors is not restricted to the area postrema (Pratt et al., 1990) and is therefore far more extensive than the observed distribution of vagal afferents identified as containing 5-HT. In addition, no evidence exists to establish any specific association between 5-HT_3 receptors and afferents identified as containing 5-HT. Nevertheless, it is interesting to suggest that the emetic response (or equivalent response in non-vomiting species) evoked by vagal stimulation (Andrews & Davidson, 1990) is mediated through sensory afferents containing serotonin.

A smaller number of vagal afferents containing 5-HT immunoreactivity were also detected in the ventral nTS, an area that receives vagal afferent innervation predominantly from the respiratory tract (Altchuter et al., 1989; Kalia & Mesulam, 1980b; Sweazey & Bradley, 1986). Therefore, this group of 5-HT-containing vagal afferents maybe associated with airway function. There is some pharmacological evidence to support this notion as studies have demonstrated that 5-HT in the nTS can influence respiration (Carter & Lightman, 1980; Henry & Sessle, 1985). However, no evidence for the involvement of vagal afferents in this response has been provided.

Serotonin has also been suggested as an integral neurotransmitter of the baroreceptor reflex. Uptake studies of [^{3}H]5-HT combined with neuronal tracing techniques reported 5-HT accumulating neurones in the nodose ganglion that project in the aortic depressor nerve (Gaudin-Chazal et al., 1983). Although microinjections into the nTS produce a dose dependent hypotension and bradycardia that is suggested to be mediated by 5-HT$_2$ receptors (Laguzzi et al.,1984; Merahi et al., 1992; Shvaloff & Laguzzi,1986), antagonists of this receptor do not block the baroreceptor reflex (Laguzzi et al., 1984). However, more recent studies using selective 5-HT$_3$ agonists has suggested that these receptors are involved in the reflex regulation of blood pressure.

The findings in this study do not support a role for 5-HT-containing vagal afferents in cardiovascular reflexes as these were not observed in areas reported to receive afferent innervation from the aortic baroreceptors but this does not exclude a role for 5-HT, or 5-HT$_3$ receptors in cardiovascular regulation.

The dorsomedial medulla is densely innervated with 5-HT-immunoreactive

structures, the majority of which (with the possible exception of the area postrema and the surrounding region) do not originate from the nodose ganglia in the rat. There are serotonergic cell groups in the medulla that probably contribute to this innervation, including interneurones in the medial subnuclei and area postrema (Calza et al., 1985; Howe et al., 1983; Steinbush et al., 1981), as well as a 5-HT-immunoreactive projection from the medullary raphe nuclei (Thor & Helke, 1987).

4:4:5 CONCLUSIONS.

This study describes a technique for investigating the neurochemical content of an identified population of afferent axonal terminals and their subsequent analysis using a laser scanning confocal microscope. Using this method immunoreactivity for substance P, CGRP and 5-HT has been demonstrated in the central ends of vagal sensory neurones in the rat suggesting a role for these neurochemicals at the first synapse in vagal reflexes. However, the data obtained in this study, together with the results from previous experiments suggest that the peptides SP and CGRP probably play a more significant role in the peripheral ends of vagal sensory neurones than they do in the central ends. The organization of the terminal fields of the different chemically identified afferents reveals some chemical coding within this system. This was most noticeable with the distribution of 5-HT-containing vagal afferents which showed some degree of correspondence with the viscerotopic organization of afferents innervating the gastrointestinal tract.

5. FINAL DISCUSSION.

The experiments in this thesis utilized a combination of neuroanatomical techniques at both the light and electron microscopic level to provide further information regarding the morphology and neurochemistry of the central terminations of vagal sensory afferents. Although previous neuronal tracing studies have provided limited data (Chazal et al., 1991; Chiba & Doba, 1976; Kalia & Richter, 1985b, 1988b, Leslie et al., 1982; Sumal et al., 1983) the present studies give a comprehensive account of the morphology and synaptic organization of these primary afferents throughout the rostrocaudal extent of the rat nTS. Vagal sensory afferents have similar morphological characteristics despite their functional heterogeneity and the varying diameters of their axonal fibres. These sensory afferents provide input to 2nd order neurones in the dorsomedial medulla that is further modulated by interactions on the common post-synaptic targets.

Although the presence of a variety of neurochemicals has been demonstrated in the sensory neurones of the nodose ganglia (Czyzyk-Krzeska et al., 1991, Gamse et al., 1979; Gaudin-Chazal et al., 1982; Helke & Hill, 1988; Katz & Karten, 1980; Ling et al., 1992; Lundberg et al., 1978; Nosjean et al., 1990; Thor et al., 1988) relatively little was known regarding the neurochemistry of the central terminals of vagal afferents. Several indirect studies had implicated glutamate as a neurotransmitter of vagal afferents (Dietrich et al., 1982; Lawrence & Jarrott, 1994; Meeley et al., 1989; Perrone, 1981; Reis et al., 1981; Schaffer et al., 1990; Streit, 1980) but the studies in this thesis, using a combination of anterograde labelling and post-embedding immunocytochemistry, were able to establish the presence of elevated levels of this EAA in these afferents. The homogeneous distribution of vagal afferents immunoreactive for glutamate is in sharp contrast to the heterogeneous distribution of

vagal afferents containing the putative neurotransmitters/neuromodulators SP, CGRP and 5-HT. Interestingly, the distribution of these chemically-identified vagal afferents suggests that the chemical expression of neurones located in the nodose ganglia may be related to its target tissue (or function). In particular, the terminal fields of the serotonergic vagal afferents showed some correlation with the previously described viscerotopic distribution of gastrointestinal afferents (see section 1:4:3 for refs.).

Comparison of the morphological features of vagal sensory afferents with other primary afferent systems may be useful in attempting to gain a further understanding of the processing and transmission of sensory impulses. Unfortunately limited data are available regarding the morphology and synaptic organization of the sensory afferents of other cranial nerves although a brief study describing the central terminations of the rat greater petrosal nerve (a branch of the IX nerve) suggests similarities to vagal afferents (Hosoya & Suguira, 1984). Therefore, the possibility that visceral sensory afferents of the cranial nerves share common ultrastructural characteristics awaits further confirmation.

In contrast, the morphology of the somatosensory system, which has a different embryological origin to that of the cranial nerves (neural crest versus epibrachial placodes) has been studied extensively. Comparison of the vagal afferent system with the somatosensory system reveals that although these two sensory systems share some common features there are also significant differences. The somatosensory system displays far greater diversity as regards the synaptic vesicle content of the afferent terminals than the vagal afferent system. Several distinct morphological classes of somatosensory afferent terminals have been described with individual zones of the spinal cord containing characteristic densities of these afferents (Beattie et al., 1978; Duncan & Morales, 1973; Narotzky & Kerr, 1978; Nolan & Brown, 1981; Snyder,1982). As there appears to be some correlation between the types of synaptic

vesicles and neurochemical content (for examples see Clements et al., 1990; Helfert et al., 1992) I suggest that the primary afferents in the somatosensory system may contain a greater diversity of neurotransmitters.

Both in the dorsal horn and nTS there appears to be specificity in the termination sites of different classes of primary afferent fibres. In the spinal cord large, myelinated fibres arborize mainly in laminae II and III whilst unmyelinated C-fibres appear to terminate predominantly in laminae I and II. Likewise, in the nTS the majority of coarse fibres of the glossopharyngeal and vagus nerves terminate in the lateral subnuclei whilst most fine, unmyelinated fibres terminate in medial and commissural subnuclei (Claps et al., 1989; Torrealba & Calderon, 1990). Therefore, in both systems the post-synaptic targets of the different classes of fibres may display some degree of segregation.

The predominant post-synaptic targets of somatosensory primary afferents are distal dendrites and spines (Snyder, 1982; Ralston, 1968) with which they form asymmetric synapses. In this respect they are similar to vagal sensory afferents. One exception are the terminals of group Ia afferents which make frequent contacts with perikarya as well as proximal dendrites (Maxwell & Bannatyne, 1983). In the vagal afferent system the presence of an anatomical substrate for presynaptic inhibition remains equivocal (see section 2:4:3 for a fuller discussion) and it is possible that in this system local ionic changes are responsible for the observed presynaptic modulation (Richter et al., 1976, 1986). In the somatosensory system there is more evidence for presynaptic inhibition (Rudomin et al., 1974) occurring at axo-axonic synaptic contacts. For example, many somatosensory afferents are post-synaptic to axons that contain GABA (Alvarez et al., 1992; Barber et al., 1978; McLaughlin et al., 1975; Todd & Lochhead, 1990) and possibly glycine (Todd, 1990). It is also possible that somatosensory afferents receive a further inhibitory input from dendrites as these

afferents have been observed post-synaptic to vesicle-containing dendrites (Gobel et al., 1980; Todd, 1988). The origin of the parent cell bodies of these putative inhibitory dendrites is presently unclear although it is known that some, at least, arise from the islet cells of laminae II (Gobel et al., 1980). A shared characteristic of both systems is the ability to form complex synaptic glomerular arrangements and thereby provide diffuse input to several neurones simultaneously. Glomerular arrangements in the dorsal horn are more complex than in the nTS with axo-axonic and dendro-axonic synaptic contacts present and can be divided into several different classes (Willis & Coggeshall, 1978).

The rather homogeneous appearance of vagal afferents throughout the nTS suggests that different classes of afferents can not be easily distinguished at the ultrastructural level. This proposition is supported by the studies of Kalia and Richter (1985b, 1988b) who were only able to distinguish between individual, functionally identified pulmonary SARs and RARs on the basis of their synaptic arrangements (simple vs glomerular, calibre of the post-synaptic dendrite etc.) in different nTS subnuclei and the absence of axosomatic synapses formed by RARs contacts. In the present study presumably all vagal sensory afferents were labelled and, therefore, it was not possible to assign these fairly subtle ultrastructural charateristics to any particular class of afferent. The greater heterogenity of the somatosensory system provides the potential for easier recognition of different classes of receptor on the basis of their ultrastructure. So far the limited studies suggest that primary afferents with large diameter, myelinated fibres (AαB) have a significantly different synaptic arrangement than fine C-fibres (Alvarez et al., 1993; Maxwell et al., 1984; Semba et al., 1984, 1985), whilst a proportion of IA spindle afferents in Clarke's column could be identified on the basis of their long axes (> 7μm; Hongo et al., 1987; Tracey & Walmsey, 1984). However, identification of functionally different afferents that all

belong to the same fibre class may be more difficult. Examination of functionally different cutaneous afferents that belong to the AαB class could only be distinguished from each other after painstaking analysis of their synaptic arrangements (Bannatyne et al., 1984; Maxwell et al., 1984; Semba et al., 1984, 1985).

The studies in this thesis establish that vagal afferents contain elevated levels of the EAA, glutamate, and therefore together with previous studies measuring EPSPs following afferent stimulation (Andresen & Yang, 1990; Brooks et al., 1992; Felder & Mifflin, 1988; Mifflin et al., 1988a,b) provide evidence that vagal sensory afferents transmit excitatory input to the dorsomedial medulla. In addition, synaptic contacts between vagal afferents and their post-synaptic targets were of the asymmetric type (with one possible exception). This type of synapse has been classically associated with excitatory input (Andersen et al., 1963; Andersen & Eccles, 1965; Eccles, 1964; Gray, 1962) and this study confirms this correlation in these primary afferents. Boutons forming asymmetric synapses have been demonstrated in known excitatory pathways in the cerebellum (Somogyi et al., 1986) and the cortex (De Felipe et al., 1988). Conversely, boutons forming symmetric synapses and containing the inhibitory amino acid, GABA, have been reported throughout the CNS (for examples see Clements et al., 1990; Izzo et al., 1992). However, some presumptive GABAergic terminals forming asymmetric synapses were reported in various regions (for refs. see Ribak & Roberts, 1990) on the basis of preembedding GAD immunostaining. This discrepancy may be a result of the indirect nature of the GAD studies where the distribution of the synthetic enzyme was demonstrated rather than GABA itself. Alternatively it may be due to the difficulties in ascertaining the types of synapses in peroxidase reacted material as more recent studies, using antisera to GABA and post-embedding techniques, have not reported immunoreactive boutons forming asymmetric synapses.

The studies in this thesis demonstrated that a proportion of the glutamate-immunoreactive boutons in the nTS originate from the nodose ganglion. Some HRP-labelled vagal afferents did not contain levels of immunogold significantly different from surrounding dendritic structures. However, interpretation of negative immunocytochemical results can be difficult and it is possible that the negative results obtained were due to known technical problems (e.g. variation in immunostaining, detrimental effect of the histochemical reaction on the immunostaining) and the difficulties in discriminating between metabolic and neurotransmitter glutamate. As glutamate is an important excitatory neurotransmitter throughout the CNS it is possible that all vagal afferents in the nTS utilize glutamate as a neurotransmitter. Alternatively, it may be that only a proportion of vagal afferents are glutamatergic but this population innervates all target organs.

It has been proposed that various EAAs are co-localized and therefore the possibility that vagal afferents contain multiple EAAs should be considered. Aspartate is one possible candidate as it suggested to be co-localized with glutamate in somatosensory afferents (Merighi et al., 1991, Tracey et al., 1991) and possibly glossopharyngeal afferents (Okada & Miura, 1992). Aspartate appears to have a role as a neurotransmitter in the nTS (Kihara et al., 1989; Lawrence & Jarrott; 1994) although it is present at much lower concentrations than glutamate (Meeley et al., 1989; Reis et al., 1981). Currently the role of aspartate in the nTS, and specifically in vagal afferents, is unclear. Nodose ganglionectomy did not reduce aspartate levels significantly (Dietrich et al., 1982; Meeley et al., 1989) although these indirect techniques are of questionable value especially when either very high or very low levels of neurochemicals are present. Elevation of arterial blood pressure with phenylephrine evoked the release of glutamate in the nTS but not aspartate (Lawrence & Jarrott, 1994) suggesting that aspartate is not a neurotransmitter of baroreceptors. However, it is

possible that aspartate may be present only in a subset of baroreceptors and was released at concentrations below the sensitivity of the technique used.

As discussed previously the nTS contains numerous glutamate-immunoreactive boutons that form asymmetric synapses with their post-synaptic targets. In a previous study Izzo and co-workers (1992) demonstrated that the nTS is also densely and fairly evenly innervated with GABA-immunoreactive boutons that contain pleomorphic synaptic vesicles and form exclusively symmetric synapses. Taken together these two studies demonstrate that GABA and glutamate are contained within separate populations of boutons and therefore it is suggested that in the nTS, at least, the hypothesis that asymmetric synapses correlate with excitatory input and symmetric synapses correlate with inhibitory input appears to be correct (Gray, 1962). In addition, as the majority of afferents in the nTS appear to have a similar morphology to those of GABA- and glutamate-immunoreactive afferents (Chazal et al., 1991; Leslie et al., 1982; personal observations) it is proposed that most boutons contain one or other of these amino acids. Interestingly, other studies have also suggested that these terminals immunoreactive for GABA and glutamate together, account for the majority of presynaptic terminals in the neuroendocrine hypothalamus (Decavel & van der Pol, 1992), lateral superior olive (Helfert et al., 1992) and terminals providing input onto sympathoadrenal neurones (Bacon & Smith, 1988; Llewellyn-Smith et al., 1992) and it is possible that future studies will reveal this pattern throughout the CNS.

Glutamate and other excitatory amino acids released by sensory afferents (and other synaptic boutons) mediate fast post-synaptic potentials by acting on multiple EAA receptors and therefore neuronal function may be related to the density of specific subtypes on individual neurones. Application of various excitatory amino acid receptor agonists and antagonists in the nTS have implicated both N-methyl-D-aspartate (NMDA) and non-NMDA receptors in baroreceptor (Guyenet et al., 1987; Le

Galloudec et al., 1989, Leone & Gordon, 1989; Talman, 1989), chemoreceptor reflexes (Zhang & Mifflin, 1993) and deglutition (Kessler et al., 1990; Kessler & Jean, 1991; Hashim & Bierger, 1989). Electrophysiological studies have demonstrated the presence of EAA receptors in the nTS including kainate, quisqualate, NMDA (Drewe et al., 1990; Miller & Felder, 1988) and possibly metabotrophic (Glaum & Miller, 1992; Pawloski-Dahm et al., 1992) with multiple subtypes present on individual nTS neurones (Drewe et al., 1990). Further *in vitro* studies have suggested that multiple EAA receptors are also involved in mediating excitatory afferent input to nTS neurones (Andresen & Yang, 1990; Miller & Felder, 1988). Intracellular recordings in the medial nTS have obtained direct evidence that afferent input is mediated primarily at non-NMDA receptors with NMDA receptors possibly having a modulatory role (Andresen & Yang, 1990).

Although different classes of vagal afferents have a similar morphology and may all utilize glutamate as a neurotransmitter the studies in this thesis have demonstrated that only some afferents contained SP, CGRP or 5-HT. The distribution of vagal afferents containing these neurochemicals in the dorsomedial medulla can be partly related to their previously described viscerotopy and therefore, the phenotype of vagal sensory neurones may be influenced by interactions with their target organ. There is some evidence for this hypothesis from investigations in the somatosensory system. Recent studies using double-labelling techniques have demonstrated that specific combinations of neuropeptides and enzymes contained within the sensory neurones are related to their peripheral targets although none of the markers examined were unique for any end organ (Gibbins et al., 1987; Lindh et al., 1989; Molander et al., 1987; O'Brien et al., 1989). Furthermore, the expression of peptides can be altered following cross-reinnervation of peripheral axons between different target tissues (McMahon & Gibson, 1987).

There is little information regarding the function of CGRP and SP in the central terminations of vagal afferents although their function in primary afferents in the dorsal horn has been more extensively investigated. However, caution must be taken as it can not be assumed that these neuropeptides will automatically play the same role in both systems especially as the levels of CGRP and SP appear to be much greater in the central terminals of somatosensory afferents (Barber et al., 1979; Chung et al., 1993; Jessell et al., 1979; Kawatani et al., 1985; Sharkey et al., 1987). Recent studies have provided strong indications that CGRP and SP contained within somatosensory afferents are involved in mediating noxious input. For example, noxious stimuli evokes the release of these neuropeptides (Brodin et al., 1987; Duggan et al, 1988; Duggan et al; 1987; Klein et al., 1990; Linderoth & Brodin, 1988; Morton & Hutchinson, 1989) whilst nociceptive spinal reflexes in rats are potentiated by intrathecal injection of CGRP (Cridland & Henry, 1988; Oku et al., 1987; Woolf & Wisenfeld-Hallin, 1986). Immunoreactivity for CGRP was also detected in a single afferent identified as having nociceptive properties (Alvarez et al., 1993). There is also evidence that CGRP and SP interact in the dorsal horn as intrathecal administration of CGRP potentiates the scratching and biting behaviour evoked by intrathecal SP in rats (Wiesenfeld-Hallin et al., 1984). However, whether these SP and CGRP-containing vagal afferents play a similar role in transmitting noxious input as somatosensensory afferents awaits further verification. At present the role of the nTS and more specifically vagal sensory afferents in pain control is poorly understood although it is known that aversive sensations can be produced following noxious stimulation of some cranial nerve afferents e.g. mild substernal burning, tightness of the chest and nausea.

Unfortunately there is little data in the literature regarding possible functions of 5-HT released from the central terminals of vagal afferents. Most studies have concentrated on the role of 5-HT in cardiovascular function (see Laguzzi, 1994) but as

5-HT-containing vagal afferents were not observed in regions of the nTS receiving projections from aortic baroreceptors in this thesis it would appear that this neurochemical does not play a role at the first synapse of this reflex. The majority of serotonergic vagal afferents were detected in or around the area postrema suggests that they must be closely associated with the functions of this structure i.e controlling body fluid regulation, food intake, metabolism and cardiovascular homeostasis (see chapter 4 for a fuller discussion of the possible role of 5-HT). It is possible that this monoamine may have a role that is unique to the vagal afferent system as 5-HT is only expressed by dorsal root ganglion perikarya following dorsal rhizotomy suggesting that its expression by these neurones may be suppressed by a neurotrophic factor transported back from the spinal cord (Cameron et al., 1987).

As glutamate-immunoreactive boutons are numerous throughout the nTS it is suggested, given the multitude of putative neurotransmitters and neuromodulators containined within this area (for review see van Giersbergen et al., 1992), that this amino acid is co-localized with various combinations of these neurochemicals. Furthermore, as a variety of putative neurotransmitters and neuromodulators have been detected in the perikarya of sensory neurones in the nodose ganglia (see section 1:7 for further details) it is likely that glutamate-immunoreactive vagal afferents also contain other neurochemicals. Indirect evidence for co-localization was obtained from the present studies. Occasionally vagal afferents with elevated levels of glutamate were observed containing dense core vesicles, which are assumed to contain neuropeptides (De Biasi & Rustioni, 1988; Merighi et al.,1989, 1991), whilst the presence of SP, CGRP and 5-HT in vagal afferent central terminations was also established. The exact effects of co-localization of glutamate and various combinations of putative neuromodulators /neurotransmitters in afferents on the pre- and post-synaptic mechanisms remains to be elucidated.

FUTURE WORK.

Although this study has added to the understanding of the morphology and particularly the neurochemistry of the central terminations of vagal sensory afferents much more research is required before a complete picture is obtained of how particular classes of vagal afferents transmit information regarding the internal environment to the dorsomedial medulla. Further studies are required to elucidate the identity of the post-synaptic targets of vagal afferents, the origin of their other synaptic inputs and their circuitary. Whilst this thesis established the presence of glutamate in at least some vagal afferents additional studies are required to determine whether all vagal afferents utilize this ammino acid or oly some fibres innervating all of the target organs. As discussed previously, it is possible that vagal afferents contain other EAAs and therefore, ultilizing similar techniques to those described in chapter 3B it should be possible to investigate whether vagal afferents also contain aspartate and/or other excitatory amino acids. Although there is a large body of literature supporting a neurotransmitter role for glutamate in sensory afferents it still remains to be established whether all or some of other neurochemicals present in sensory systems are also involved in synaptic transmission or have other roles i.e. as neurotrophic agents. As only three neurochemicals were examined in the present study it would be of interest to determine if other neurochemicals in the vagal afferent system are related to specific subsets of afferent axons and if the central afferent terminals of other sensory systems also display a similar 'chemical coding'. Using similar double-labelling techniques to those developed in chapter 4 it is anticipated that further studies will be able to verify the suggestions made in this thesis regarding the peripheral projection of the SP, CGRP and 5-HT-containing vagal afferents. Finally, an interesting finding in this thesis was the observation of a discrete group of immunoreactive perikarya in the medial area of the intermediate nTS. Studies are now underway in this laboratory to establish whether these neurones provide excitatory input to the RVLM.

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