

**Investigations into the organisation and morphology
of vagal preganglionic neurones and the anatomical
identification of the chemistry and origin of some
of their synaptic inputs.**

BY

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To Sue and My Family.

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Abstract.

The investigations in this thesis involve the use of neuroanatomical techniques in the study of vagal preganglionic neurones (VPNs) in the medulla oblongata of the rat and cat. Light microscopic examination of VPns identified by the injection of horseradish peroxidase (HRP) into the cervical vagus nerve of the rat revealed that they lay mainly in two areas of the medulla - the dorsal vagal nucleus (DVN) and the nucleus ambiguus (NA). Labelled cells in the DVN (median diameter 18 μ m) were arranged in a tightly packed group. In contrast, labelled neurones in the NA were arranged in two identifiable groups. The most dorsal of these groups consisted of tightly packed cells with median diameter of 30 μ m (compact group - cNA) whereas the other group were smaller (median D 25 μ m) and were scattered in ventrolateral regions of the medulla (vINA). Neurones in each group were identified as being statistically different in diameter and soma area from those in other groups (Student's t-test). Cardiac vagal preganglionic neurones (CVPNs), retrogradely labelled by the injection of cholera toxin-HRP into the right atrium, were located mainly in the vINA with median soma diameter 25 μ m. Ultrastructural examination of the groups of VPns revealed that they shared some morphological characteristics.

VPns in the NA, particularly those in the vINA, were observed to be intermingled with neurones retrogradely labelled from the phrenic motor nucleus in the spinal cord and were often

morphologically indistinguishable. VPNs in the NA were also intermingled with neuropeptide Y immunoreactive neurones.

The chemistry of inputs to VPNs was examined using a combination of retrograde tracing to identify VPNs, immunocytochemistry to detect various neurotransmitter chemicals, and electron microscopy. Serotonin, substance P and neuropeptide Y were identified in boutons forming asymmetric type synaptic contacts with VPNs in the nucleus ambiguus of the rat which were retrogradely labelled from the heart or the cervical vagus nerve.

The origin of inputs to VPNs was investigated by the ionophoretic injection of HRP into regions of the cat ventral medulla where antidromic potentials were recorded to stimulation of the cervical vagus nerve. Retrogradely labelled cells were localised both contralaterally and ipsilaterally in the nucleus tractus solitarius, raphe nuclei, parabrachial nucleus, periaqueductal gray matter and the NA. To determine if the NTS was a source of synaptic input to VPNs in the NA, VPNs were identified by retrograde tracing followed by the ionophoretic injection of the anterograde tracer biocytin into regions of the NTS where evoked potentials were recorded to stimulation of the carotid sinus or cervical vagus nerve. Light microscopic examination revealed anterogradely labelled boutons and fibres in the NA, some of which were in close association with retrogradely labelled VPNs. Subsequent electron microscopic examination revealed some of these boutons forming synaptic specialisations with retrogradely labelled VPNs.

Abbreviations.

A 1	A1 region of the caudal ventrolateral medulla.
C1	C1 region of the rostral ventrolateral medulla.
CHAT	choline acetyl transferase.
cNA	compact region of the nucleus ambiguus.
CT-HRP	cholera-toxin conjugated to horseradish peroxidase.
CVLM	caudal ventrolateral medulla.
CVPN(s)	cardiac vagal preganglionic neurone(s).
DAB	diamino benzidine tetrahydrochloride.
DVN	dorsal vagal nucleus.
E.M.	electron microscopy.
5-HT	5-hydroxytryptamine.
GABA	gamma-aminobutyric acid.
HRP	horseradish peroxidase.
L.M.	light microscopy.
NA	nucleus ambiguus.
NPY	neuropeptide Y.
NTS	nucleus tractus solitarius.
PHA-L	phaseolus vulgaris leucoagglutinin.
PNMT	phenylethanolamine-N-methyl transferase.
RVLM	rostral ventrolateral medulla.
scNA	semi-compact region of the nucleus ambiguus.
SP	substance P.
S-HRP	Streptavidin-horseradish peroxidase.
TMB	Tetramethyl benzidine.
TS	tractus solitarius.
VLM	ventrolateral medulla.
vINA	ventrolateral nucleus ambiguus.

VLDNs ventrolateral depressor neurones.

VLPNs ventrolateral pressor neurones

VPN(s) vagal preganglionic neurone(s).

VRG ventral respiratory group.

WGA-HRP .wheat germ agglutinin conjugated to cholera-toxin.

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Chapter One.

General Introduction.

Section 1:1. Introduction.

Vagal preganglionic neurones play a major role in the control of many autonomic functions (see Loewy and Spyer, 1990). They innervate parasympathetic ganglia that lie near, on the surface, or within the muscular walls of cervical, thoracic and abdominal visceral organs (Kalia and Mesulam, 1980a,b). In order to gain an understanding into the factors which control vagal activity and thus affect the functions of their target organs, vagal preganglionic neurones have been the subject of many neuroanatomical investigations in recent years.

The distribution of vagal preganglionic neurones (VPNs) has been studied utilising the retrograde transport of substances applied to the cervical vagus nerve in the cat (DeVito *et al*, 1974; Kalia and Mesulam, 1980a,b; Kalia, 1981; Nomura and Mizuno, 1983), rat (Contreras *et al*, 1980; Karim and Leong, 1980; Kalia and Sullivan, 1982; Ritchie *et al*, 1982; Stuesse, 1982), dog (Chernicky *et al*, 1983), monkey (DeVito *et al*, 1974; Hamilton *et al*, 1987; Gwyn *et al*, 1985), pigeon (Katz and Karten, 1983), ferret (Withington-Wray and Spyer, 1988), golden hamster (Miceli and Malsbury, 1985) and pig (Hopkins *et al*, 1984). In general these studies have found retrograde labelling in the medulla oblongata in the region of the ipsilateral dorsal vagal nucleus (DVN) and nucleus ambiguus (NA), as well as in the intermediate zone between the two nuclei.

Section 1:2. The Dorsal Vagal Nucleus.

The dorsal vagal nucleus (DVN) is located in the dorsomedial portion of the caudal medulla oblongata, close to the floor of the fourth ventricle and ventromedial to the Nucleus Tractus Solitarius (see figure 1.1). Injection of HRP into end organs or branches of the vagus has revealed that VPNs in the DVN innervate mainly sub-diaphragmatic organs such as the stomach, small intestine, liver, gall bladder, pancreas, ascending and transverse colon (Cat - Kalia and Mesulam, 1980b; Pagani, Norman and Gillis, 1988. Rat - Fox and Powley, 1985; Shapiro and Miselis, 1985b; Norgren and Smith, 1988; Altschuler *et al*, 1991; Berthoud, Carlson and Powley, 1991) but some also innervate the pharynx, larynx, trachea, bronchi, lungs, heart, oesophagus (Kalia and Mesulam, 1980b; see Kalia, 1981 for review; Bieger and Hopkins, 1987).

1:2.1. Topographical Arrangement of Vagal Preganglionic Neurones in the Dorsal Vagal Nucleus.

Selective application of retrograde tracers to specific branches of the sub-diaphragmatic vagus has demonstrated that VPNs are arranged topographically in the DVN and are organised in a series of longitudinal columns running the entire rostrocaudal length of the nucleus (Rat - Fox and Powley, 1985; Norgren and Smith, 1988). A large medial column of cells, lying immediately adjacent to the midline, was labelled following application of retrograde tracers to the gastric branches of the vagus nerve (Rat - Fox and Powley, 1985; Norgren and Smith, 1988).

Fig. 1:1. A diagrammatic representation of three sections of the medulla oblongata of the rat at three different rostrocaudal levels. The dorsal vagal nucleus (DVN) is represented by the blacked area on the right hand side of the sections.

Abbreviations: Cu-cuneate nucleus; GR - gracilis nucleus; TS - tractus of the solitarius; NTS - nucleus of the tractus solitarius; DVN - dorsal vagal nucleus; nX11 - hypoglossal nucleus; NA - nucleus ambiguus; RP - raphe pallidus; LRN - lateral reticular nucleus; IOD - inferior olive dorsalis; IOM - inferior olive medialis; IO inferior olive.

A column of cells in the lateral aspects of the DVN, of much smaller mediolateral extent, was labelled following application of tracers to the coeliac and accessory coeliac branches of the vagus nerve (Rat - Fox and Powley, 1985; Norgren and Smith, 1988). Retrograde tracing from the hepatic branch of the vagus labelled a few scattered cells in the medial DVN on the left side only, suggesting that the distribution of VPNs in the DVN was branch specific. (Fox and Powley, 1985; Norgren and Smith, 1988). However, other studies have shown that branches of the vagus innervate many structures in the GI tract (Berthoud, Carlson and Powley, 1991). Following sub-diaphragmatic vagotomies which left only specific branches intact, a fluorescent anterograde tracer (Dil) was injected into the DVN of rat and the gastrointestinal tract searched for anterogradely labelled fibres. It was concluded that the gastric branches innervated the stomach and the first 2cm of the duodenum, the hepatic branch the duodenum with minor projections to the distal antral stomach and the intestines, and the coeliac branches innervated the duodenum, jejunum, ileum, caecum and entire colon (Berthoud, Carlson and Powley, 1991).

Other workers have investigated the possibility of topographic organisation of VPNs in the DVN by injecting retrograde tracers into end organs in the abdomen of the rat (Kalia and Mesulam, 1980b; Shapiro and Miselis, 1985b; Bieger and Hopkins, 1987; Altschuler *et al*, 1991). These techniques have revealed that VPNs in the medial DVN innervate mainly the stomach (Shapiro and Miselis, 1985b), whereas the lateral DVN contains many neurones which innervate the caecum as well as a smaller number of neurones projecting to the jejunum, ileum and ascending colon (Altschuler *et al*, 1991). The

lateral DVN may also contain neurones projecting to the heart, trachea, larynx, oesophagus and lungs, although their numbers and precise distribution remains uncertain (Kalia and Mesulam, 1980b; Bieger and Hopkins, 1987). The location of VPNs in the DVN which project to the heart is investigated in Chapter Three.

1:2.2. Morphology of Vagal Preganglionic Neurones in the Dorsal Vagal Nucleus.

Examination of the DVN in Nissl stained sections revealed that neurones in the DVN are arranged in a tightly packed group consisting mainly of medium-sized fusiform neurones and some smaller neurones (Cat - Getz and Sirnes, 1949; Taber, 1961). Two types of DVN neurones have also been identified in Golgi-impregnated material (Cat - McLean and Hopkins, 1981). One of these types consisted of fusiform, medium-sized neurones which had three to four primary dendrites branching within the confines of the DVN, whereas the other were small neurones which were round or fusiform with two to four infrequently branching dendrites with few spines (McLean and Hopkins, 1981). Light and electron microscopic analysis of neurones in the DVN of the rabbit, following sectioning of the vagus nerve, revealed a population of degenerated medium sized neurones which were thus presumed to be VPNs, and intact smaller neurones (Rabbit - Aldskogius, 1978). In cat (McLean and Hopkins, 1981,1982) and monkey (McLean and Hopkins, 1985a) 80-85% of medium sized neurones in the DVN were identified as VPNs (mean diameter = 20 μ m X 26 μ m) as they were labelled retrogradely by injection of HRP into the cervical vagus nerve. In the cat, smaller neurones (mean diameter =10 μ m X 17 μ m) were labelled

following injections of HRP into the pons, hypothalamus and amygdala and were thus not vagal preganglionic neurones (McLean and Hopkins, 1982).

At the electron microscopic level VPNs were characterised by abundant organelles and regions of the cytoplasm which contained clumped rough endoplasmic reticulum (Rabbit - Aldskogius, 1978. Cat - McLean and Hopkins, 1981,1982. Monkey - McLean and Hopkins, 1985b). The nucleus was usually oval and non-invaginated with an eccentrically located nucleolus. VPNs had from three to five synapses per $100\mu\text{m}^2$ of membrane, described as a relatively low number of synapses in comparison to neurones in other regions of the brain (McLean and Hopkins, 1985b). Terminals with round vesicles which made asymmetric or symmetric contacts with the post-synaptic membrane were most common, and those with pleomorphic vesicles were the least common. Symmetrical synapses were more common on the somata of VPNs (McLean and Hopkins, 1985b). In contrast, small neurones which projected within the CNS were characterised by a scanty cytoplasm which contained a paucity of organelles and clumped cytoplasmic reticulum. The nuclei of these small neurones had many deep invaginations (McLean and Hopkins, 1981, 82, 85b).

Although Golgi impregnation studies suggested that vagal preganglionic neurones in the DVN had a limited dendritic architecture (McLean and Hopkins, 1981), those labelled retrogradely by injection of cholera toxin-HRP into sub-diaphragmatic organs had extensive dendrites which spread dorsally into the NTS (Rat - Shapiro and Miselis, 1985b; Rinaman *et al*,

1989; Altschuler *et al*, 1991).

1:2.3. Neurochemical Content of Vagal Preganglionic Neurones in the Dorsal Vagal Nucleus.

Since the classic demonstration by Loewi (1921) that the vagus nerve released 'Vagusstoff', and consequently that 'Vagusstoff' was probably acetylcholine (Loewi and Navratil, 1926), vagal preganglionic neurones have been presumed cholinergic. Direct evidence that VPNS were cholinergic was obtained on the observations that the vagal motor nuclei were stained upon incubation of the medullary sections with antibodies to choline acetyltransferase, the enzyme responsible for the final step in the production of acetylcholine (Rat - Armstrong *et al*, 1983; Houser *et al*, 1983), as well as histochemically demonstrated to contain the enzyme acetylcholinesterase, which is responsible for the degradation of acetylcholine (Palkovits and Jacobowitz, 1974; Butcher and Woolf, 1984).

Recently, it has been claimed that some vagal preganglionic neurones in the DVN also contain catecholamines (Rat - Ritchie *et al*, 1982; Manier *et al*, 1987; Tayo and Williams, 1988). In the study by Ritchie *et al* (1982), VPNS were labelled retrogradely by the injection of HRP into the cervical vagus nerve and contained punctate reaction product. In the same material, dopamine-B-hydroxylase, the enzyme responsible involved in the conversion of dopamine to noradrenaline or ultimately adrenaline, was immunocytochemically localised using HRP conjugated antibodies and contained diffuse HRP reaction product. Some cells in the

rostral DVN appeared to contain both types of reaction product, suggesting that they were catecholamine containing VPNs (Rat - Ritchie *et al*, 1982). However, Blessing, Willoughby and Joh (1985) injected the cervical vagus nerve of the rabbit with the fluorescent retrograde tracer Fast Blue and localised catecholaminergic cells with an antibody to tyrosine hydroxylase (an enzyme contained in all catecholaminergic cells) visualised with a rhodamine labelled secondary antibody. No double labelled cells were found and they suggested that earlier studies using punctate versus diffuse HRP reaction products to identify double labelled cells produced false positive results. Nevertheless, using double fluorescent techniques others have since immunocytochemically localised tyrosine hydroxylase and choline acetyltransferase in neurones in caudal portions of the DVN (Rat - Manier *et al*, 1987), and tyrosine hydroxylase in labelled retrogradely neurones also in the caudal DVN (Rat - Tayo and Williams, 1988). Thus catecholamines have been localised only in VPNs in the rostral DVN (Ritchie *et al*, 1982) or only in VPNs in the caudal DVN (Manier *et al*, 1987; Tayo and Williams, 1988) and further investigations are required.

In addition to acetylcholine and catecholamines, retrogradely labelled VPNs in the DVN have also been shown to contain immunoreactivity, for neuropeptide Y, but only at levels rostral to the obex (Rabbit - Blessing *et al*, 1986).

1:2.4. Functions of Vagal Preganglionic Neurones in the Dorsal Vagal Nucleus.

The functions of the vagal preganglionic neurones in the DVN have been studied by chemical or electrical stimulation of the nucleus, which elicits increases in gastric acid secretion (Cat - Kerr and Pershaw, 1969. Rat - Laughton and Powley, 1987; Tache, 1988 for review), gastric motility (Cat - Pagani, Norman, Kaskebar and Gillis, 1985), glucagon and insulin secretion (Rat - Laughton and Powley, 1987; Berthoud, Fox and Powley, 1990) and decreases in ileal water absorption (Martin *et al*, 1989). In addition, many studies have ascribed cardiovascular functions to VPNS in the DVN, but the evidence for this is controversial and is reviewed in the introduction to chapter 3.

1:2.5. Chemistry of Synaptic Inputs to Vagal Preganglionic Neurones in the Dorsal Vagal Nucleus.

Numerous neurochemicals have been identified in the DVN, including amino acids, peptides and biogenic amines (see Palkovits, 1985 and Leslie, 1985). However, as the DVN also contains neurones projecting to the pons, hypothalamus and amygdala (McLean and Hopkins, 1982) it is essential to identify the postsynaptic targets of these neurotransmitters. When identification of VPNS has been carried out, those projecting to the stomach were demonstrated to be innervated by synaptic boutons containing Thyrotrophin Releasing Hormone immuno-reactivity (Rat - Rinaman and Miselis, 1990) and those to a cardiac branch of the vagus nerve were innervated by GABA immunoreactive boutons (Cat - Maqbool *et al*, 1991).

1:2.6. Origins of Synaptic Input to Vagal Preganglionic Neurones in the Dorsal Vagal Nucleus.

Injections of retrograde tracers into the DVN has identified projections to this area from the paraventricular nucleus of the hypothalamus, the lateral hypothalamic area, the dorsomedial hypothalamic nucleus and the nucleus tractus solitarius (Rat - ter Horst, Luiten and Kuipers, 1984), as well as projections from the mesencephalic central gray matter, the medullary reticular formation and the raphe obscurus nucleus (Rat - Rogers et al, 1980; terHorst et al, 1984). In addition, anterograde tracing studies have shown projections to the DVN from the insular region of the cerebral cortex (mouse - Shipley, 1982), the central nucleus of the amygdala (Cat - Hopkins and Holstege, 1978. Monkey - Price and Amaral, 1981. Rabbit - Schwaber et al, 1982), the A5 catecholamine cell group (Rat - Loewy et al, 1979) and the lateral hypothalamic area (Rat - Berk and Finkelstein, 1982). However, despite these studies, VPNs have not been identified as post-synaptic targets of neurones projecting to the DVN.

VPNs in the DVN have been demonstrated to be innervated by vagal afferents (Rat - Neuhuber and Sandoz, 1986; Rinaman *et al*, 1989). HRP was applied to the cervical vagus nerve of rats and reaction product identified at the ultrastructural level in boutons innervating VPNs in the DVN (Rat - Neuhuber and Sandoz, 1986). Similarly, following injection of CT-HRP into the stomach in rats, labelled vagal afferent terminals were observed in the parvocellular sub-nucleus of the Nucleus Tractus Solitarius in synaptic contact with dendrites extending from VPNs in the DVN (Rinaman *et al*, 1989).

Section 1:3. The Nucleus Ambiguus.

The nucleus ambiguus, so called as it is a diffuse nucleus with indistinct borders (Krause, 1876, quoted in Mohlant, 1910), lies in the ventrolateral portion of the medullary reticular formation beginning posterior to the facial motor nucleus and extending caudally to the first cervical segment of the spinal cord (see figures 1.1 and 1.2). There have been various schemes used to describe the architecture of the Nucleus Ambiguus, probably due the difficulties in precisely determining its borders.

1:3.1. Organisation and nomenclature of the Nucleus Ambiguus.

Until recently one of the most widely used descriptions of the NA was that proposed by Taber (1961) which was based on Nissl staining in the cat medulla. A compact group of cells rostral to obex but caudal to the facial nucleus was termed the nucleus ambiguus, the retrofacial nucleus ventral to the NA, and the nucleus retroambiguus caudal to the NA, "occupying a medullary position comparable to the intermediolateral cell column of the spinal cord".

In 1966 the nucleus ambiguus in the rabbit was defined by the distribution of degenerated cell bodies in the medulla on cutting branches of the vagus and glossopharyngeal nerves (Lawn, 1966a,b). The most rostral part of the NA was described as a 'compact' formation consisting of a **principal** column of medium sized tightly packed cells innervating the oesophagus, and a **medial** column which contained degenerated smaller neurones upon sectioning of

the glossopharyngeal nerve. Approximately 1.5mm caudal to the rostralmost point of this medial column, the cells became scattered in a region termed the **diffuse** formation, which contained medium sized and larger neurones innervating the laryngeal musculature except the cricothyroid muscle.

In 1987 the Nucleus Ambiguus was redefined in the rat by Bieger and Hopkins on the basis of the distribution of neurones retrogradely labelled from branches of the vagal or glossopharyngeal nerves. In this description these authors used terms employed at the turn of the century (see Molhant, 1910 for review) which described the NA as comprising of two major divisions (Bieger and Hopkins, 1987; see figure 1.2.). The first division began caudal to the facial nucleus, extended caudal to the first cervical spinal cord segment and was defined as comprising of three rostro-caudally arranged sub-divisions. The rostralmost sub-division, immediately caudal to the facial nucleus, was termed the **compact** group and consisted of tightly packed medium sized cells labelled retrogradely from the oesophagus (max. diameter (M.D) = 30 μ m). An intermediate sub-division began around obex and extended rostrally in a position ventral to the compact formation and was termed the **semi-compact** formation as it comprised of a scattered group of larger neurones innervating the striated muscle of the pharyngeal constrictors and cricothyroid muscle (M.D. = 45 μ m). The third most caudal sub-division, called the **loose** formation of the nucleus ambiguus, extended from just rostral to obex caudally almost to the spinal cord and contained medium sized neurones (M.D. = 30 μ m) innervating striated muscle of the laryngeal musculature (except the cricothyroid muscle) which were widely scattered throughout

the reticular formation. The second major division of the NA was called the **external formation** and reported to contain small cells labelled retrogradely from the glossopharyngeal and superior laryngeal nerves, the lungs and the heart (M.D = 20 μ m). At rostral levels neurones of the external formation were ventral to the compact NA and at caudal levels were intermingled with the neurones of the loose formation. There were no labelled neurones in the NA labelled retrogradely from the stomach and earlier reports of such a projection were ascribed to spillage of tracer onto the oesophagus.

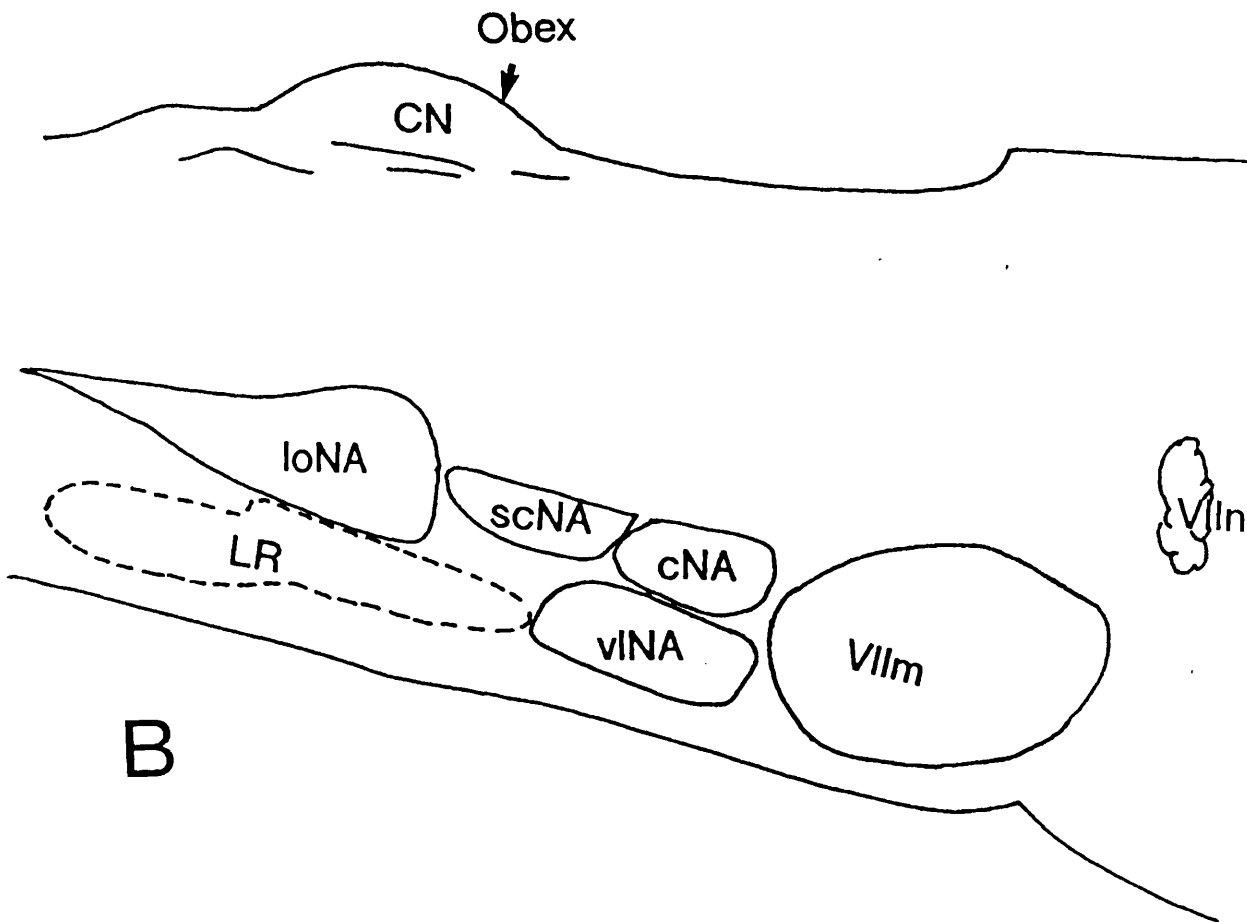
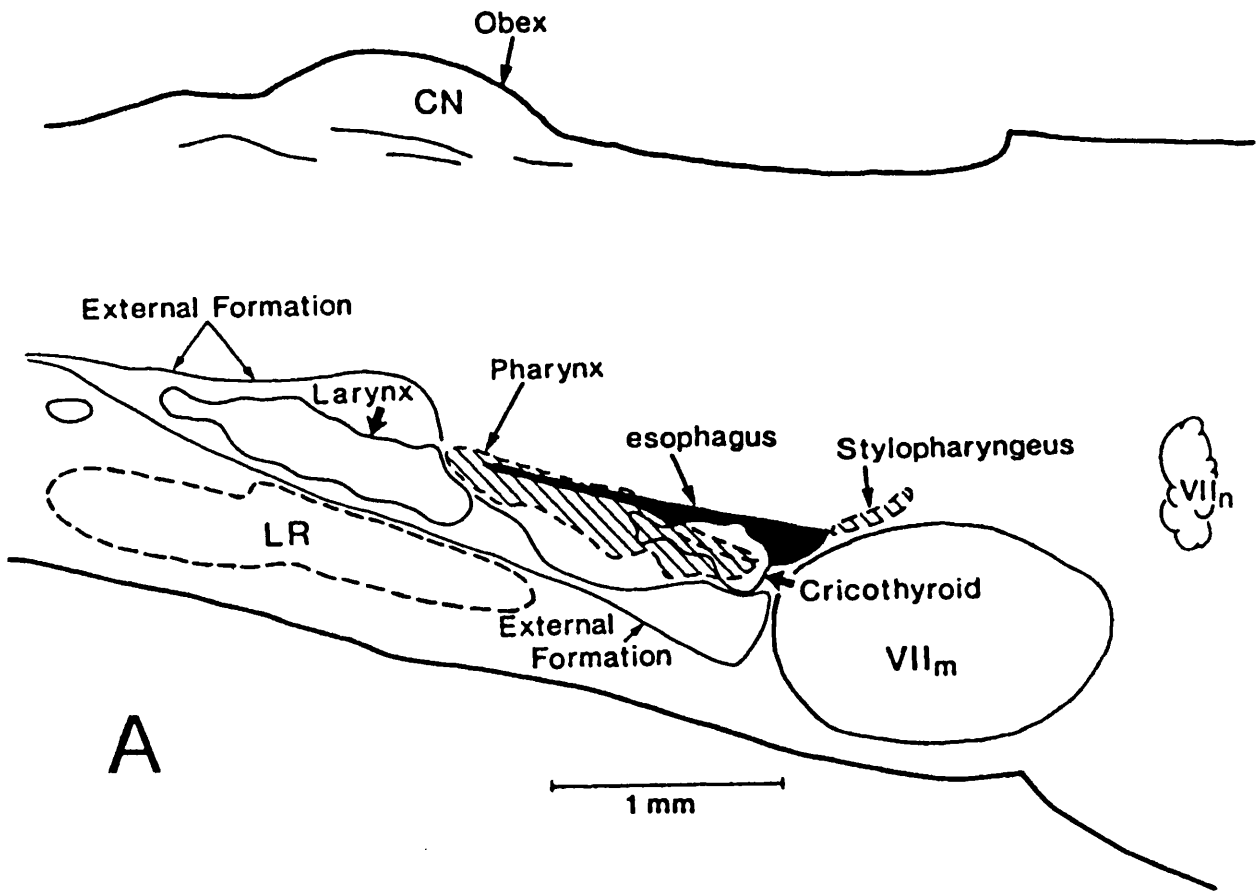
As the nomenclature of this latest re-definition of the nucleus ambiguus appears to have been widely accepted, these terms will be largely used in this thesis. However, for ease of writing the rostral portion of the external formation ventral to the compact nucleus ambiguus will be referred to as the ventrolateral nucleus ambiguus (vINA), and the caudal portion will be considered to be part of the semi-compact and loose formations (see figure 1.2.).

Figure 1:2.

Figure 1:2.A: Diagrammatic representation of the projections of vagal preganglionic neurones in the nucleus ambiguus according to Bieger and Hopkins (1987). In this model the compact NA contains neurones innervating the oesophagus, the semi-compact formation those projecting to the pharynx and cricothyroid muscle, the loose formation VPNS innervating the larynx and the external formation contains VPNS innervating the heart and lungs, as well as some with unidentified targets with axons in the superior laryngeal and glossopharyngeal nerves.

Figure. 1:2.B: A diagrammatic representation of the nomenclature used in this thesis to describe the nucleus ambiguus. The model is basically the same as that of Bieger and Hopkins although the external formation ventral to the compact NA is termed the ventrolateral NA (vINA) and at caudal levels it is included in the definition of the scNA and loNA.

Abbreviations: LR - lateral reticular nucleus; VIIIm - facial motor nucleus; loNA loose formation of the nucleus ambiguus; scNA - semi-compact formation of the NA; cNA - compact formation of the nucleus ambiguus; vINA - ventrolateral nucleus ambiguus.



1:3.2. Morphology of Vagal Preganglionic Neurones in the Nucleus Ambiguus.

Unlike VPNS in the DVN, those in the NA display a wide range of sizes (Bieger and Hopkins, 1987). However, despite many studies on the distribution of VPNS within the NA, there have been very few morphological examinations of these neurones. Electron microscopic examination of VPNS in the NA has been carried out in the cat and monkey (McLean and Hopkins, 1985b). Retrogradely labelled vagal preganglionic neurones appeared similar to motoneurones as the cytoplasm contained abundant organelles such as ribosomes, smooth and rough endoplasmic reticulum, Golgi complexes, lysosomes, microtubules and a non-invaginated nucleus (McLean and Hopkins, 1985b).

Electron microscopy also revealed that the distribution of morphologically distinct synapses on the membrane of VPNS varied widely (McLean and Hopkins, 1985a). Although specific numbers were not given in the Author's text it appears that they were evenly divided between asymmetric or symmetric synapses with round vesicles and symmetric synapses with pleomorphic vesicles, whereas asymmetric synapses with pleomorphic vesicles were rare. VPNS were reported to have fewer synapses than other neurones in the CNS. The number of synapses on VPNS was reported to increase with size of neurone and from cat to monkey, although the maximum of 25 synapses per $100\mu\text{m}^2$ of membrane was observed in monkey VPNS in the middle range of sizes (McLean and Hopkins, 1985a).

In addition to retrogradely labelled VPNs the NA also contained many unlabelled neurones (McLean and Hopkins, 1985a). These were morphologically diverse, though generally smaller than VPNs (M.D. = 30µm). The smallest of these neurones (M.D. = 20µm) had markedly invaginated nuclei and a paucity of organelles. In general unlabelled neurones had greater numbers of impinging synapses than VPNs of a comparable size (McLean and Hopkins, 1985b). However, some of the larger unlabelled neurones appeared similar to VPNs, and it was not clear whether these were unlabelled VPNs or neurones with other functions.

1:3.3. Neurochemical Content of Vagal Preganglionic Neurones in the Nucleus Ambiguus.

Vagal preganglionic neurones in the NA were shown to be cholinergic as they stained histochemically for the enzyme acetylcholinesterase (Palkovits and Jacobowitz, 1974; Butcher and Woolf, 1984), and were immunoreactive for choline acetyltransferase (Armstrong *et al*, 1983; Houser *et al*, 1983).

Recently, it has been claimed that some vagal preganglionic neurones in the NA also contain catecholamines as retrogradely labelled VPNs in the NA also contained immunoreactivity for dopamine-B-hydroxylase (Rat - Ritchie *et al*, 1982). However, peroxidase methods were used to localise both VPNs and immunoreactivity and double labelled cells may have been false-positive results (Ritchie *et al*, 1982). Others have found no double labelled cells using fluorescent retrograde tracers to label VPNs and fluorescent secondary antibodies to localise tyrosine hydroxylase,

the enzyme present in all catecholaminergic cells (Rabbit - Blessing, Willoughby and Joh,1985).

VPNs in the NA of the rat labelled retrogradely from the heart, stomach, oesophagus, abdominal vagus and recurrent laryngeal nerve have been shown to contain galanin and calcitonin-gene related peptide (CGRP; Sawchenko *et al*, 1987). However, in the cat CGRP has been shown to be absent from VPNs projecting to the abdomen and thorax and present only in VPNs projecting to the striated muscle of the larynx and pharynx (McWilliam *et al*, 1989). This apparent species difference may be due to the fact that Sawchenko *et al* pretreated their animals with colchicine, which disrupts axoplasmic flow allowing neurochemicals to accumulate in cell soma and thus cells with low levels of transmitter can be detected, whereas McWilliam *et al* did not. This requires further investigations.

In the rat, VPNs projecting to the larynx have also been shown to be immunoreactive for glutamate and choline acetyltransferase (Saji and Miura, 1991).

1:3.4. Functions and Properties of Vagal Preganglionic Neurones in the Nucleus Ambiguus.

1:3.4.A. Cardiac Vagal Preganglionic Neurones.

In anaesthetised preparations, the spontaneous discharge of cardiac vagal preganglionic neurones appears to vary between species. Vagal 'tone' has been reported to be low in the cat, but activity appears to be higher in the dog (Jewett, 1964; Katona *et al*, 1970) and rabbit (Jordan *et al*, 1982), although vagal efferent discharge rarely exceeds 20 action potentials per second (Katona *et al*, 1970; Kunze, 1972). CVPNs are reported to have axons which conduct in the B-fibre range ($<2.5\text{m.s}^{-1}$; Middleton, Middleton and Grundfest, 1950; McAllen and Spyer, 1978a; Jordan *et al*, 1982), but in the rat there also appears to be some C-fibres in the vagus nerve which slow the heart on electrical stimulation (conduction velocity $3\text{-}15\text{m.s}^{-1}$; Nosaka *et al*, 1979).

CVPNs receive an excitatory input from the baroreceptors and discharge with a pulse-synchronous rhythm (McAllen and Spyer, 1978a,b; Jordan *et al*, 1982). This input is from baroreceptors in both the carotid sinus and aortic arch, since CVPNs were excited by stimulation of the afferent nerves from both these regions (McAllen and Spyer, 1978b; Jordan *et al*, 1982). The pathway from the baroreceptors to CVPNs appears to be multi-synaptic since stimulation of the carotid sinus nerve in the cat evoked excitation of CVPNs with a delay of 20-50ms (McAllen and Spyer, 1978b). However, on correlating CVPN activity to carotid sinus nerve activity the same authors estimated that the central delay for

conduction from baroreceptors to CVPNs was 20-110ms, and that most responded in the range 80-100ms. Further studies suggested that two pathways may be involved since excitation of CVPNs occurred 20-60msec or 70-110msec after natural stimulation of the baroreceptors (McAllen and Spyer, 1978b).

As well as a pulse-synchronous activity, an underlying respiratory rhythm has been obtained from recordings of the cardiac branch of the vagus nerve (Rijlant, 1936; Jewett, 1964; Katona *et al*, 1970; Neil and Palmer, 1975; Davidson, Goldner and McCloskey, 1976) and from recordings of antidromically identified CVPN cell bodies (McAllen and Spyer, 1976; Gilbey *et al*, 1984), and CVPNs appear to discharge in post-inspiration.

1:3.4.B. VPNs Innervating the Airways.

It is now established that a certain level of airway smooth muscle tone is due to vagal activity, since injections of atropine increase anatomical dead space (Severinghaus and Stupfel, 1975) and decrease tracheal muscle tension (Mitchell *et al*, 1985). These bronchoconstrictor neurones are located in the NA since microinjection of excitatory amino acids into the NA of dogs elicited increases in total lung resistance which were blocked by prior intravenous application of atropine (Dog - Connelly, McCallister and Kaufman, 1987; Haselton, Padrid and Kaufman, 1991). Similarly, topical application of excitatory amino acids to the VLM surface increased tracheal muscle tone by a vagally mediated effect (Cat - Haxhiu, Deal, Norcia, VanLunteren, and Cherniack, 1987).

The properties of VPNS innervating the airways have been identified by recording from the cell bodies of neurones in the NA antidromically activated by stimulation of pulmonary projecting branches of the vagus nerve (McAllen and Spyer, 1978a). These neurones had calculated axon conduction velocities in the B-fibre range. Some had ongoing activity but others were induced to fire by ionophoretic application of excitatory amino acids, and most fired mainly in inspiration (McAllen and Spyer, 1978a).

1:3.4.C. VPNS Innervating the Gastro-Intestinal Tract.

VPNS in the NA have also been suggested to be involved in control of the gastro-intestinal tract since excitation of VPNS by microinjections of glutamate into the NA caused contractions in the pharynx and oesophagus (Rat - Bieger, 1984). However, VPNS in the NA do not appear to control functions of the gastrointestinal tract below the oesophagus. Electrical stimulation of the NA elicited contractions of the antrum, pylorus and duodenum which were abolished by vagotomy, but these responses failed to follow repetitive stimuli at high frequency, suggesting that they were mediated through a multi-synaptic pathway and not by a direct effect of stimulation of VPNS (Rat - Pagani, Norman, Kaskebar and Gillis, 1984).

1:3.5. Functional Heterogeneity of the Nucleus Ambiguus.

Despite the NA often being named with respect to distribution of VPNs, it is important to note that it contains cells with projections other than the vagus nerve. Neurones in the NA have been labelled retrogradely by the injection of HRP into the cervical spinal cord (Cat - Rikard-Bell et al, 1984; Portillo and Pasaro, 1988; Tohyama et al, 1979. Rabbit - Ellenberger et al, 1990. Rat - Onai et al, 1987; Portillo and Pasaro, 1988; Ellenberger and Feldman, 1990b), thoracic regions of the spinal cord (Cat - Tohyama et al, 1979); parabrachial nucleus (Cat - King, 1980), the NTS (Cat - Kalia et al, 1979; Rat - Ellenberger and Feldman, 1990a), the facial nucleus (rabbit - Bystrzycka and Nail, 1984) and other parts of the contralateral and ipsilateral NA (See table 1:1.). Some of these neurones have respiratory related activity and are termed the Ventral Respiratory Group (VRG; see below) and others may be involved in central control of the sympathetic nervous system (see section 1:3.5.B.).

1.3.5.A. Relationship of Ventral Respiratory Group to the Nucleus Ambiguus.

Neurones in the ventral medulla which display respiratory related firing patterns are functionally defined as the ventral respiratory group and some of these neurones have been located in the nucleus ambiguus (see figure 1.3; Merrill, 1970, 1984; for review see Feldman, 1986). At the most rostral part of the VRG in the vicinity of the cNA, in a region also known as the Botzinger Complex, cells fire with an expiratory related pattern (Merrill, 1984). Caudal to

this group of cells there is a group of inspiratory neurones, which overlaps further caudally with another group of expiratory neurones (Merrill, 1984; see fig. 1.3).

The functional definition of the VRG also includes some vagal preganglionic neurones in the NA, since neurones with respiratory related activity have been antidromically activated by electrical stimulation of branches of the vagus nerve projecting to the larynx (Cat - Delgado-Garcia et al, 1983), pharynx (Cat - Grelot et al, 1988; Bianchi et al, 1988), heart and lungs (Cat - McAllen and Spyer, 1978a,b). In other studies VPNs have been shown as a component of the VRG by intracellular recording from neurones with respiratory related activity which were confirmed as projecting to the vagal nerve tract by dye filling (Cat - Kreuter *et al*, 1977. Rat - Zheng *et al*, 1991).

In addition to vagal preganglionic neurones the VRG consists of neurones which are activated antidromically by electrical stimulation of the NTS, NA and spinal cord (Cat - Merrill, 1970; Fedorko and Merrill, 1984). Those activated antidromically by stimulating the spinal cord have been located within the nucleus ambiguus by intracellular recording combined with dye filling, which also confirmed their axonal trajectories (Cat - Kreuter *et al*, 1977. Rat - Zheng *et al*, 1991).

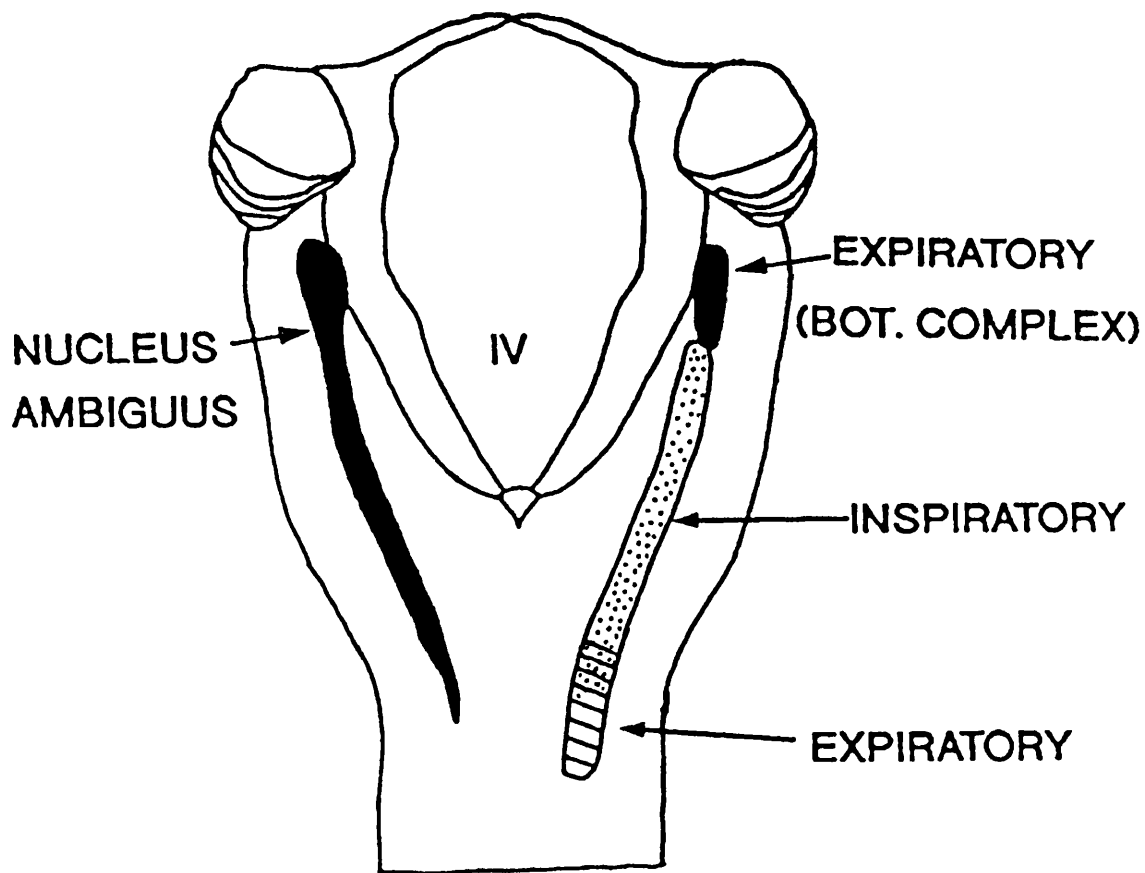


Figure 1.3 Schematic of a horizontal view of the caudal brainstem of the rat, indicating on the right hand side the rostral group of neurones with expiratory related activity (Botzinger Complex; blacked area), the middle group of inspiratory neurones (stippling) which overlaps with the caudal most group of expiratory neurones (lines). The left hand side represents the Nucleus Ambiguus (blacked area). Adapted from Ellenberger and Feldman (1990b).

Anatomical studies have also confirmed that neurones in the VRG are located in the nucleus ambiguus since retrograde labelling from areas in which electrical stimulation could antidromically activate neurones in the VRG resulted in labelled neurones in the NA (Cat - Kalia *et al*, 1979; Rikard-Bell *et al*, 1984; Portillo and Pasaro, 1988. Rabbit - Ellenberger *et al*, 1990 Rat - Ellenberger and Feldman, 1990b) . In this way, neurones in the NA have been shown to project to the phrenic motor nucleus in the spinal cord (Cat - Rikard-Bell *et al*, 1984; Portillo and Pasaro, 1988. Rabbit - Ellenberger *et al*, 1990 Rat - Ellenberger and Feldman, 1990b), the dorsal respiratory group in the ventrolateral NTS (Cat - Kalia *et al*, 1979) and the contralateral VRG (Rat - Ellenberger and Feldman, 1990b).

Neurones in the ventral respiratory group have been shown by anatomical studies to lie amongst vagal preganglionic neurones in the NA (Cat and Rat - Portillo and Pasaro,1988; Rat - Ellenberger and Feldman, 1990b). In cat and rat neurones labelled retrogradely by the injection of different fluorescent tracers into the cervical spinal cord and intrinsic laryngeal muscles were intermingled within the NA (Cat and Rat - Portillo and Pasaro,1988). In addition, injection of fluorescent tracers into the phrenic motornucleus and the VRG in one side of the medulla in rats retrogradely labelled cells in the contralateral VRG which were intermingled with labelled retrogradely VPNS, especially in the vINA (Rat - Ellenberger and Feldman, 1990b). In this study neurones projecting to the vagus and phrenic motor nucleus were found to be indistinguishable on the basis of size or morphology at the light microscopic level, whereas those projecting to the contralateral VRG were smaller (Ellenberger

and Feldman, 1990b). Thus vagal preganglionic neurones in the NA are indistinguishable at the light microscopic level from some neurones in the region with respiratory related functions.

1:3.5.B. Neurones Involved in Central Sympathetic Control.

In addition to vagal preganglionic neurones and other neurones with respiratory related functions, the ventrolateral medulla contains neurones implicated in the central control of the sympathetic nervous system (for review see Guyenet, 1990). These neurones are located in regions of the ventrolateral medulla (VLM) which are traditionally functionally divided into a rostral and a caudal portion (Alexander, 1946; Guertzenstein and Silver, 1974; see figure 1:4).

1.3.5.B.1. Rostral Ventrolateral Medulla.

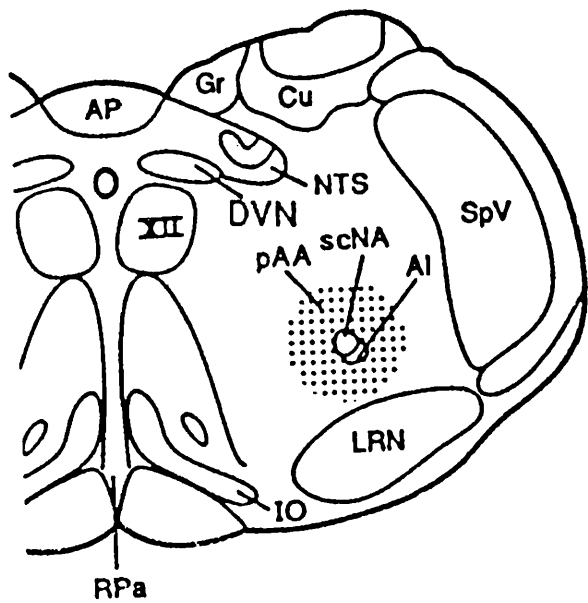
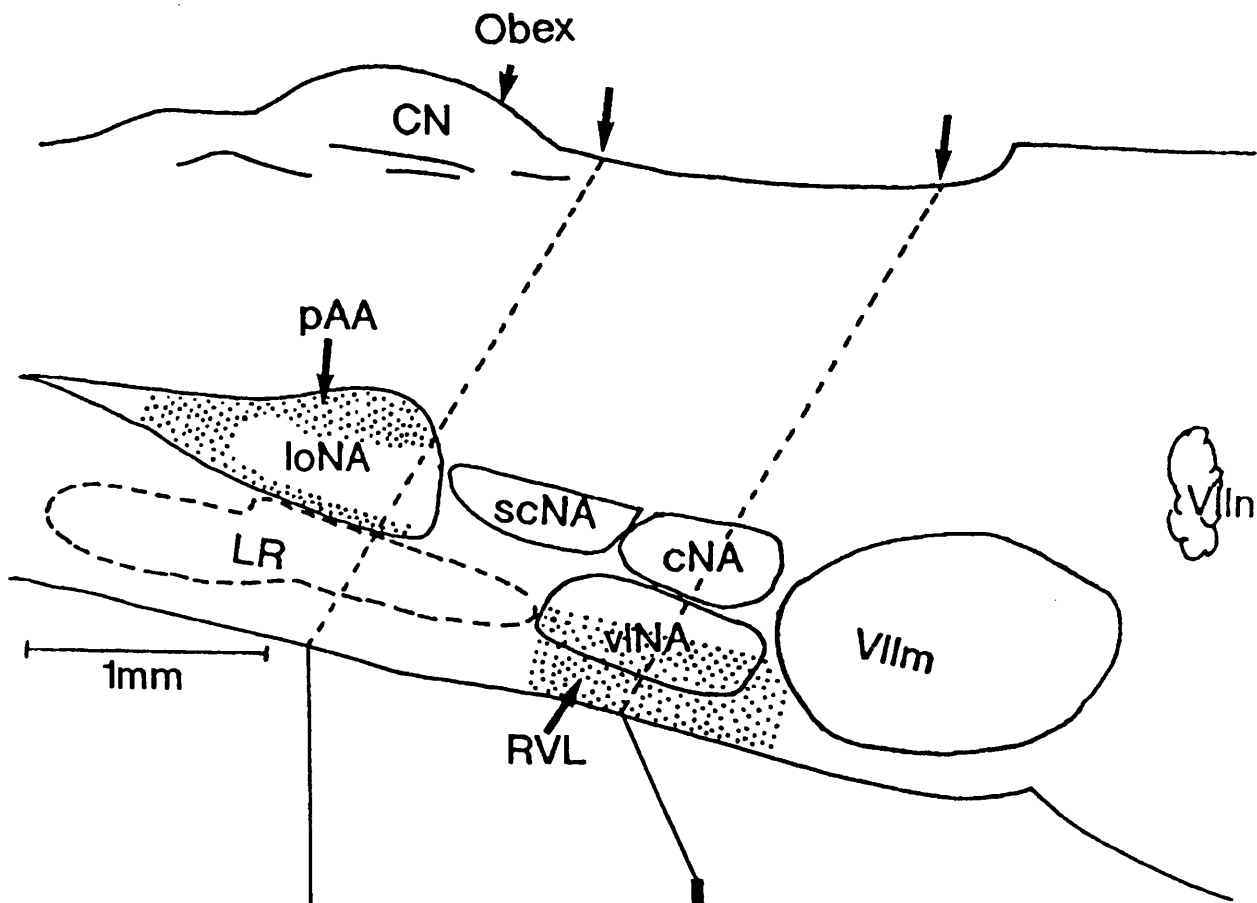
The rostral portion of the VLM contains the functionally defined 'pressor region' which consists of a group of neurones considered by some to be responsible for the maintenance of vasomotor tone and arterial blood pressure (Cat - Dampney and Moon, 1980; Calaresu and Yardley, 1988; Gebber, Barman and Kocsis, 1990). Anatomically, this region has been termed the rostral ventrolateral medulla (RVLM) and lies rostral to the lateral reticular nucleus, caudal to the facial nucleus and extends to the ventral surface of the medulla, overlapping with the vlNA (see Guyenet, 1990; Figure 1.4.).

Figure 1:4. (adapted from Guyenet, 1990).

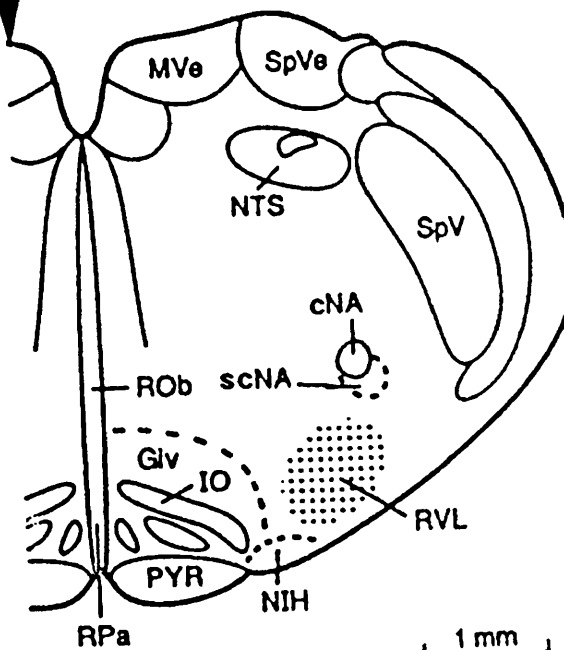
Top - Diagrammatic representation of a para-sagittal section of the rat medulla similar to that in figure 1.2. The peri-ambigular area (dashed section in the region of the scNA) is often called the caudal ventrolateral medulla and contains the A1 noradrenergic and C1 adrenergic neurones, non-catecholaminergic neurones involved in the control of sympathetic outflow and ventral respiratory group neurones. RVL represents the rostral ventrolateral medulla which contains C1 adrenergic neurones, VRG neurones, non-catecholamine neurones and overlaps with the vINA.

Below: Frontal sections of the medulla oblongata of the rat taken from the levels indicated by the arrows and dashed lines on the upper diagram, indicating the positions of the periambigular area and the rostral ventrolateral medulla with respect to the NA.

Abbreviations: Top - same as figure 1. except pAA = periambigular area and RVL = rostral ventrolateral medulla.
Bottom: Cu - cuneate nucleus; DVN - dorsal vagal motor nucleus; Giv - gigantocellular reticular nucleus; GR - gracile nucleus; IO - inferior olive; MVe - medial vestibular nucleus; NTS - nucleus tractus solitarius; PYR - pyramidal tract; RPa - raphe pallidus; Rob - raphe obscurus; RVL - rostral ventrolateral medulla; SPV - spinal trigeminal nucleus; SpVe - spinal vestibular nucleus.



+0.5mm



+2.0mm

Stimulation of the rostral ventrolateral medulla (RVLM) by micro-injection of glutamate elicits pressor effects which are thought to be mediated by increases in the activity of the sympathetic nervous system (Rat - Willette *et al*, 1983a). However, the identity of the neurones which mediate these effects is not clear (for review see Guyenet, 1990).

Anatomical studies have shown that the RVLM contains a heterogenous population of neurones. Neurones chemically identified in the RVLM include those known as the C1 group, which contain immunoreactivity for phenylethanolamine-N-methyl transferase (PNMT), the synthesising enzyme for adrenaline (Hokfelt *et al*, 1974). Neurones in the C1 group have various projections as they have been labelled retrogradely from the spinal cord (Rat - Ross *et al*, 1981a, 84), the NTS (Rat - Thor and Helke, 1987) and the hypothalamus (Rat - Sawchenko *et al*, 1985; Tucker *et al*, 1987).

PNMT containing neurones in the C1 group neurones in the rat have also been shown to contain other neurotransmitters such as substance P (Lorenz *et al*, 1985), neuropeptide Y (Everitt *et al*, 1984) and enkephalin (Ceccatelli *et al*, 1989; Murakami *et al*, 1989). Some of these substances are also probably not co-localised with PNMT in neurones in the RVLM. Immunocytochemical studies have shown that neurones in the RVLM contain 5-HT, substance P and thyrotrophin releasing hormone, sometimes co-localised (Rat - Johansson *et al*, 1981). In addition, GABA (Rat - Millhorn *et al*, 1987), enkephalin (Rat - Hokfelt *et al*, 1974; Menetrey and Basbaum, 1987) and somatostatin (Rat - Strack *et al*, 1989) have also been demonstrated in neurones in this region.

1:3.5.B.2. Caudal Ventrolateral Medulla.

The CVLM, which is also known as the peri-ambigular area, overlaps with the loNA and the scNA (Guyenet, 1990; Fig.1:4). The CVLM was functionally defined as the area of the VLM containing the 'depressor' region of the VLM, which consists of neurones which mediate sympatho-inhibition upon stimulation by glutamate (Rat - Willette *et al*, 1983a; Bonham and Jeske, 1989; Smith and Barron, 1990. Rabbit - Blessing and Reis, 1982). Inhibition of the neurones in this region by microinjection of the GABA agonist muscimol caused pressor responses which were blocked by phentolamine, indicating that the pressor responses were due to increased sympathetic activity and suggesting that neurones at the injection site in the CVLM inhibited sympathoexcitatory neurones (Willette *et al*, 1983b).

Depressor responses upon stimulation of the CVLM have been suggested to be mediated by noradrenaline containing neurones in the region, which are termed the A1 group (Dahlstrom and Fuxe, 1964, Howe *et al*, 1980), since the anatomy of the A1 neurones known to date partially agrees with physiological evidence (see Guyenet, 1990). Injections of retrograde tracers into the spinal cord labelled retrogradely few (Rat - McKellar and Loewy, 1982) or none (Rat - Westlund *et al*, 1983,84) of the A1 neurones. In addition, injection of PHAL into the CVLM resulted in terminal like labelling in the RVLM in close apposition to cells labelled trans-neuronally from the adrenal gland (Rabbit - Li and Blessing, 1989). However, it is not certain that these terminals were axons of A1 neurones since injection of retrograde tracers into the pressor region of the RVLM

labelled retrogradely cells in the CVLM which were not catecholamine immunoreactive (Rabbit - Blessing and Li, 1989), but which contained the GABA synthesising enzyme glutamate decarboxylase (Rabbit - Blessing, 1990).

1:3.5.B.3. Relationship of the Ventrolateral Medulla to the Vagal Preganglionic Neurones in the Nucleus Ambiguus.

It has been described above that the ventrolateral medulla contains neurones with a wide diversity of functions and chemistry, and that despite first suggestions, the A1 and C1 cell groups may not be the neurones which mediate these functions. However, the locations of the A1 and C1 neurones define the areas where stimulation elicits changes in blood pressure through actions on the sympathetic nervous system (see Guyenet, 1990). The close proximity of neurones in these areas to vagal preganglionic neurones of the nucleus ambiguus has been demonstrated by immunocytochemistry and retrograde labelling (Ritchie *et al*, 1982). A1 and C1 neurones were identified as immunoreactive for dopamine-beta-hydroxylase, the enzyme involved in the conversion of dopamine to noradrenaline, and were found to be intermingled with retrogradely labelled VPNS in the NA, particularly in the area defined as the vINA in section 1:3.1. (Rat - Ritchie *et al*, 1982).

1:3.6. Chemistry of Synaptic Input to Vagal Preganglionic Neurones in the Nucleus Ambiguus.

There have been two approaches to studying the chemical nature of inputs to VPNS: neuropharmacological experiments investigating the sensitivity of VPNS to different putative transmitters and immunocytochemical studies investigating the neurochemical content of terminals in synaptic contact with VPNS in the NA and the DVN. Many of these studies have focussed on cardiac vagal preganglionic neurones due to their importance in the control of heart rate and blood pressure.

1:3.6.1. Neuropharmacology.

Numerous putative neurotransmitters have been suggested to excite or inhibit CVPNS when microinjected into the NA, as their effects are prevented by vagotomy or prior intravenous administration of atropine. Excitation of CVPNS, resulting in a vagally mediated cardiac slowing, was produced by microinjection into the NA of the opioid peptides fentanyl and met-enkephalin amide (Dog - Laubie *et al*, 1979. Rat - Agarwal and Calaresu, 1991), excitatory amino acids (Rat - Willette *et al*, 1983a; Agarwal and Calaresu, 1991), serotonin (Cat - Izzo *et al*, 1988), neuropeptide Y (Rat - MacRae and Reid, 1988), substance P (Rat - Urbanski *et al*, 1988; Agarwal and Calaresu, 1991), Atrial Natriuretic Factor (Rat - Ermirio *et al*, 1991) and acetylcholine (Rat - Agarwal and Calaresu, 1991). In contrast, GABA appears to inhibit CVPNS since injection of the GABA antagonist bicuculline into the nucleus ambiguus in anaesthetised artificially ventilated dogs evokes a dose-dependent fall in heart

rate due to an increase in vagal activity (DiMicco *et al*, 1979). Other neurotransmitters which may inhibit CVPNs upon microinjection into the NA and elicit vagally mediated increases in heart rate include vasopressin and oxytocin (Rat - Kiriakopolous and Caverson, 1991) and glycine (Chitravanshi *et al*, 1991). However, these studies fail to distinguish if the action of injected drugs is directly on CVPNs or is mediated through other neurones with CVPNs the final output.

The ionophoretic application of some of these compounds directly onto antidromically identified CVPNs suggests that they have direct actions on CVPNs (McAllen and Spyer, 1978a; Gilbey *et al*, 1984,85). In the cat, CVPNs were inhibited by ionophoretic application of GABA, an inhibition that was specific since it was prevented by the application of the GABA antagonist bicuculline (Cat - Gilbey *et al*, 1985). Using identical techniques CVPNs were also inhibited by application of acetylcholine, an effect antagonised by simultaneously applied atropine (Gilbey *et al*, 1984), and excited by application of excitatory amino acids (Cat - McAllen and Spyer, 1978b; Gilbey *et al*, 1984).

1:3.6.2. Immunocytochemistry.

There have been many studies demonstrating the presence of neuroactive substances in the NA (see Loewy and Spyer, 1990). However, in studies of the input to neurones in the NA it is particularly important to identify post-synaptic targets due to the diffuse nature of the nucleus and the functional heterogeneity of neurones in this region (see section 1:3.5.), and this has been carried

out only rarely. In the cat, 5-HT and substance P immunoreactive boutons were observed in close apposition to VPNs in the NA labelled retrogradely from the larynx (Cat - Holtman, 1988). In the rat, enkephalin and somatostatin immunoreactive boutons have been observed in the vicinity of VPNs labelled retrogradely by injections of tracers into the oesophagus (Rat - Cunningham and Sawchenko, 1989; Cunningham *et al*, 1990b). However, no electron microscopy was carried out to establish synaptic associations between labelled retrogradely and immunoreactive structures. Electron microscopy has been used in this way only once, to identify GABA immunoreactivity in boutons in synaptic contact with neurones in the NA of the cat labelled retrogradely from a cardiac branch of the vagus nerve (Maqbool *et al*, 1991).

1:3.7. Origin of Synaptic Inputs to Vagal Preganglionic Neurones in the Nucleus Ambiguus.

There have been few studies primarily concerned with identifying the origin of synaptic inputs to VPNs in the NA. However, there have been many studies which have injected the ventrolateral medulla with retrograde tracers and noted the distribution of labelled cells (see below and table 1:1). Since VPNs are located in the VLM, these studies may indicate areas which contain cells innervating VPNs. Using these techniques retrogradely labelled cells have been observed in various nuclei of the NTS (see table 1.1), the medullary reticular formation, other levels of the NA and VLM, lateral and medial parabrachial nuclei, Kolliker-Fuse nucleus, paraventricular nucleus of the hypothalamus, lateral hypothalamic nuclei, dorsal medial hypothalamus, ventral and lateral parts of the peri-

aqueductal gray, central nucleus of the amygdala, bed nucleus of the stria terminalis, substantia inominata, medial prefrontal cortex, inferior colliculus and locus coeruleus (see table 1.1; Bystrzycka, 1980; Stuesse and Fish, 1984; terHorst *et al*, 1984; Lovick, 1985; vanBockstaele *et al*, 1989; Loewy and Burton, 1978; Ross *et al*, 1985; Dampney *et al*, 1987; Cunningham and Sawchenko, 1990; Nunez-Abades *et al*, 1990; Smith *et al*, 1989; Takayama *et al*, 1990). However, as the NA contains a wide diversity of neurons this type of study cannot determine the post-synaptic targets of neurones labelled retrogradely from this region.

Use of anterograde tracers has shown that the VLM receives projections from cells in the NTS (Cat - Loewy and Burton, 1978. Monkey - Beckstead *et al*, 1980. Rat - Ross *et al*, 1985; vanBockstaele *et al*, 1989; Cunningham and Sawchenko, 1990a,b), the area postrema (Rat - Shapiro and Miselis, 1985a), the raphe nuclei (Cat - Bobillier *et al*, 197. Rat - Nicholas and Hancock, 1990), the peri-aqueductal gray matter (Cat - Holstege, 1989; Holstege *et al*, 1983; Lovick, 1985. Rat - vanBockstaele *et al*, 1989), the parabrachial nucleus (Rat - Herbert *et al*, 1990), the central nucleus of the amygdala (Cat - Hopkins and Holstege, 1978; Price and Amaral, 1981. Rat - Ross *et al*, 1981a), and the paraventricular nucleus and lateral area of the hypothalamus (Rat - Berk and Finkelstein, 1982). However, many of these studies traced pathways with tritiated amino acids and it is impossible to determine if the silver grain labelling indicative of anterograde transport is contained in axons of passage or terminal boutons (Bobillier *et al*, 1976; Hopkins and Holstege, 1978; Loewy and Burton, 1978;

Table 1:1. - The distribution of retrogradely labelled cells following the injection of retrograde tracers into the nucleus ambiguus (NA), ventrolateral medulla (VLM) or paragigantocellularis lateralis (PGCL).

Abbreviations - RF-reticular formation; NTS - nucleus tractus solitarius; mNTS - medial NTS; vNTS - ventral NTS; vINTS - ventrolateral NTS; commNTS - commissural NTS; LRN - lateral reticular nucleus; CRN - caudal raphe nuclei; AP - area postrema; LPBN - lateral parabrachial nucleus; MPBN - medial parabrachial nucleus; KF - Kolliker - Fuse nucleus; PVN - paraventricular nucleus; LHA -lateral hypothalamic area; DMH - dorsomedial hypothalamus; PAG -periaqueductal gray matter; CG - central gray; V & L CG - ventral and lateral CG; Cdl - central gray dorsal and lateral; CAN - central nucleus of the amygdala; BST - bed nucleus of the stria terminalis; SI - Substantia Inominata; MPC - medial prefrontal cortex; IC - inferior colliculus; LC - locus coeruleus.

First Authors.	Area, Species.	Medulla.	Pons.	HYP.	PAG.	Others.
Stuesse (1984)	NA, Rat.	mNTS, vINTS, RF, NA.	L&M PBN KF.	PVN.		
terHorst (1984)	NA, Rat.	NTS, RF, NA, LRN, CRN.	L&M PBN	PVN, LHA, DMH	OG	CAN, BST, SI.
Bockstaele (1990).	PGCL, Rat.	NTS, RF, VLM, CRN, AP.	LPBN, KF.	LHA, PVN.	V, L, CG	MPC, IC, LG.
Portillo (1990).	NA, Rat.	mNTS, DVN, LRN, FN.	MPBN, KF.			
Lovick (1985).	PGCL, Cat.			LHA, DMH.	CdL	
Dampney (1987).	VLM, Cat.	M&V&Comm NTS.	KF.	PVN,	LHA.	
Smith (1989)	NA, Cat.	vI&mNTS, NA, RF, CRN	L&MPBN, KF, RTN.			
Takayama (1990).	NA, Cat.	NTS, VLM.	PBN.	PVN.		CAN.

Price and Amaral, 1981; Ross *et al*, 1981; Berk and Finkelstein, 1982; Holstege *et al*, 1983; Holstege, 1989). Furthermore, when PHAL or WGA-HRP were used as anterograde tracers, no electron microscopy was carried out to determine if anterograde labelling was contained in synaptic boutons (Ross *et al*, 1985; Shapiro and Miselis, 1985a; vanBockstaele *et al* , 1989; Cunningham and Sawchenko, 1990a,b; Herbert *et al*, 1990; Nicholas and Hancock, 1990).

Despite the diverse population of cells in the ventrolateral medulla, only a few studies have attempted to identify vagal preganglionic neurones as post-synaptic targets of these projections. Injections of the anterograde tracer PHAL into the nucleus tractus solitarius has resulted in terminal-like labelling in the region of retrogradely labelled VPNS (Ross *et al*, 1985; Cunningham *et al*, 1989). However, no close associations between retrogradely labelled neurones and anterogradely labelled structures were reported, and no electron microscopy was carried out to determine the synaptic relationship between these structures.

Section 1:4. Summary

Vagal preganglionic neurones in the medulla oblongata are located in the dorsal vagal nucleus and the nucleus ambiguus. All VPNs appear to be cholinergic, but some may also contain catecholamines, neuropeptide Y, galanin, calcitonin gene related peptide or glutamate.

VPNs in the DVN form a tightly packed group and innervate mainly sub-diaphragmatic structures. They are organised viscerotopically with respect to their projections. In contrast, VPNs in the NA predominantly innervate structures above the diaphragm. They are loosely arranged and are intermingled with neurones with other functions. The precise organisation and morphology of VPNs in the NA with respect to these other neurones is unclear.

The chemical content of boutons in synaptic contact with vagal preganglionic neurones is largely unknown. In the DVN, VPNs were observed to be innervated by thyrotrophin releasing hormone containing boutons in the rat, and GABA containing boutons in the cat. GABA has also been demonstrated in boutons in synaptic contact with VPNs in the NA of the cat.

There have been many studies using anterograde and retrograde tracing to determine projections to the DVN and the NA. However, it is not possible to identify if these projections are onto VPNs since the DVN and the NA also contain neurones other than vagal preganglionic neurones.

Section 1.5. Aims.

The experiments in this thesis were carried out to develop a sensitive technique to retrogradely label vagal preganglionic neurones, with specific reference to those innervating the heart, in order to study their organisation and morphology at the light and electron microscopic levels. These techniques were then combined with immunocytochemistry to determine if 5-HT, substance P and neuropeptide Y were contained in boutons in synaptic contact with vagal preganglionic neurones, and with anterograde tracing in the medulla oblongata to determine if the nucleus tractus solitarius was a source of input to vagal preganglionic neurones.

Chapter Two

Materials and Methods

The experiments in this thesis have employed neuroanatomical techniques in the study of the organisation and morphology of vagal preganglionic neurones and the investigation of the chemistry and origin of their synaptic inputs. These procedures have involved retrograde tracing from peripheral nerves, retrograde and anterograde tracing in the CNS, and immunocytochemistry. All the techniques were applied at both the light and electron microscopic levels. This chapter describes the basic methodology, technical considerations and all necessary preparative work. Greater details and variations to these basic methods are given in the relevant chapters. A summary diagram of procedures used is shown at the end of the chapter in figure 2.1.

2:1. Preparation of Animals.

2:1.1. Retrograde Tracing from the Cervical Vagus Nerve

Retrograde tracing was carried out using the enzyme horseradish peroxidase (HRP) as a neuronally transported substance. When injected into the CNS or into peripheral nerves this is taken up by cell bodies, dendrites and axons and transported throughout the cell (Lynch *et al*, 1974). HRP can then be detected by a variety of histochemical reactions (see section 2.2).

Male Sprague-Dawley rats (220-350g) or female cats (2.2-2.6Kg) were used in these studies. The surgical procedures required recovery of the animals and were undertaken using aseptic conditions. Prior to surgery the animals were anaesthetized with

intraperitoneal injections of sodium pentobarbitone in 0.9% NaCl (Sagatal. Rats - 60mg/kg; cats - 40mg/kg). The femoral vein was cannulated and supplemental doses of anaesthetic (6mg/kg in 0.9% NaCl) were given via this route as required. An incision was made through the skin dorsally and lateral to the trachea and the muscles were reflected to reveal the carotid artery and the cervical vagus nerve. The vagus nerve was carefully dissected free from the carotid artery until just below the nodose ganglion and separated from the sympathetic chain if possible. Ten to twenty microlitres of HRP (20% in 0.1M phosphate buffer) was injected into the nerve using a hand held adaptation of a nanolitre syringe (Saper, 1983). Any spillage of tracer was mopped up with cotton buds. At the end of the surgical procedures the femoral cannula was removed, the vein tied off, the muscles and skin sutured and the animals allowed to recover for 36-72 hours before perfusion.

2:1.B. Retrograde tracing in the CNS.

Retrograde tracing was carried out in the central nervous system by injections of HRP into the spinal cord (chapter 3) or the medulla oblongata (chapter 7). Full details of these procedures will be given in the relevant chapters.

2:1.C. Anterograde Tracing in the CNS.

Anterograde tracing was carried out in cats using biocytin (see chapter 7). It has been demonstrated that biocytin ionophoretically injected into the CNS is taken up by neurones and

dendrites but not axon terminals at the injection site and transported in their efferent axons (King *et al* , 1989; Izzo, 1991). As little as one hour later biocytin can be detected in axons 3-4mm from the injection site (Izzo, 1991). This fast transport time allowed short pathways to be studied under acute experimental conditions, allowing for accurate and elaborate physiological identification of the injection site.

2:1.2. Perfusion and Fixation

36-72 hours after the initial surgery or 6-12 hours after an injection of biocytin the animals were re-anaesthetised and pinned on a cork board over a sink within a fume hood. An incision was made laterally across the stomach, up each side of the ribcage and across the diaphragm to expose the heart. The left ventricle was pierced by a needle attached by rubber tubing to the perfusate via a peristaltic pump. Immediately upon entry of the needle into the ventricle the animal was perfused with 0.9% NaCl and the right auricle cut to flush the blood from the vascular system. Once the exiting fluid was clear of blood, the animal was perfused with 750ml of an ice cold mixture of glutaraldehyde and paraformaldehyde in 0.1M phosphate buffer over a period of 15-30 minutes. The concentration of aldehyde in the fixative was determined by the subsequent procedures which were being carried out on the material.

After perfusion the brains were removed from the skull and post-fixed for 1-3 hours in the perfusing fixative, following which they were washed with several changes of 0.1M PB. The brainstem and other relevant areas were blocked and sectioned at 60-80 μ m in phosphate buffer (0.1M, PB) using a vibrating microtome (Vibratome). The sections were collected in soda glass vials in which all further processing was carried out.

2.2. HRP Histochemistry

Four different histochemical reactions were used to visualise injected and transported HRP. These methods all used the same principle of an electron donor (e.g. DAB) and substrate for the peroxidase enzyme (always H₂O₂).

2:2.1. DAB method.

Introduced by Graham and Karnovsky (1966). Sections were washed in Tris HCl buffer (Tris buffer, pH 7.4), preincubated in diaminobenzidine tetrahydrochloride (DAB; 50mg/100ml) in Tris buffer for 10 minutes then 0.01% H₂O₂ was added to the same solution for 5-12 minutes. This results in a brown punctate reaction product localised mainly in the cytoplasm of the cell soma when viewed at the light microscopic level and to lysosomes when viewed at the electron microscopic level.

2:2.2. TMB method at pH 6.0. (Marfurt *et al*, 1988).

The sections were washed in ice cold 0.1M phosphate buffer (PB) made at pH 6.0 for ten minutes. They were then preincubated at room temperature for 20 minutes in a solution of which each 100ml contained 250mg of ammonium molybdate, 97.5ml 0.1M PB pH6.0 and 2.5ml of a tetramethyl benzidine (TMB) solution (25 mg TMB in 12.5ml of absolute ethanol). 10 μ l of 1%H₂O₂ was added to each millilitre of incubation medium for 20 minutes at room temperature followed by incubation in 5% ammonium molybdate in 0.1M PB pH6.0 at 0-4°C for one hour. This produces a soluble reaction product which is stabilised by incubating the sections with a solution of 0.1M PB pH 6.0 which contained 0.05% DAB, 0.05% cobalt chloride and 0.01% hydrogen peroxide which results in either a black crystalline product or a punctate product in the cytoplasm and proximal dendrites of the labelled cells.

2:2.3. O-tolidine method. (Somogyi *et al*, 1979).

The tissue was pre-incubated for 30 minutes in a 1:1 solution containing 0.1M citric acid, 0.1M ammonium acetate then incubated for 2 hours in the same solution but with the addition of 30mg/100ml of substrate O-tolidine (3,3'-dimethylbenzidine), 0.05% sodium nitroprusside and 0.003% hydrogen peroxide at 4°C. This reaction product was stabilised by transferring the sections into a mixture of 0.05% DAB in 0.1M PB, 0.02% cobalt chloride and 0.01% hydrogen peroxide at 4°C for 5-10 minutes. The final product is black and amorphous and appears to completely fill the cytoplasm and dendrites of labelled cells.

2:2.4. Hanker-Yates Method (Hanker *et al*, 1977).

Two solutions were made up prior to the reactions: solution A contained 0.4g ammonium nickel sulphate and 0.6g cobalt chloride in 100ml of distilled water and solution B contained 2.15g sodium cacodylate in 100ml of distilled water altered to pH 5.1-5.2 with HCl, with the further addition of 100mg catechol and 50mg phenylenediamine. Sections were preincubated in a mixture containing 100ml solution A and 20ml solution B for 15 minutes, rinsed in 0.1M phosphate buffer and then incubated in solution B with the addition of one drop of 30% hydrogen peroxide. Following the reaction the sections were washed in 0.1M phosphate buffer.

2.3. Immunocytochemistry.

Immunocytochemical techniques were employed to determine the chemical content of structures forming synaptic associations with VPNS. These techniques provide a sensitive and specific method involving the use of labelled antibodies as specific reagents for the localisation of tissue constituents *in situ*. The general procedure will be described here and details of antibodies used given in relevant chapters.

Immunostaining was carried out using pre-embedding procedures. In such procedures high glutaraldehyde concentrations limits penetration of antibodies into the material and may alter the antigen beyond recognition by the antibody. However as all material was prepared for electron microscopy, glutaraldehyde

was necessary for ultrastructural preservation. In these studies a fixative containing 0.1-0.5% glutaraldehyde and 3% paraformaldehyde was found to provide a suitable compromise for all the antibodies used.

Following the histochemical demonstration of HRP (or directly after sectioning) sections were incubated with a high dilution (1:10,000-1:15,000) of the specific primary antiserum made up in phosphate buffered saline (PBS). A detergent, triton x-100, was added to some vials to enhance antibody penetration into the tissue. This was followed by incubation with an excess (1:50-1:200 dilution) of an antiserum raised in a second species against IgG of the species in which the primary antiserum was raised. This secondary antibody was usually conjugated to HRP. The peroxidase attached to the antibody was visualised using the DAB method as described in section 2.2.1. The reaction time was terminated before background staining was too high and specific staining was most intense. This resulted in an electron dense brown amorphous product distinguishable from the punctate reaction product seen upon localisation of retrogradely transported HRP.

2.3.1. Specificity and Controls. (See also Appendix One: p. 288).

The specificity of primary antisera was tested in various ways. The pattern of staining obtained with a particular anti-serum was always compared with previous reports of distribution of immunoreactive structures. In addition, some sections were treated in the same way except the primary antibody was omitted

or replaced by an antibody which was either raised in a different species to the primary antibody or which was known to result in a different pattern of staining.

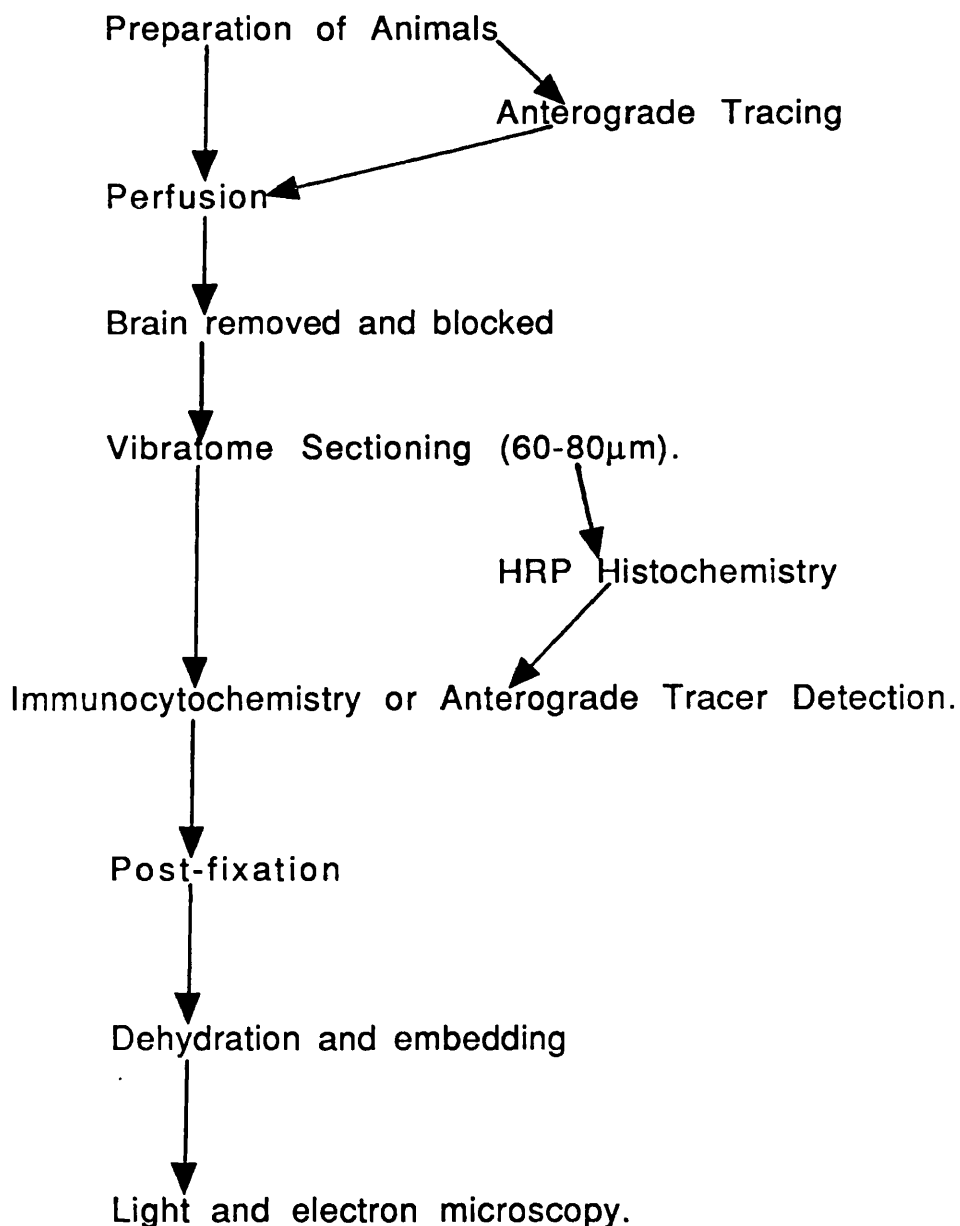
2.4. Preparation of Sections for Light Microscopy.

After the previous procedures the sections were washed for 30 minutes in 0.1M PB. Sections for light microscopic examination only were air dried on chrome alum-gelatin coated slides before mounting with coverslips using Xam. Those for light and electron microscopic examination were post-fixed in 1% osmium tetroxide in PB for 30-40 minutes and washed in distilled water for 10 minutes before being dehydrated through a series of 50%, 70%, 95% ethanol followed by two washes in 100% ethanol. The sections were kept in 70% ethanol with added uranyl acetate (1%) for 40 minutes to enhance contrast for electron microscopy. After the ethanol treatment the sections were washed twice for 15 minutes in propylene oxide prior to immersion in freshly prepared Durcupan ACM resin (Fluka), in which they were left overnight which ensured that the resin thoroughly infiltrated the material and residual propylene oxide evaporated. The sections were then mounted on microscope slides, covered with a coverslip and cured for 48 hours at 60°C. Once cured structures of interest were photographed and these photographs served as permanent records which were used to correlate the structures at the light and electron microscopic levels.

2.5. Electron Microscopy

After light microscopic examination and photography relevant areas on the sections were selected for electron microscopic examination. The coverslip was removed by inserting a razor blade underneath it and the slide then placed on a hot-plate. Once the resin was heated the required area was cut out with a scalpel and glued to the top of a Durcupan block which had been made in a Beem capsule. In this manner the section can be viewed at the light microscope, allowing continuous observation of the material while sectioning. The blocked area was trimmed until it contained just the relevant structures (usually the edges of the block were just visible at X40 magnification). 65-70nm serial sections were cut using an ultramicrotome (Reichert-Jung) and a glass or diamond knife (Diatome). The sections were collected on double distilled water and picked up on Pioloform coated single slot grids. Before viewing on a Phillips 201 electron microscope the ultrathin sections were stained with lead citrate (Reynolds, 1963) for 1-2 minutes.

Figure 2.1. Flow diagram illustrating how retrograde labelling, immunocytochemistry and anterograde labelling were combined in these studies. Note that not all procedures were carried out in all animals. Full details are given in the text.



Materials

Ammonium Molybdate (Sigma Chemical Co.).

Ammonium Nickel Sulphate (BDH).

Biocytin (Sigma Chemical Co.).

Catechol (Sigma Chemical Co.).

Cholera toxin B chain (Sigma Chemical Co.).

Cobalt chloride (BDH).

Diaminobenzidine tetrahydrochloride (Sigma Chemical Co.).

Durcupan ACM resin (Fluka).

Glutaraldehyde (Taab).

Horseradish peroxidase (Sigma Chemical Co.).

Hydrogen peroxide (H₂O₂) (Sigma Chemical Co.).

Osmium tetroxide (Taab).

O-tolidine (Sigma Chemical Co.).

Paraformaldehyde (Sigma Chemical Co.).

Phenylenediamine (Sigma Chemical Co.).

Pioloform (Bio-Rad).

Propylene oxide (BDH).

Sagatal (May and Baker Ltd.).

Sodium cacodylate (Agar Scientific LTD).

Sodium nitroprusside (Sigma Chemical Co.).

Streptavidin-horseradish peroxidase (Amersham International).

Tetramethyl benzidine (Sigma Chemical Co.).

Triton X-100 (BDH).

Chapter Three.

**Investigations into the organisation and morphology
of vagal preganglionic neurones in the rat, with
specific reference to vagal preganglionic neurones
innervating the heart.**

Section 3.1. - Introduction.

The morphology of vagal preganglionic neurones has been described at light and electron microscopic levels in the cat and monkey (McLean and Hopkins, 1981,82, 85b) but not in the rat, despite the rat being the species of choice for many anatomical and physiological studies on VPNS (see Chapter One). The experiments in this chapter were undertaken to study the organisation and morphology of vagal preganglionic neurones in the rat, with specific reference to vagal preganglionic neurones innervating the heart.

Cardiac vagal preganglionic neurones (CVPNS) play an important role in the control of cardiac functions (see Levy and Martin, 1984). These cardiac functions have been revealed by electrical stimulation of the vagus nerve, which decreases heart rate, increases the time for atrioventricular conduction and depresses ventricular contractility (see Levy and Martin, 1984). However, although crucial to a full understanding of the control of the cardiovascular system, the location of CVPNS is still uncertain.

Early workers disagreed over the location of cardiac vagal preganglionic neurones and thought that they were preferentially located in either the dorsal vagal nucleus or the nucleus ambiguus in the medulla oblongata (Rabbit - Bunzl-Federn, 1899; Getz and Sirnes, 1949. Monkey - Mitchell and Warwick, 1955; Dog - Kosaka and Yagita, 1908; Kitchell *et al*, 1956. Cat - Smolen and Truex, 1977). The first studies favoured the NA as the source of cardiac innervation. Following sectioning of cardiac branches of the vagus nerve in rabbits, degenerated cell bodies were observed only in the

NA, and particularly in the ventrolateral NA (Rabbit - Bunzl-Federn, 1899; Dog - Kosaka and Yagita, 1908). However, similar studies since then have observed degenerated CVPNs only in the DVN in rabbit (Getz and Sirnes, 1949), monkey (Mitchell and Warwick, 1955), sheep, dog and goat (Kitchell *et al*, 1956), cat (Kitchell *et al*, 1956; Smolen and Truex, 1977) and pigeon (Cohen *et al*, 1970). The location of CVPNs was not clarified using the converse technique of anterograde degeneration, as in some studies lesions in the DVN resulted in degenerated fibres in the cardiac vagus (Rabbit - Mohlant, 1910; Cat - Calaresu and Cottle, 1965) , but not in others (Cat - Szentagothai, 1952; Kerr, 1969), where degenerated fibres in the cardiac vagus were found only after destruction of the NA (Cat - Szentagothai, 1952).

Electrophysiological studies have also reported that either the NA or DVN was the exclusive location of CVPNs. Thus, vagally mediated bradycardia has been elicited only by electrical stimulation in the NA (Cat - Gunn *et al*, 1968; Borison and Domjan, 1970; Thomas and Calaresu, 1975a,b; Chen and Chai, 1976; Geis, Kozelka and Wurster, 1981) but not in the DVN (Calaresu and Pearce, 1965b; Achari *et al*, 1968; Gunn *et al*, 1968; Geis *et al*, 1981), and the NA was the sole site of neurones antidromically activated by electrical stimulation of a cardiac branch of the vagus nerve (Cat - McAllen and Spyer, 1976). However, in other studies vagally mediated bradycardia was obtained by stimulation only in the DVN (Cat - Miller and Bowman, 1916; Chiurugi and Mollica, 1954; Dugin *et al*, 1976. Dog - Miller and Bowman, 1916; Weiss and Priola, 1972. Rabbit - Schwaber and Schneiderman, 1975) and this was the location of the majority of antidromically activated CVPNs in the rabbit (Jordan *et al*, 1982).

In contrast to previous reports that CVPNs were located in either the DVN or the NA, recent experiments have suggested that CVPNs are located in both nuclei. Electrical stimulation in the DVN and NA elicited a vagally mediated bradycardia (Dog - Gunn *et al*, 1968. Rabbit - Ellenberger *et al*, 1983. Rat - Nosaka *et al*, 1979b; Stuesse, 1982) and neurones antidromically activated by stimulation of a cardiac branch of the vagus were recorded in both nuclei (Rat - Nosaka *et al*, 1982; Cat - Ford *et al*, 1990).

In addition to the DVN and the NA, the external cuneate nucleus has been suggested to contain CVPNs since electrical stimulation of the external cuneate nucleus elicited a vagal bradycardia (Cat - Ciriello and Calaresu, 1978) and was the site of antidromic activity recorded on stimulation of a cardiac vagus branch (Ciriello and Calaresu, 1980). However, this appears to have been due to activation of the vagal afferent fibre tract which runs close to this area in the cat (Kalia and Mesulam, 1980a,b), as no others have reported this nucleus to contain CVPNs. These studies illustrate that electrical stimulation or recording antidromic activity can be an inaccurate method to localise CVPNs, due to the possibility of stimulating or recording from fibres of passage.

In recent years the precise distribution of CVPNs has been studied by application of anatomical tracing techniques (Cat - Todo *et al*, 1977; Sugimoto *et al*, 1979; Geis and Wurster, 1980a; Kalia and Mesulam, 1980b; Bennett *et al*, 1981; Geis *et al*, 1981; Kalia, 1981; Miura and Okada, 1981; Ciriello and Calaresu, 1982. Dog - Hopkins and Armour, 1982, 84; Plecha *et al*, 1988. Rat - Nosaka *et al*, 1979a; Miselis *et al*, 1989. Pig - Hopkins *et al*, 1984) . The

first study to employ retrograde tracing by application of HRP to the myocardium observed retrograde labelling in the nucleus tractus solitarius as well as throughout the DVN (Cat - Todo, 1977; Todo *et al*, 1977). However, no evidence was presented that retrograde tracer was detected within cells and reaction product within the NTS was probably due to endogenous peroxidase activity observed in this region in subsequent studies (Bennett *et al*, 1981). In all other studies, following application of retrograde tracers to the myocardium or to cardiac branches of the vagus nerve, CVPNs were localised in both the DVN and NA, as well as an area in the reticular formation between the two nuclei known as the intermediate zone (Cat - Sugimoto *et al*, 1979; Geis and Wurster, 1980a; Kalia and Mesulam, 1980; Bennett *et al*, 1981; Geis *et al*, 1981; Kalia, 1981; Miura and Okada, 1981; Ciriello and Calaresu, 1982. Dog - Hopkins and Armour, 1982, 84; Plecha *et al*, 1988. Rat - Nosaka *et al*, 1979a; Miselis *et al*, 1989. Pig - Hopkins *et al*, 1984). In addition, injections of tritiated amino acids into the DVN or NA anterogradely labelled vagal fibres in the myocardium, indicating that both these nuclei contained neurones projecting to the heart (Ciriello and Calaresu, 1982).

Despite localisation of CVPNs to the NA and DVN, anatomical studies have differed on the precise distribution of CVPNs within the vagal nuclei. Most studies using retrograde tracing techniques report that the DVN and the intermediate zone contain few CVPNs, and those in the DVN are located mainly on the lateral edges of the nucleus (Cat - Sugimoto *et al*, 1979; Bennett *et al*, 1981; Miura and Okada, 1981; Ciriello and Calaresu, 1982. Rat - Nosaka *et al*, 1979a; Miselis *et al*, 1989). However, although there is general agreement that the NA

is the main site of CVPNs, their precise distribution within the NA remains uncertain. Application of retrograde tracers to cardiac branches of the vagus nerve or to the myocardium has labelled neurones in both the cNA and the vNA (Cat - Sugimoto *et al*, 1979; Kalia and Mesulam, 1980b; Bennett *et al*, 1981; Kalia, 1981; Miura and Okada, 1981; Ciriello and Calaresu, 1982;. Rat - Stuesse, 1982) or only within the vNA (Rat - Nosaka *et al*, 1979a; Hopkins and Armour, 1982; Bieger and Hopkins, 1987; Miselis *et al*, 1989. Pig - Hopkins *et al*, 1984).

Thus modern anatomical techniques have localised CVPNs mainly in the NA, although it is uncertain whether they are located only in ventrolateral NA, or in the ventrolateral and compact regions of the NA. One reason for the differences in the location of CVPNs may have been whether CVPNs were retrogradely labelled from the myocardium or a cardiac branch of the vagus nerve, since cardiac branches also contain axons innervating the lungs (McAllen and Spyer, 1978b). However, this does not appear to be the case since CVPNs have been located in the vNA and cNA following retrograde labelling from the myocardium (Cat - Kalia and Mesulam, 1980b; Geis, Kozelka and Wurtser, 1981; Plecha *et al*, 1988. Rat - Stuesse, 1982) as well as cardiac branches of the vagus nerve (Cat - Sugimoto *et al*, 1979; Bennett *et al*, 1981; Miura and Okada, 1981; Ciriello and Calaresu, 1982), and both techniques have also resulted in retrogradely labelled neurones only in the vNA (Myocardium - Rat - Miselis *et al*, 1989. Branches - Rat - Nosaka *et al*, 1979a; Dog - Hopkins and Armour, 1982. Pig - Hopkins *et al*, 1984). Another possible explanation for differing locations of CVPNs is that some labelling is due to spillage of tracer onto other structures in the

thorax which are innervated by the vagus nerve, but there is no clear evidence if this is the case.

In addition to uncertainty regarding the location of CVPNs there has been little study of their morphological characteristics, both at the light and electron microscopic levels. The few studies which comment on the size of CVPNs report conflicting results (see table 3.1.), which may be a reflection of observed differences in their location as VPNs in different parts of the NA differ in size (see section 1:3.1). Ultrastructural examination of CVPNs in the DVN of the cat have suggested that they have characteristics similar to other VPNs (Maqbool *et al*, 1991; see sections 1:2.2 and 1:3.2 for description of ultrastructure of vagals). However, there is no information regarding the morphology of CVPNs in the rat.

Thus the precise distribution and morphology of CVPNs in the vagal nuclei of the rat remains uncertain. The aim of this study was to develop a technique to retrogradely label CVPNs and determine their location and morphology in comparison to other VPNs retrogradely labelled from the cervical vagus nerve.

As there is no available information on the morphology of vagal preganglionic neurones in the rat, initial experiments were undertaken to determine the location and morphology of vagal preganglionic neurones retrogradely labelled by the application of horseradish peroxidase (HRP) to the cervical vagus nerve, which contains the axons of VPNs innervating thoracic and abdominal organs (see Kalia and Mesulam, 1980a,b). HRP was used as a retrograde tracer as it has been visualised at the light and electron

microscopic levels in VPNs following application to the cervical vagus nerve of the cat and monkey (McLean and Hopkins, 1981, 82, 85a,b). To determine a sensitive method for light and electron microscopic localisation of retrogradely transported HRP, three histochemical reactions were tested, all of which have previously been applied at the light and electron microscopic levels - the DAB reaction (Graham and Karnovsky, 1966), the TMB reaction at pH6.0 (Marfurt *et al*, 1988) and the *o*-tolidine reaction (Somogyi *et al*, 1979).

The morphology and precise location of cardiac vagal preganglionic neurones was then determined by comparison to that of VPNs retrogradely labelled from the cervical vagus nerve. To enable this comparison to be made, experiments were undertaken to establish a technique to specifically retrogradely label VPNs innervating the heart which was compatible with both light and electron microscopy.

To label neurones innervating the heart, retrograde tracers were injected into the myocardium in preference to cardiac nerves, since cardiac branches of the vagus nerve contain axons innervating the lungs as well as the heart (McAllen and Spyer, 1978a), and these VPNs cannot be distinguished on the basis of position or morphology (Bennett *et al*, 1981). In addition, to determine if previous differences in distribution of CVPNs could be attributed to spillage of tracer, retrograde tracers were also intentionally spilled into the thorax and not injected into the myocardium.

To determine a sensitive retrograde tracer for labelling neurones from the myocardium, the degree of retrograde labelling was compared using cholera toxin HRP (CT-HRP) or HRP itself as retrograde tracers. HRP was used since it was applied to retrograde labelling of VPNs from the cervical vagus nerve, and has also been shown to retrogradely label neurones following injections into the myocardium (Cat - Kalia and Mesulam, 1980b; Geis, Kozelka and Wurtser, 1981. Plecha *et al*, 1988. Rat - Stuesse, 1982). However, since cholera-toxin B chain has been suggested to be a superior retrograde tracer to HRP itself, especially when injected into end-organs (Trojanowski, Gonatas and Gonatas, 1982; Wan *et al*, 1982), this was conjugated to HRP for use as a retrograde tracer. The conjugation was undertaken as cholera toxin itself is detected by immunocytochemical means, which results in deterioration in ultrastructure, whereas conjugated HRP could be detected with the histochemical reactions suitable for ultrastructural preservation.

Having established sensitive and specific techniques to retrogradely label VPNs projecting to the heart or cervical vagus nerve, experiments were undertaken to determine if VPNs had particular characteristics which could distinguish them from other neurones in their vicinity. The distribution and morphology of VPNs was compared to that of neurones identified by retrograde tracing from the phrenic motor nucleus in the cervical spinal cord, which have been shown to be intermingled with VPNs (see section 1:3.5.A; Cat and Rat - Portillo and Pasaro, 1988. Rat - Ellenberger and Feldman, 1990b).

Table 3:1 - Average measured diameters of CVPNs in the medulla oblongata of various species.

Asterisks indicate measurements not distinguished between cNA and vINA. (Abbreviations. DVN - dorsal vagal nucleus; cNA - compact nucleus ambiguus; vINA - ventrolateral nucleus ambiguus).

First Author	Species	DVN	cNA	vINA
Bennett <i>et al</i> (1981)	Cat	15.9µmX28µm	17µm X 30µm. *	
Miura and Okada (1981)	Cat.	<30µm	30-60µm.*	
Ciriello (1982)	Cat.	15-25µm	20-30µm.*	
Stuesse (1982).	Rat.			25µm X 15µm.
Hopkins (1984).	Dog.		30µm X 39µm	21µm X 33µm.

Section 3.2. - Methods.

3:2.1. Retrograde Tracing From the Cervical Vagus Nerve.

Vagal preganglionic neurones were retrogradely labelled by the application of HRP to the cervical vagus nerve as described in section 2:1.A.1.

3:2.2. Retrograde Tracing from the Heart.

Rats were anaesthetised and a femoral vein cannulated. The trachea was cannulated and the animals artificially ventilated at a rate of 100 breaths/minute X 2.5ml/breath. An incision was made in the midline of the chest and the sternum exposed by reflecting the skin and muscles. The sternum was divided using a mini circular saw (RS Components) and the heart exposed. The heart was manipulated until easy access could be gained to the right atrium. Occasionally acetylcholine was applied topically to the right atrium to slow heart rate. Using a nanolitre injection device (Saper, 1983), 10 μ l of horseradish peroxidase (HRP) or cholera toxin conjugated to HRP, which was conjugated using the procedure of McIlhinney, Bacon and Smith (1988), were placed into the wall of the right atrium near the entry of the vena cavae. After injection the surrounding area was swabbed with a cotton bud to minimise spillage. The chest wall was tightly sewn up and the muscle and skin stitched up in layers over the sternum to provide an airtight seal. The pneumothoractomy was reduced by inserting a small bore needle attached to an empty syringe through the skin, and air extracted. Positive pressure was

applied to re-inflate the lungs and the animal taken off the ventilator. The tracheal cannula was removed and the two ends of the trachea stitched together. Animals were allowed to recover for 36-72 hours.

3:2.3. Retrograde Tracing From the Phrenic Motor Nucleus.

Ten rats were anaesthetised and the femoral vein cannulated for maintenance of anesthesia. The animals were placed in a stereotaxic head frame (Kopf Instruments) and supported by a polystyrene block. An incision was made in the skin from the level of the second cervical spinal cord segment (C2) caudally to the second thoracic spinal cord segment (T2). The cutaneous maximus, acromiotrapezius and the spinotrapezius muscles were cut. Muscle was cleared from the surface of the vertebral column using a bone scraper. T2 was clamped and used to elevate the animal so that the spinal cord was almost horizontal. The bone was removed from C4 and C5 using fine rongeurs, the dura incised and the cord flooded with parafin oil to keep it moist. A nanolitre injection device (Saper, 1983) was attached to a three dimensional micro-manipulator (Narishige) and manoeuvred into the spinal cord just medial to the entry of the dorsal spinal roots to a depth of approx. 1mm, in the region of the phrenic motor nucleus. 10 μ l of 10% HRP was injected into this region in small steps of approx 0.5-1 μ l. After the injection the electrode was left in place for 5 minutes to minimise the spread of HRP up the electrode tract. The muscles and skin were sutured and the animal allowed to recover for 36-72 hours.

3:2.4. Perfusion and Histochemistry.

Following recovery animals were perfused with fixative containing 2% glutaraldehyde and 2% paraformaldehyde, the brains removed and 60-80 μ m medullary sections cut on a vibrating microtome. Retrograde tracer in sections in which VPNs had been retrogradely labelled from the heart or the cervical vagus nerve was localised using the DAB, TMB or o-tolidine reactions described in section 2.2., while sections in which bulbospinal neurones were retrogradely labelled were reacted using the DAB or TMB pH6 reactions.

3:2.4. Light Microscopy.

Following the histochemical reactions sections were prepared for light microscopy only or for both light and electron microscopy as described in sections 2.4.

All sections were examined at the light microscope for retrogradely labelled neurones. In resin embedded sections the somata of neurones which were wholly contained within the sections were drawn using a drawing tube and the diameter of the neurones measured in the short axis. The area of the drawings of the cells were also measured by tracing around the drawings on a digitizing pad linked to an IBAS image analysis system (Kontron). This provided a more sensitive measure of cell size as it allowed comparison of sizes between groups of neurones of different shapes, and also removed problems associated with determining

between which points of the cell membrane diameter should be measured. Statistical analysis to compare measurements of identified groups was carried out with the IBAS system or an Apple MacIntosh Statworks programme using Student's t-test.

3:2.5. Electron Microscopy.

Selected labelled neurones identified at the light microscopic level were photographed and the photographs used for correlation of the neurones at light and electron microscopic level. The procedures used to correlate selected cells at the light and electron microscopic levels are described in section 2.5.

Section 3.3. - Results.

3:3.1. Retrograde Tracing From the Cervical Vagus Nerve.

3:3.1.A. Sensitivity of Histochemical Reactions.

Vagal preganglionic neurones retrogradely labelled by the injection of HRP into the cervical vagus nerve were localised using the DAB, TMB and *o*-toluidine reactions. These reactions differed in degrees of sensitivity.

DAB reaction - this was the least sensitive of the reactions used. It resulted in a brown punctate reaction product within the cytoplasm of labelled cell soma but rarely within the dendrites (figure 3.5.C). At the light microscope level the reaction product was visible only at high magnifications. At the electron microscopic level morphological preservation was excellent (Fig. 3.5). The reaction product was electron dense and associated with lysosomes and multivesicular bodies in the cytoplasm of labelled cells (figure 3.5.D).

TMB pH6 reaction - this was more sensitive than the DAB reaction. It resulted in a black punctate reaction product visible in somata and primary dendrites of labelled neurones at the light microscopic level (figures 3.4.C and 3.6.A). Ultrastructural preservation was good. The reaction product appeared electron dense and crystalline, or similar to DAB reaction product in lighter labelled regions of cells, and could be observed in somata and dendrites of labelled cells (Fig. 3.6). It often obscured the

organelles with which it was associated (figure 3.6.C&D).

O-tolidine reaction - this was the most sensitive of the reactions used. It resulted in a dense black amorphous reaction product within the cytoplasm and dendrites of the labelled cells (figure 3.1 and 3.2). Reaction product could be visualised in somata and primary dendrites (figure 3.2). At the electron microscopic level amorphous reaction product was so dense that it filled the cell soma and often obscured internal organelles. Ultrastructural preservation was often poor.

3:3.1.B. Location and Morphology of Neurones Retrogradely Labelled From the Cervical Vagus Nerve.

Cells labelled by the injection of HRP into the vagus nerve were located mainly in the dorsal vagal nucleus (DVN) and the nucleus ambiguus (NA), and only a very small number of cells were detected in the intermediate zone (figure 3.1 and 3.2). Light microscopy of VPNS in the NA and DVN revealed that cells in each area displayed specific characteristics.

Dorsal Vagal Nucleus - Retrogradely labelled cells in the dorsal medulla were restricted to the DVN. Labelled cells were in a tightly packed population of spindle shaped cells (figure 3.1, 3.2.A and 3.3.A). Measurement of 250 cell perikarya contained entirely within the section revealed that they had cell diameters in the range of 12-28 μm with a median diameter of 18 μm (see table 3.1) and cell soma areas in the range 85-250 μm^2 (median 160 μm^2). Statistical analysis using Student's t-test

demonstrated that these cells were significantly different in size to those in the cNA and the vNA (table 3.2).

Nucleus Ambiguus - Retrogradely labelled cells in the NA could be divided into two groups. One group was located in the compact region of the NA (figure 3.1, 3.2 C & D) and cell body diameters of 150 neurones measured in the range 15-35 μm (median 30 μm) and soma area 123-511 μm^2 (median 350 μm^2 ; table 3.1). The other group lay in the ventrolateral NA (figure 3.1, 3.2C,D and 3.5). The soma diameter of 43 of these neurones measured 22-30 μm with a median size of 25 μm and areas 105-370 μm^2 with a median of 230 μm^2 (table 3.2). Cells in the vNA were statistically significantly smaller than those in the cNA (table 3.2).

Correlated light and electron microscopic examination of 15 retrogradely labelled VPNS in the DVN (Fig. 3.3), 15 in the cNA (Fig. 3.4) and 10 in the vNA (Fig. 3.5) revealed that they shared some common characteristics (figure 3.6): smooth cell bodies with occasional somatic spines; large round nuclei approximately 12 μm diameter lying close to the cytoplasmic membrane on one side of the cell (usually with one prominent nucleolus); mitochondria ranged in shape from small round to long rod-like structures and were evenly distributed through the cytoplasm; lysosomes and multivesiculate bodies; a small number of golgi bodies; a scattering of free ribosomes and an area particularly abundant in rough endoplasmic reticulum often at the perimeter of the cell membrane. Labelled dendrites also contained mitochondria of various shapes, as well as lysosomes and multivesiculate bodies which frequently contained granules of

reaction product. Synapses on cell somata were counted in 5-10 serial sections and were often sparse (fewer than five per cell for the sections examined) but appeared to be greater on the dendrites of labelled cells.

Table 3.2. Statistics of the perikarya of neurones in the medulla oblongata retrogradely labelled from the cervical vagus nerve, the heart and the cervical spinal cord.

Cell Type (medullary site)	Diameter Range (μm)	Median D (μm)	Area Range (μm^2)	Median Area (μm^2)	Statistical difference when compared to cNA cells.
VPNs (DVN)	12-28	18	85-280	160	$p < 0.001$
VPNs (cNA)	15-35	30	123-511	350	-----
VPNs (vINA)	22-30	25	105-370	230	$p < 0.001$
spinals (NA)	17-35	28	100-450	290	Not significant
CVPNs (NA)	20-28	25	98-350	220	$p < 0.001$

Figure 3:1. Location of labelled neurones in the medulla oblongata of the rat following injection of horseradish peroxidase into the cervical vagus nerve.

Right Side: Diagrammatic representation of transverse sections of the medulla oblongata of the rat at approximately the same levels as those on the left hand side. Major nuclei in the region are indicated to aid identification of the location of labelled neurones in the adjacent photomicrographs.

Left Side: Low magnification light micrographs of one side of transverse sections of the medulla oblongata of the rat. Following injection of horseradish peroxidase into the cervical vagus nerve labelled neurones are found in the nucleus ambiguus (thick black arrows), dorsal vagal nucleus (thin black arrows) and the intermediate zone (open arrow).

Abbreviations: NA - nucleus ambiguus; DVN - dorsal vagal nucleus; NTS - nucleus tractus solitarius; TS - tractus solitarius; X11 - hypoglossal motor nucleus; LRN - lateral reticular nucleus; RP - raphe pallidus; IO - inferior olivary complex.

Scale bar: 500 μ m.

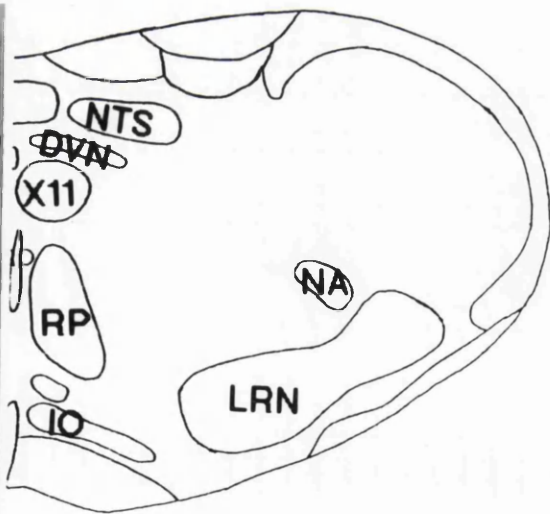
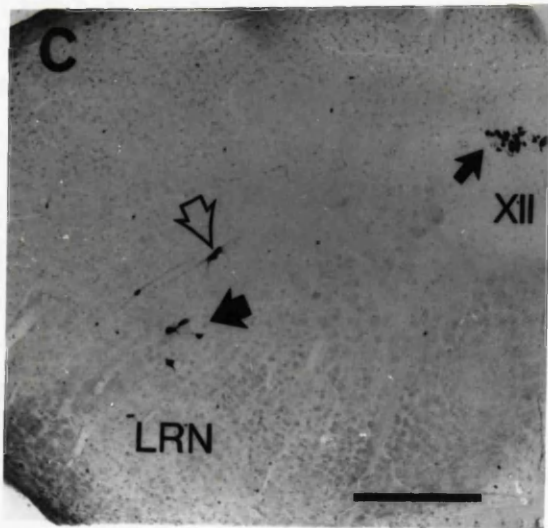
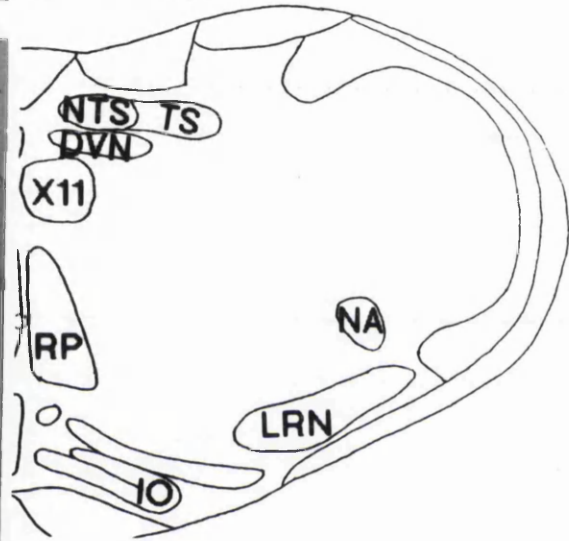
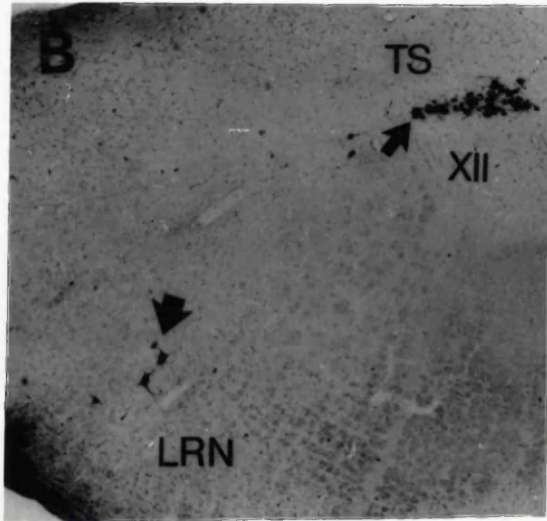
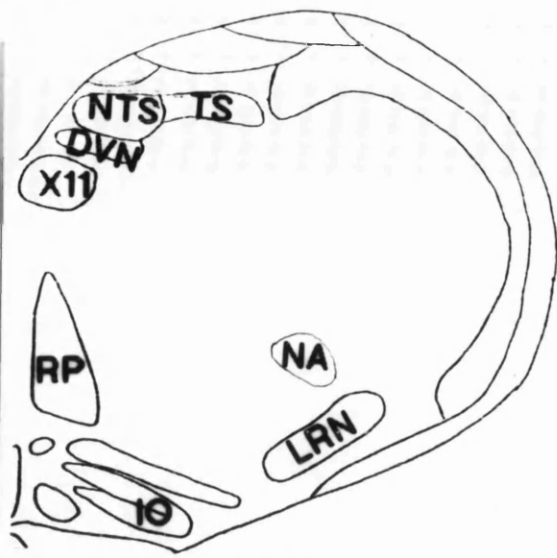
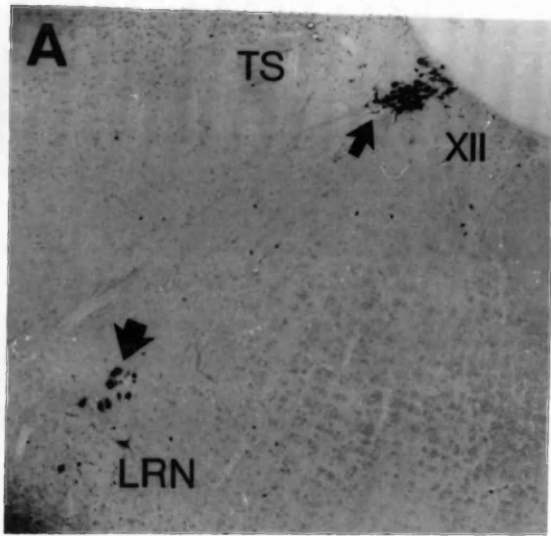


Figure 3:2. High magnification photomicrographs of labelled neurones in the medulla oblongata following injection of HRP to the cervical vagus nerve.

3:2.A. - Retrogradely labelled neurones in the DVN, at approximately the same level as those shown in figure 3:1.B.

3:2.B. - Higher magnification of the labelled neurones in the NA shown in figure 3:1.C. The open arrow points to labelled neurones in the intermediate zone and the black arrows to o-tolidine reaction product in axons of the vagal nerve tract (VT).

3:2.C. - Higher magnification of the labelled neurones shown in figure 3:1.A. This indicates the location of labelled neurones in the compact region of the nucleus ambiguus (cNA) and also some labelling in the ventrolateral nucleus ambiguus (vNA). The o-tolidine reaction product can again be seen in the vagal nerve tract (VT, arrow).

3:2.D. - Illustrates the location of vagal preganglionic neurones, localised in the cNA and the vNA with the TMB reaction.

Abbreviations: AP - area postrema; NTS - nucleus tractus solitarius; X11 - hypoglossal nucleus; CC - central canal; cNA - compact region of the nucleus ambiguus; vNA - ventrolateral nucleus ambiguus; V - ventral surface of the sections; VT - vagal nerve tracts.

Scale Bar: 200 μ m.

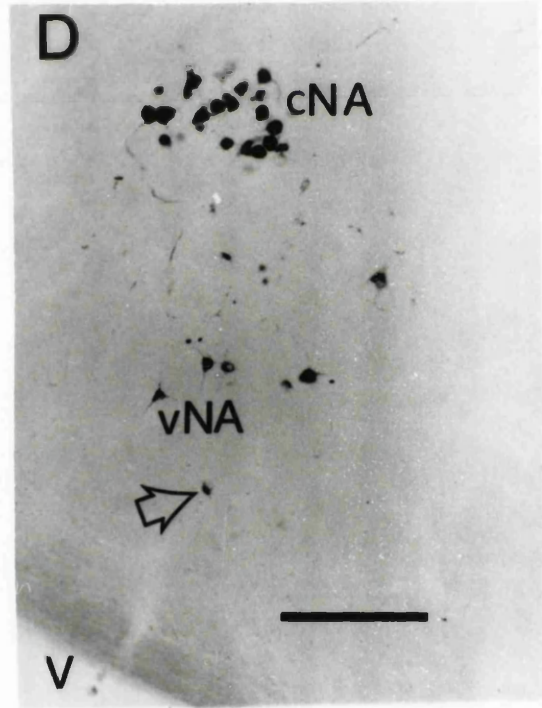
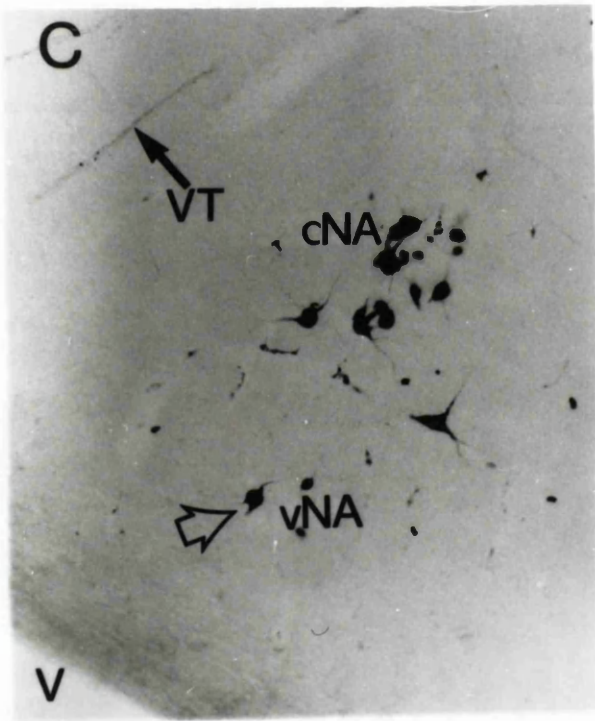
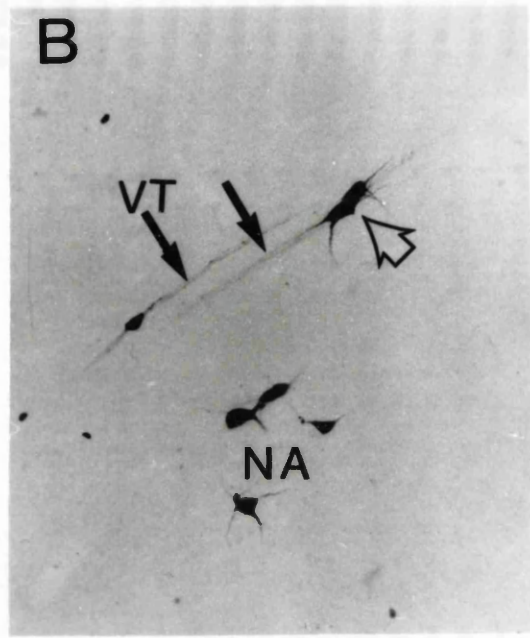
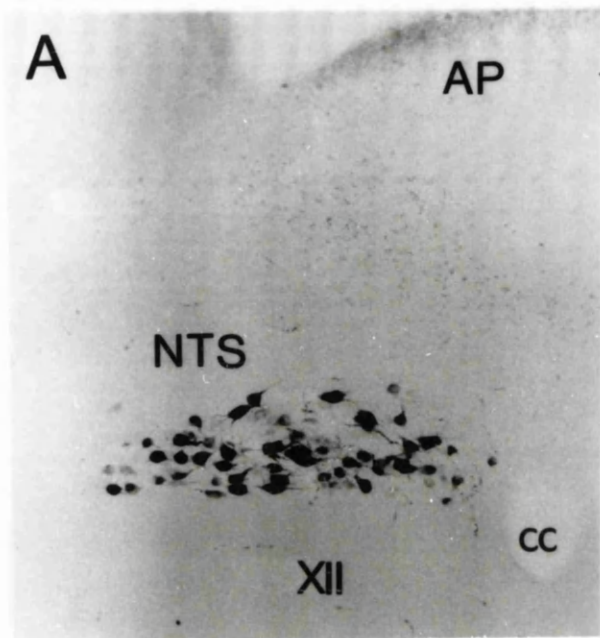


Figure 3:3. Correlated light and electron microscopic micrographs of a vagal preganglionic neurone in the DVN retrogradely labelled by the injection of HRP into the cervical vagus nerve and localised using the DAB reaction.

3:3.A. Light micrograph of retrogradely labelled neurones in the DVN. A blood vessel (C1) is used to locate a labelled neurone (arrow).

3:3.B. Higher magnification indicating that the structure depicted by the arrow in 3:3.A is indeed a labelled neurone.

3:3.C. High power of the cell in **A** and **B**. The DAB reaction product in the cell is punctate and restricted to the cytoplasm of the soma and initial portions of dendrites. The structures indicated by letters are used for correlation in **D**. C2 and C3 are blood vessels, G a glial cell and N the nucleus of the labelled cell.

3:3.D. Low power electron micrograph of the labelled vagal preganglionic neurone, correlated by the locations of the same structures highlighted in photograph **C**. The cell has a round nucleus which lies to one side of the cytoplasm and which has a prominent nucleolus. The DAB reaction product is visualisable as black punctate structures localised to the cytoplasm of the cell.

Scale Bars: A. 100 μ m B. 25 μ m C. 20 μ m D. 4 μ m.

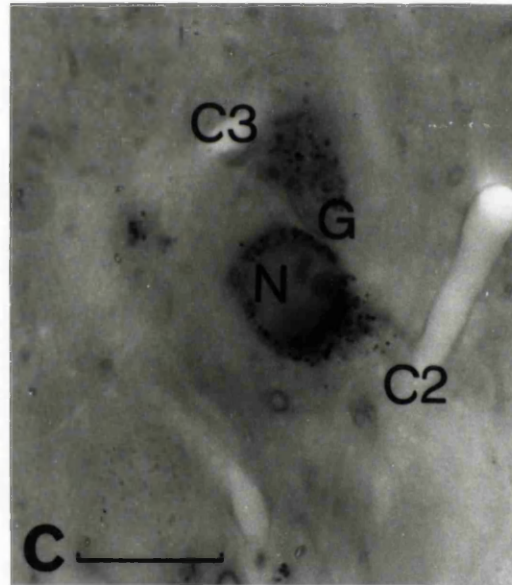
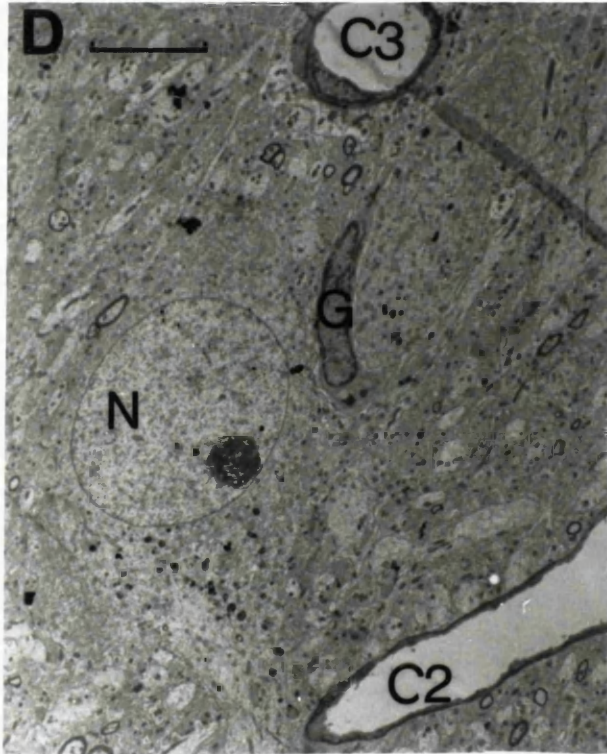
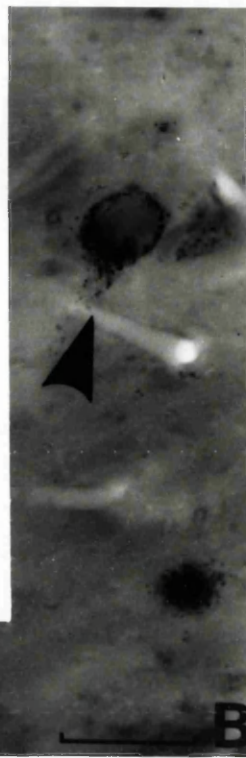
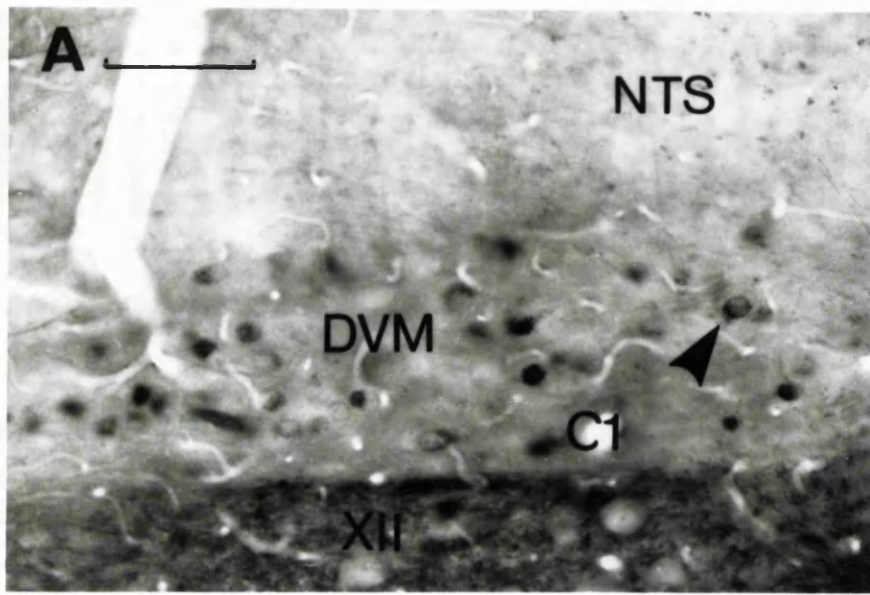


Figure 3:4. Correlated light and electron microscopic micrographs of a retrogradely labelled vagal preganglionic neurone in the compact region of the nucleus ambiguus, localised with the TMB reaction.

3:4.A. Low power light micrograph of the neuropil of the nucleus ambiguus (NA).

3:4.B. Higher magnification micrograph of the same area in **A**, illustrated by the presence of the blood vessel marked C1. A retrogradely labelled neurone can be seen in the nucleus ambiguus (arrow).

3:4.C. High power light micrograph of the labelled neurone in **B**. The punctate TMB reaction product is localised to the cytoplasm and primary dendrites of the neurone. The structures marked are for correlation in **D** - N is the nucleus of the labelled cell, C2 and C3 blood vessels used to locate the cell and U is an unlabelled cell.

3:4.D. Low power electron micrograph containing the labelled VPN. The marked structures are those in **C**. At this level it is possible to see that the labelled neurone has a round, uninvaginated nucleus which lies eccentrically in the soma and which contains a prominent nucleolus. The cell has abundant cytoplasm which contains punctate and crystalline TMB reaction product.

Scale Bars: A. 100 μ m B. 50 μ m C. 20 μ m D. 10 μ m.

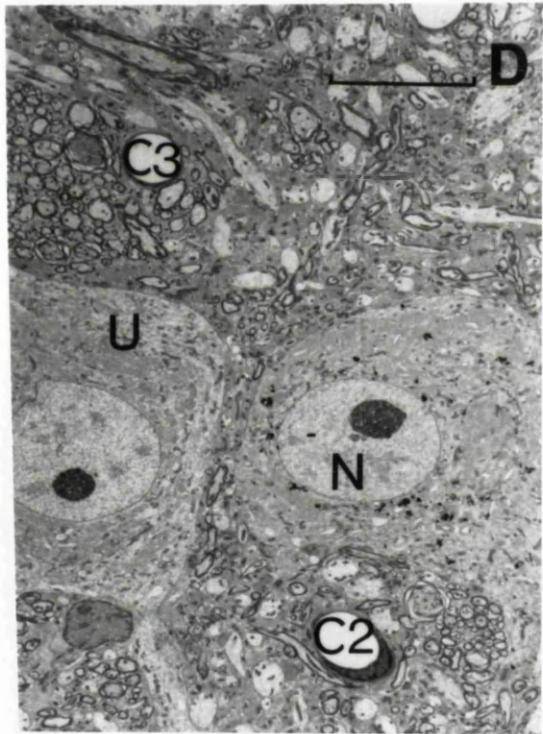
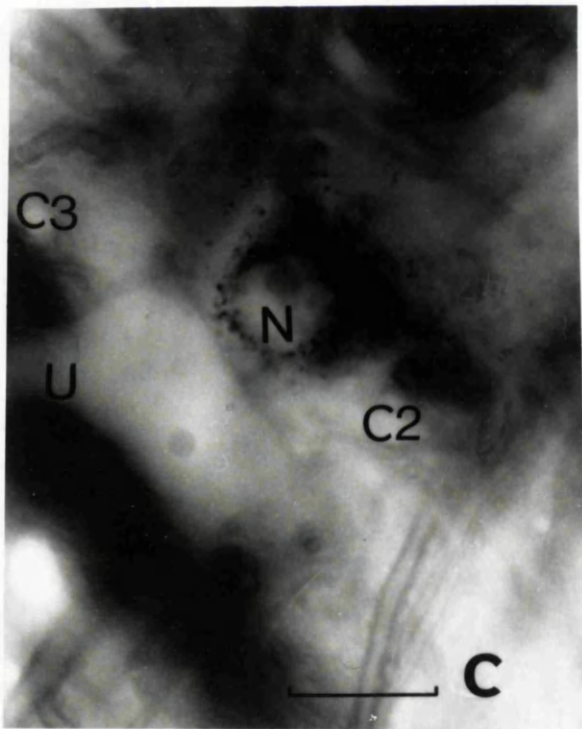
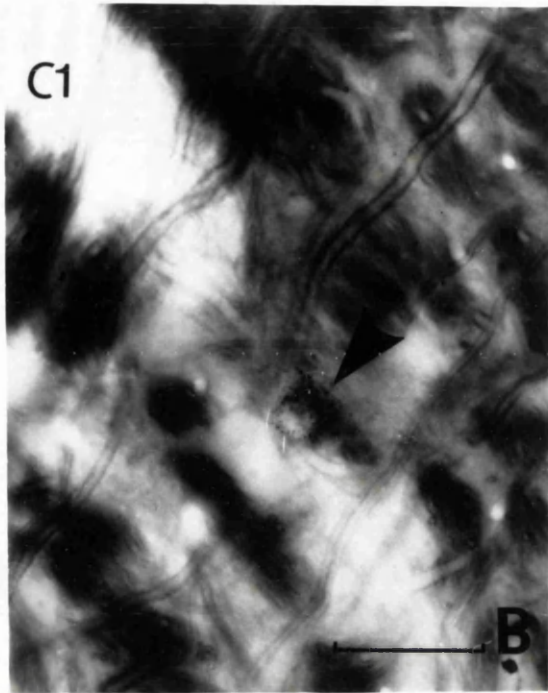
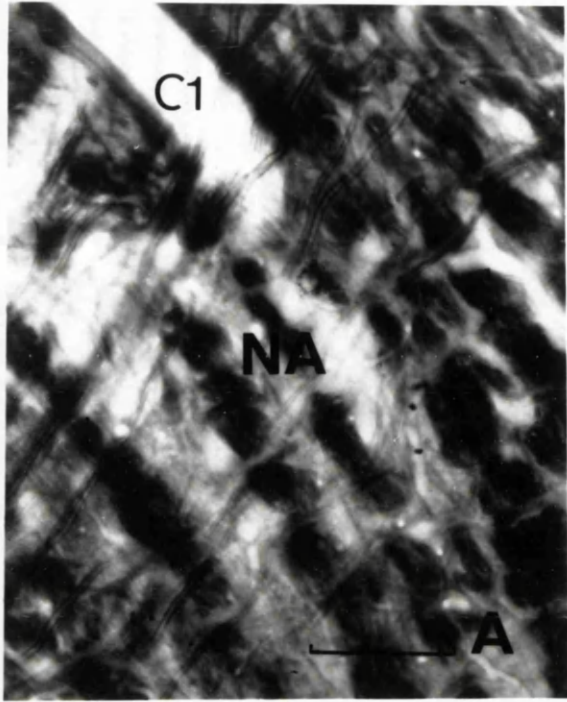


Figure 3:5. Light and electron microscopic correlation of a vagal preganglionic neurone in the ventrolateral nucleus ambiguus, retrogradely labelled by the injection of HRP into the cervical vagus nerve and localised with the DAB reaction.

3:5.A. Low power light micrograph of a region of the ventral medulla containing the compact region of the nucleus ambiguus (NA). C1 is a blood vessel which is contained in the region of the ventrolateral nucleus ambiguus.

3:5.B. A labelled neurone (arrow) is just visible at this higher magnification of the area around the blood vessel (C1).

3:5.C. High power light micrograph of the labelled vagal preganglionic neurone. DAB reaction product is punctate and restricted to the soma of the neurone. The marked structures are used to correlate the neurone at the ultrastructural level in **D**: N - the nucleus of the retrogradely labelled neurone; U - unlabelled neurone; d - unlabelled dendritic structure; C2 and C3 - blood vessels used to locate the labelled neurone.

3:5.D. Low power electron micrograph of the cell shown in **C**, correlated by the positions of the marked structures. The cell has a large, round, non-invaginated nucleus lying to one side of the cytoplasm which contains one prominent nucleolus. The cell appears to have a small amount of cytoplasm which contains punctate reaction product.

Scale Bars: A. 100 μ m B. 50 μ m C. 20 μ m D. 5 μ m.

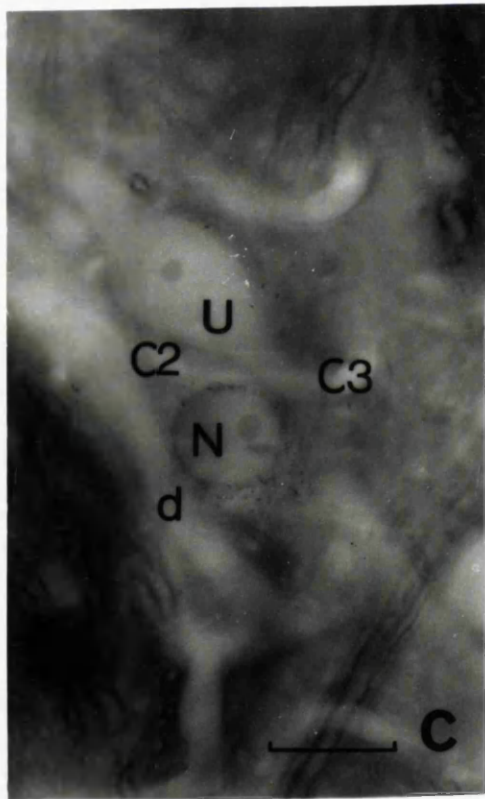
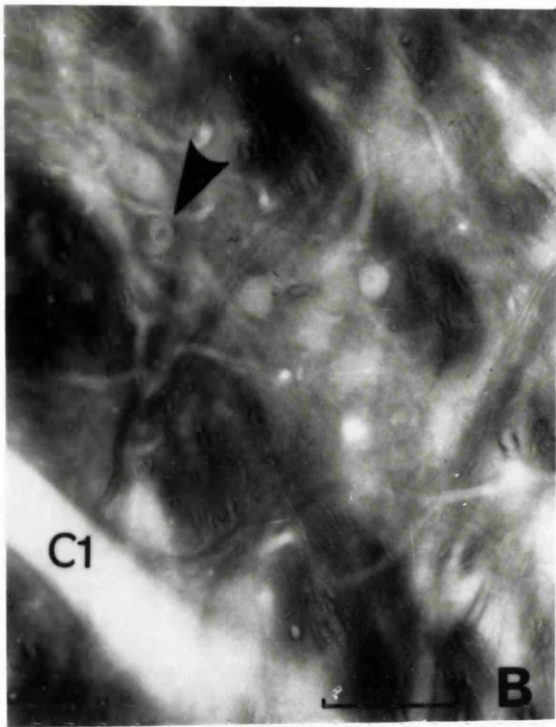
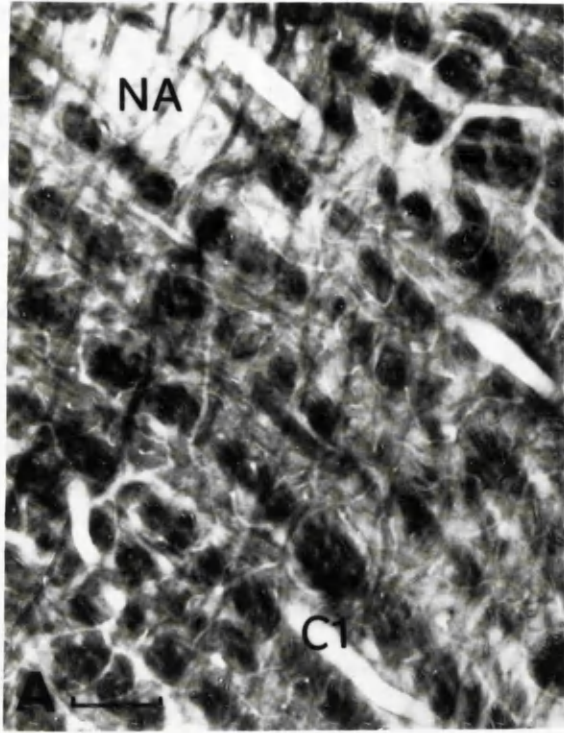


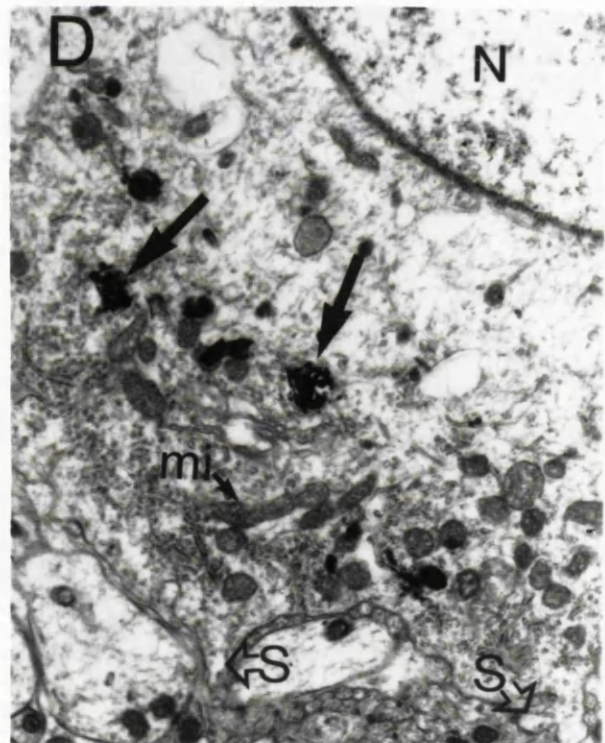
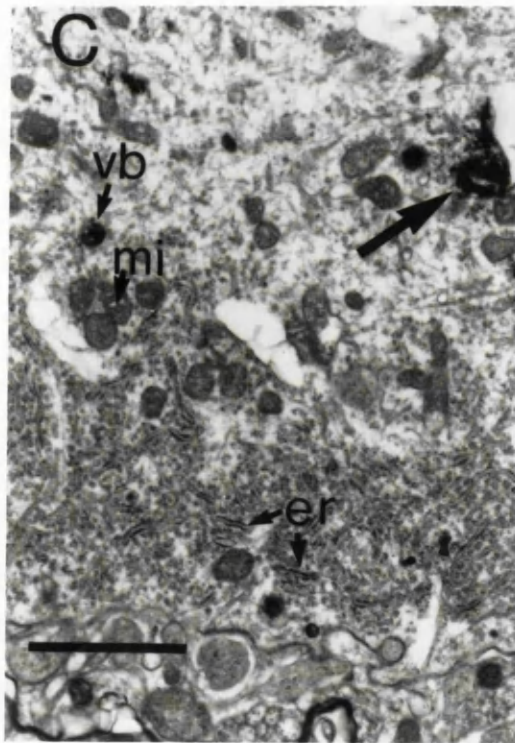
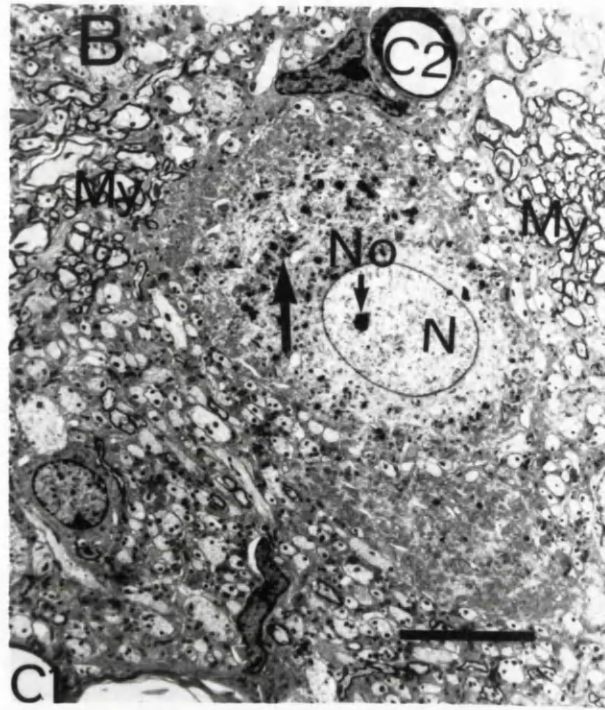
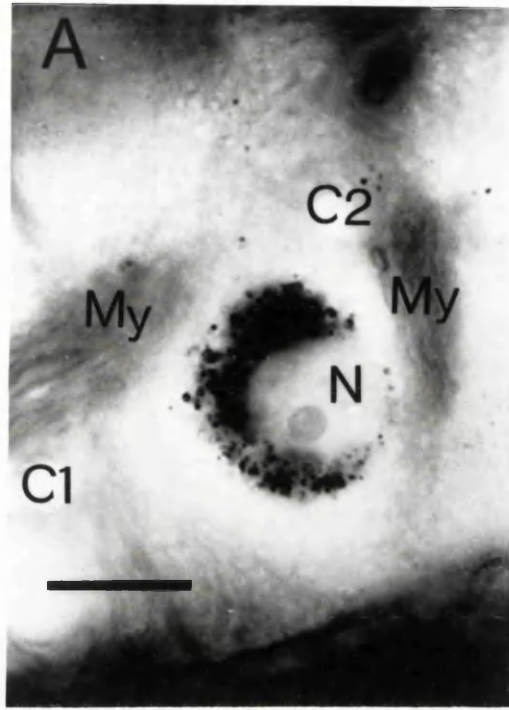
Figure 3:6. Light and electron microscopic correlation of a typical vagal preganglionic neurone retrogradely labelled by the injection of HRP into the cervical vagus nerve, to show the ultrastructural characteristics of VPNs.

3:6.A. High magnification light micrograph of a labelled VPN in the nucleus ambiguus. The marked structures are used to correlate the neurone at the ultrastructural level: N - nucleus of the labelled neurone; MY - myelinated nerve tracts; C1 and C2 - blood vessels.

3:6.B. Electron micrograph of the labelled neurone, localised by the structures in **B**. The labelled neurone has a round nucleus (N) which lies to one side of the cytoplasm and contains one prominent nucleolus (No). Crystalline TMB reaction product can be seen within the cytoplasm of the neurone.

3:6.C and D. Electron micrographs highlighting the cytoplasmic contents of the cell: vb - multi-vesiculate bodies; er - endoplasmic reticulum; s - spine-like obtrusions from the cell soma; N - nucleus. The large black arrows point to TMB reaction product which can be clearly seen as crystalline.

Scale Bars: A. 17 μ m B. 8 μ m C and D. 2 μ m.



3:3.2. Retrograde Tracing From the Heart.

3:3.2.A. Sensitivity of Retrograde Tracers and Histochemical Reactions.

Neurones were retrogradely labelled by injections into the heart only when cholera-toxin-HRP was used as the retrograde tracer, and not by HRP itself. In addition, labelled neurones could only be localised using the TMB and *o*-tolidine reactions, and not by the DAB reaction.

Using the TMB reaction CT-HRP was visualised in the somata and some way along the dendrites of labelled cells at the light microscopic level (Fig. 3.9.A). At the ultrastructural level crystalline reaction product was observed in the soma and dendrites of labelled cells, where it occasionally ran along the inner surface of the plasma membrane (Fig. 3.9.C,D). Ultrastructural preservation was good. (Fig. 3.9.B).

When the *o*-tolidine reaction was used to localise CT-HRP, reaction product appeared to fill the cytoplasm, proximal dendrites and sometimes the more distal portions of the dendritic tree, 200-500 μ m along the dendrites, giving a Golgi-like appearance to the cells (Fig. 3.8). At the electron microscopic level dense amorphous reaction product obscured the post-synaptic membrane. Ultrastructural preservation was poor.

3:3.2.B. Location and Morphology of Neurones Retrogradely Labelled From the Heart.

The majority of neurones retrogradely labelled by the injection of CT-HRP into the right atrium were situated in the ventrolateral region of the NA (figure 3.7; table 3.3). Thirty seven of these neurones were measured and had a diameter 20-28 μ m (median 25 μ m), median area 220 μ m² and were significantly different in size from those in the cNA labelled from the cervical vagus nerve (table 3.2), but not from those in the vINA. They were multipolar with dendrites extending in all directions and occasionally to the ventral surface (figure 3.8). In some animals labelled cells in the cNA were observed with a median diameter 30 μ m. These cells were lightly labelled with limited dendritic labelling (figure 3.8).

The intermediate zone contained a few heavily labelled neurones (table 3.3), which were spindle shaped with a median cell diameter 25 μ m, and their dendrites were orientated in the dorsal-ventral plane. In the DVN retrogradely labelled cells were rare (table 3.3), but were heavily labelled and were usually situated on the lateral edges of the nucleus. These cells had medium sized perikarya (median diameter 18 μ m) and dendrites which coursed mainly in the mediolateral plane within the DVN, and occasionally into the NTS.

In control animals in which CT-HRP was injected into the thorax and not into the myocardium, some lightly labelled neurones were observed in the vINA and the cNA, and very few in the DVN. All labelled cells appeared similar to lightly labelled neurones in the cNA following injections into the myocardium (Fig. 3.8).

Electron microscopic examination of ten neurones retrogradely labelled from the heart revealed that they were morphologically similar to VPNs retrogradely labelled from the cervical vagus nerve (Fig. 3.9).

Figure 3:7. Diagram of transverse medullary sections at four rostro-caudal levels, illustrating the positions of retrogradely labelled neurones (black dots) following injection of cholera-toxin-HRP into the wall of the right atrium of the heart in the rat.

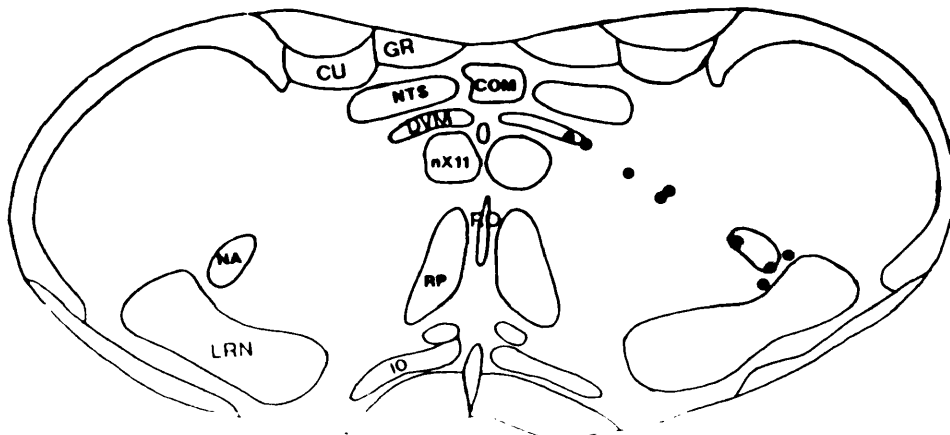
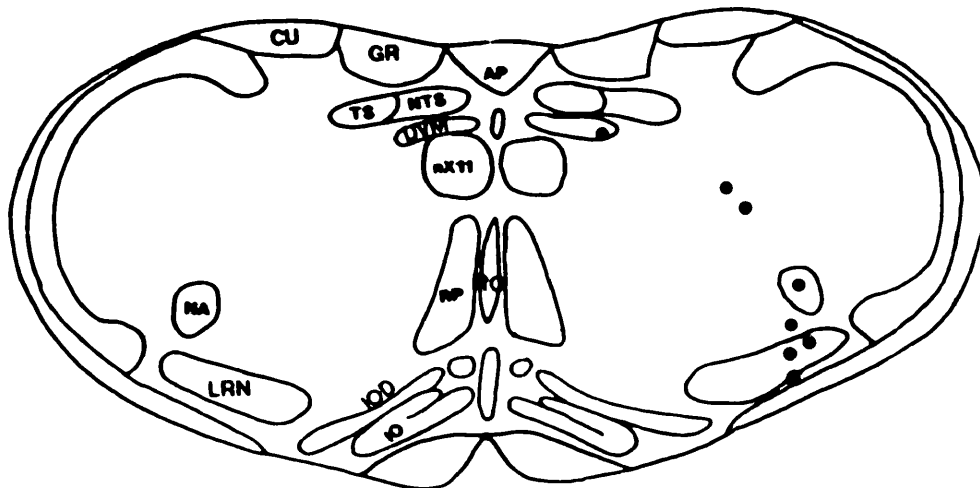
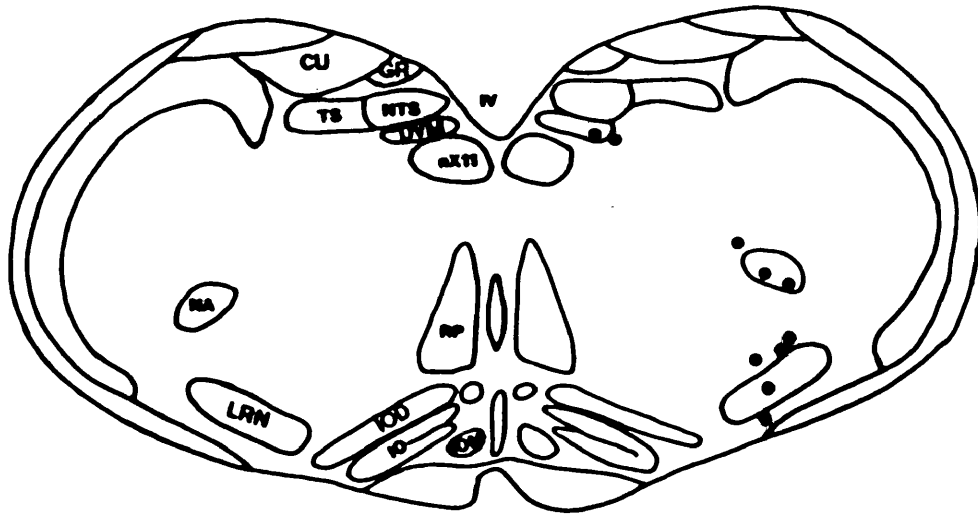
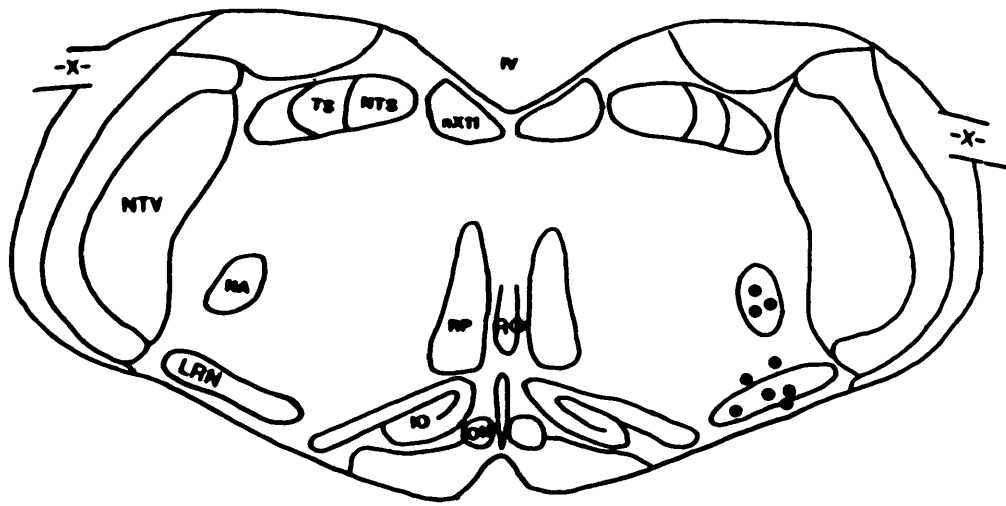


Figure 3:8. Retrogradely labelled neurones in the medulla of the rat following injection of CT-HRP into the wall of the right atrium, visualised with the o-tolidine reaction. In each case the labelled neurones (large, thin black arrows) are situated in the ventrolateral nucleus ambiguus. and their dendrites (small black arrows) sometimes extend to the ventral surface (VS) of the section. Labelled neurones in the compact region of the NA (large, thick black arrows) do not appear to have labelled dendrites.

Scale Bar: 200 μ m.

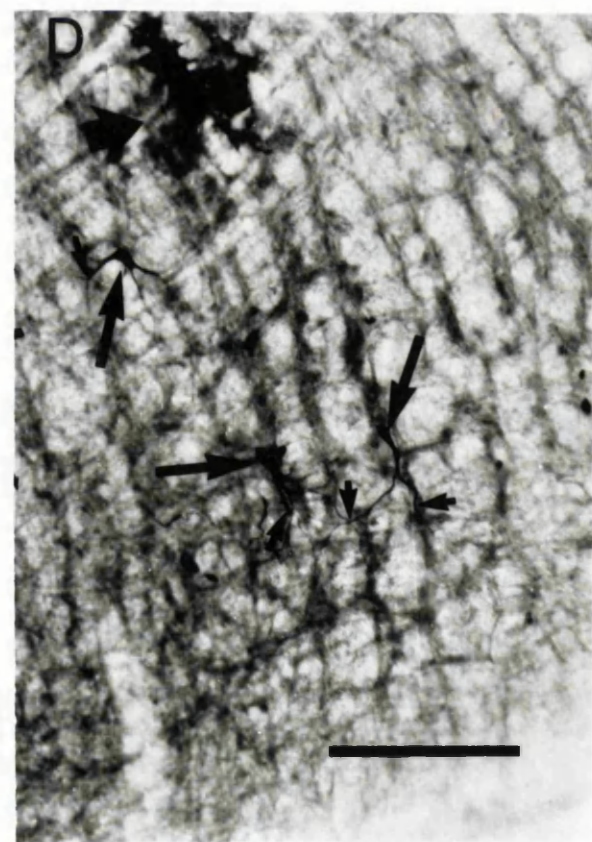
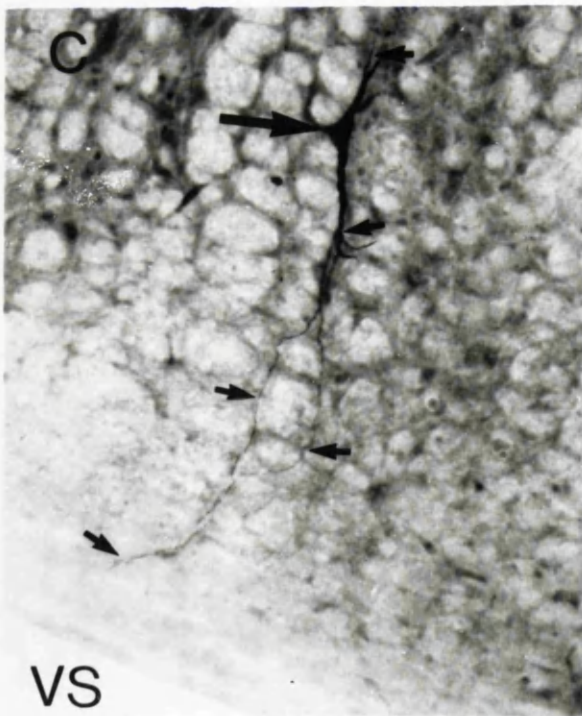
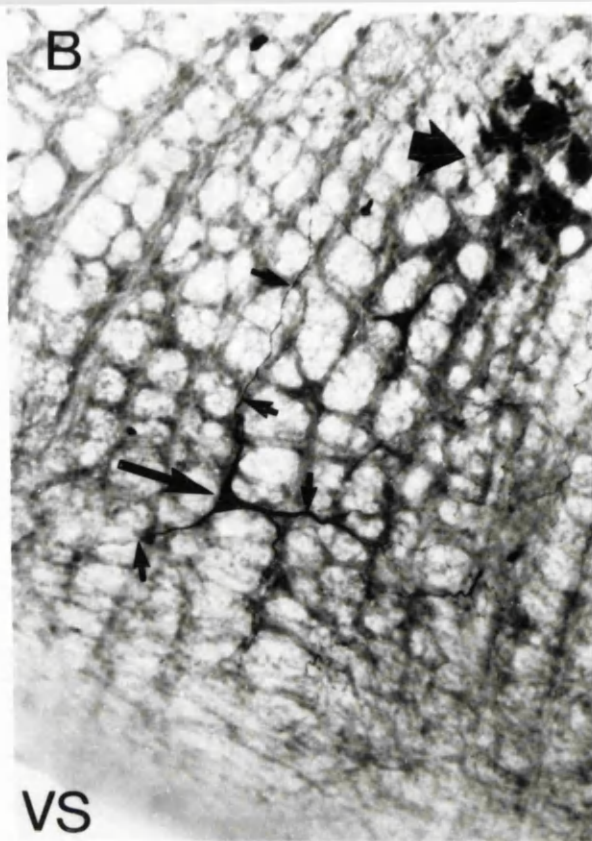
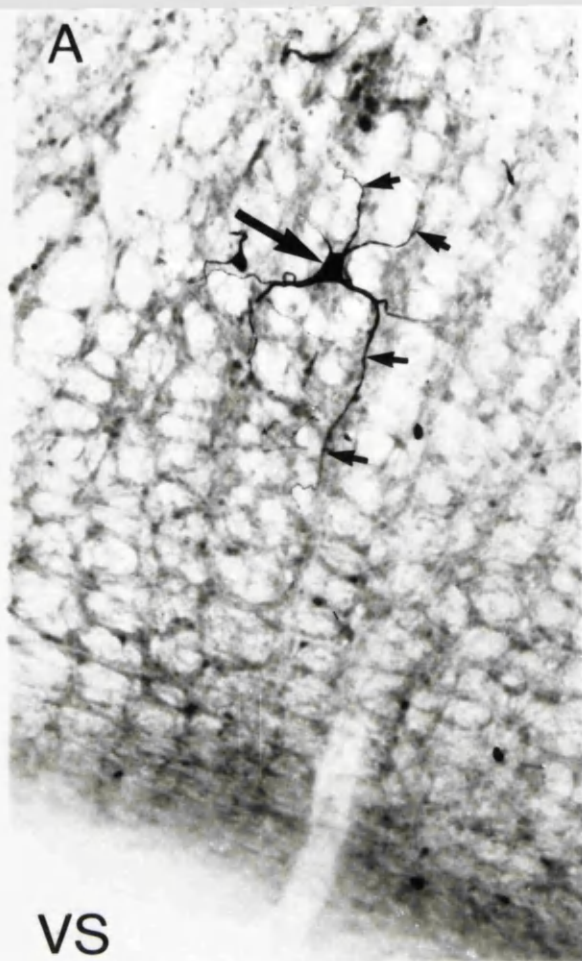


Figure 3:9. Correlated light and electron micrographs of a vagal preganglionic neurone retrogradely labelled by the injection of CT-HRP into the wall of the right atrium and localised using the TMB reaction.

3:9.A. Light micrograph of a retrogradely labelled cardiac vagal preganglionic neurone. Reaction product can be identified in the dendrites of the labelled cell. The marked structures are for correlation of the cell in **B**: My - myelinated nerve bundle; C - blood vessel; N - nucleus; No - nucleolus.

3:9.B. Low power electron micrograph of the labelled CVPN. The cell is correlated by the structures marked in **A**. Reaction product (arrows) is localised to the cytoplasm of the cell.

3:9.C and D: Electron micrographs of the cytoplasmic contents of the labelled neurone: mi - mitochondria; er - endoplasmic reticulum; ly - lysosomes; vb - vesiculate bodies. TMB reaction product within the cytoplasm of the cell is crystalline (black arrows) and can sometimes be identified running along the plasma membrane (open arrows).

Scale Bars: A. 20 μ m B. 10 μ m. C and D. 1.5 μ m.

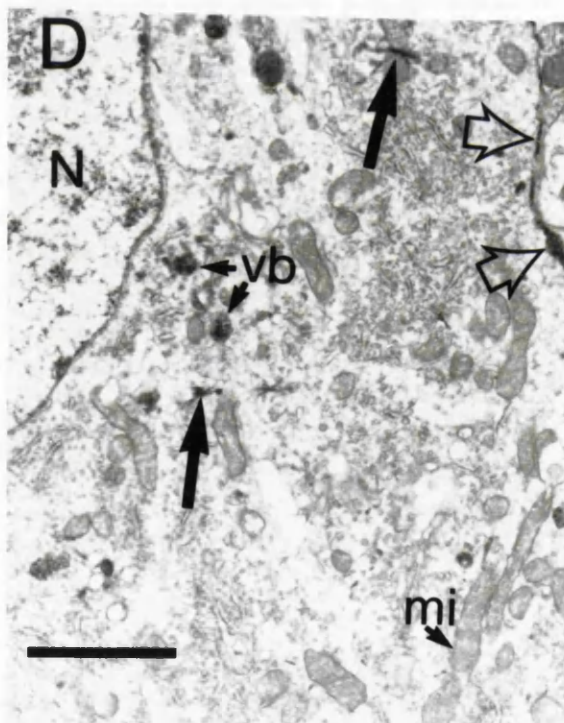
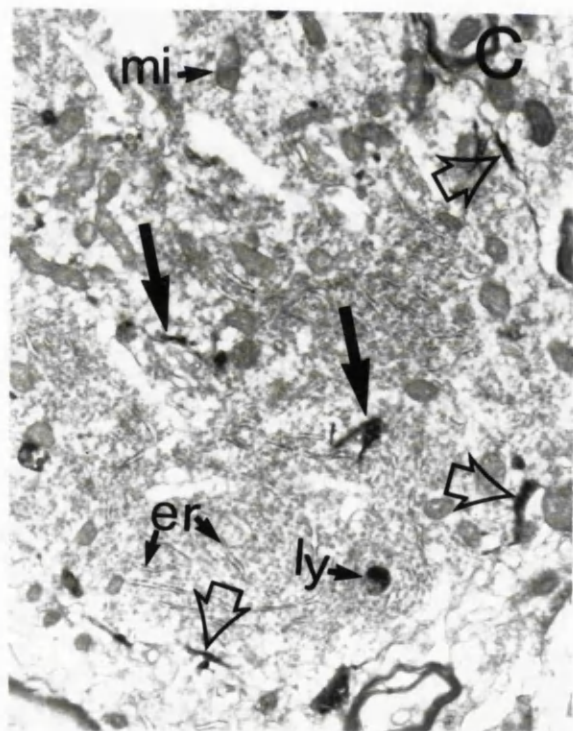
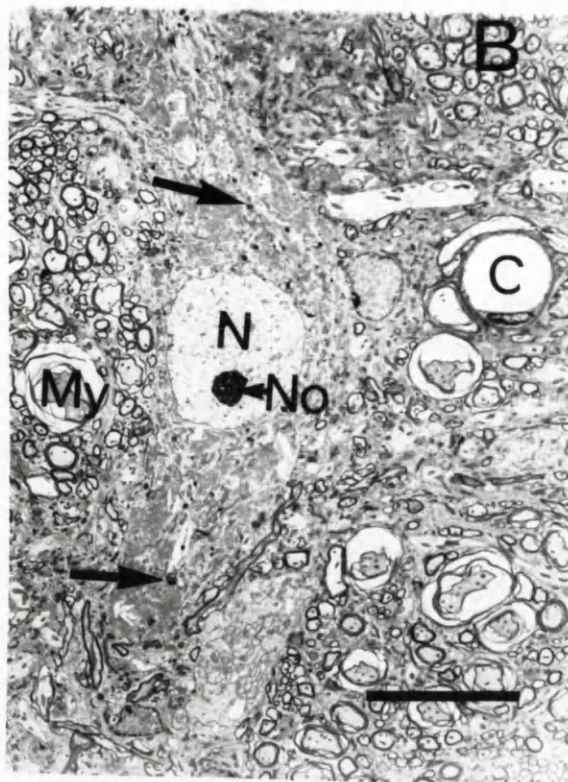
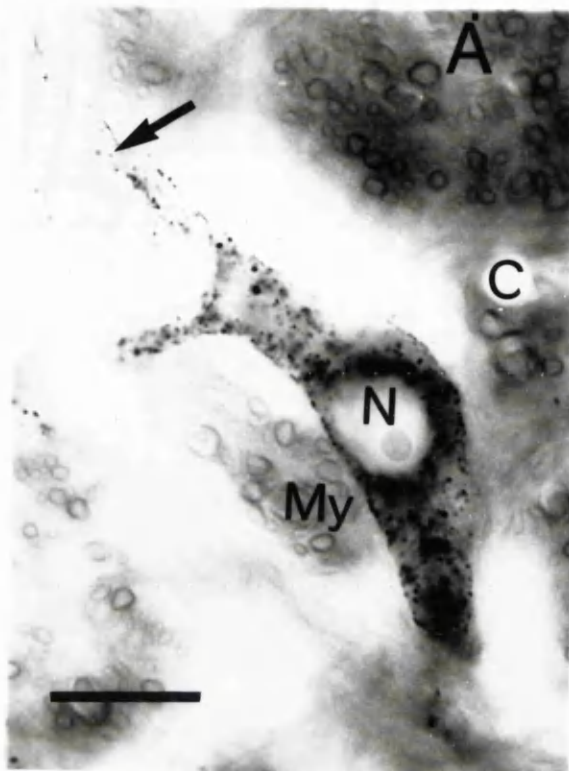


Table 3:3. - The number of retrogradely labelled neurones found in the dorsal vagal motor nucleus (DVN), the intermediate zone (IZ) and the nucleus ambiguus (NA) in 4 rats following the injection of CT-HRP into the muscle of the right atrium.

	DVN	IZ	NA	Total
Rat 1	8	30	94	132
Rat 2	5	8	61	74
Rat 3	3	9	20	32
Rat 4	10	12	81	103
Total (%)	26(7.6%)	59(17.3%)	256(75.1%)	

3:3.3. Retrograde Tracing from the Phrenic Motor Nucleus.

Injections of HRP into segments C4 and C5 of the spinal cord, combined with the DAB or TMB methods to localise HRP, resulted in retrograde labelling of cells bilaterally in many areas of the medulla (figure 3.10). The major groups were in the midline nuclei, cuneate nuclei, reticular formation and in the ventral medulla in the region of the NA (figure 3.10). Within the NA few retrogradely labelled cells were found within the cNA and many were located in the vINA (figure 3.10 and 3.11). Analysis of the diameter and soma area of 150 cells in the region of the NA revealed a diverse population (table 3.1) ranging in diameter from 17 μ m to 38 μ m (median 28 μ m) and area 100-450 μ m² (median 280 μ m²). Labelled cells were often multipolar with large round cell bodies. Statistical analysis did not detect any difference between labelled cells in the region of the NA and VPNs in the NA (table 3.1)

Electron microscopic examination of 11 of these cells revealed that although some of them shared common features with vagal cells, they were diverse in nature (see figure 3.12): cell bodies were round or fusiform with occasional somatic spines; large round nuclei with prominent nucleoli; similar proportions to VPNs of cytoplasmic organelles such as mitochondria, lysosomes, ribosomes, golgi bodies, ribosomes and rough endoplasmic reticulum. Generally spinally projecting neurones appeared to have more synaptic input onto their cell bodies than VPNs (at least ten per cell when counted in 5-10 serial sections). Smaller labelled cells generally had less cytoplasm and their nuclei had more indentations.

Figure 3.10. Diagrams of transverse sections of the medulla oblongata at four rostro-caudal levels, illustrating the distribution of labelled neurones following injection of HRP into the phrenic motornucleus in the cervical spinal cord of the rat. Each large black dot represents a group of ten cells and small dots represent a single cell.

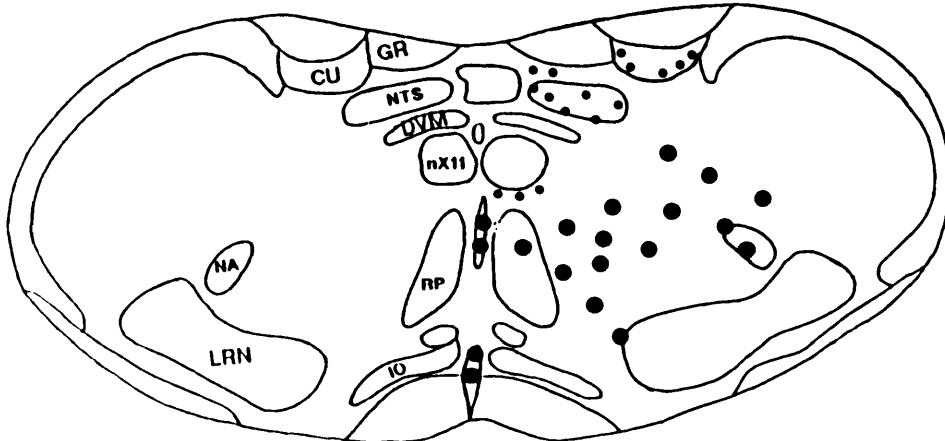
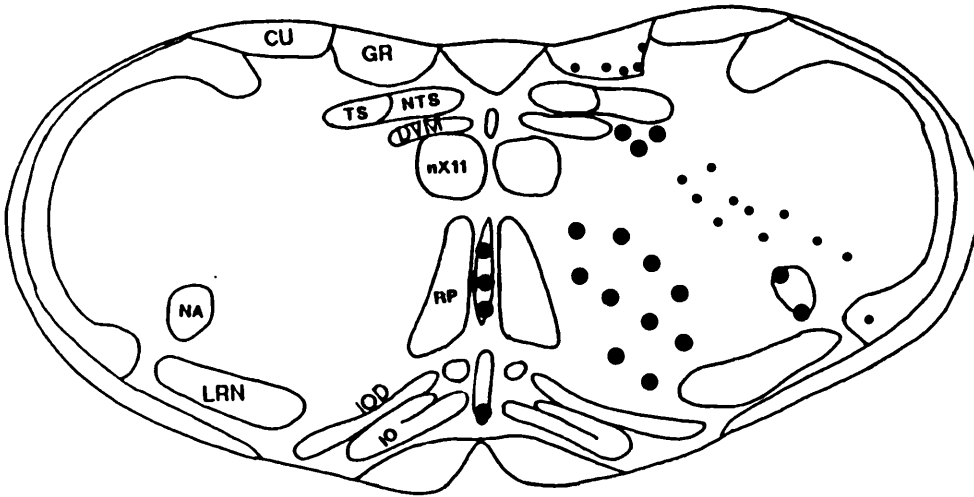
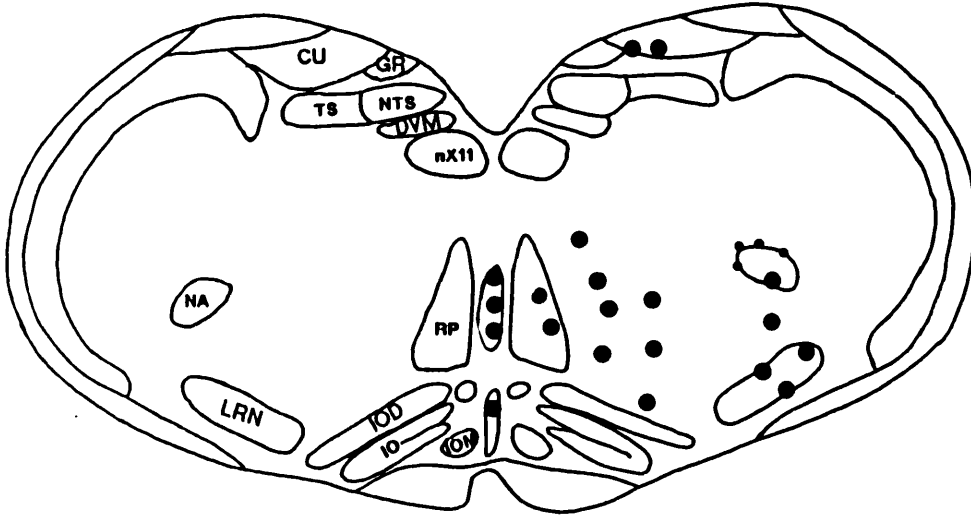
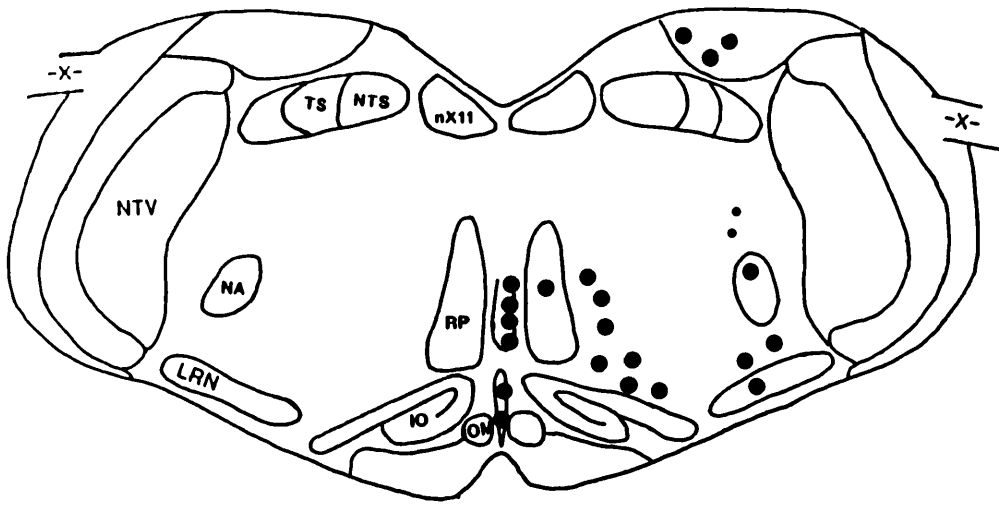


Figure 3.11. Photomicrographs of labelled neurones in the NA following the injection of HRP in the region of the phrenic motornucleus in the cervical spinal cord of the rat.

3.11.A. Low power light micrograph of a transverse section of the medulla oblongata. The nucleus ambiguus (NA) is located between two blood vessels (C1 and C2) used to identify the same region at higher power.

3.11.B. At this magnification labelled structures can be seen in the nucleus ambiguus (NA), located by the blood vessels C1 and C2.

3.11.C. At this magnification retrogradely labelled structures can be clearly identified as neurones which are located in and around the edges of the nucleus ambiguus (NA).

Scale Bars. A. 500 μ m B. 185 μ m C. 100 μ m.

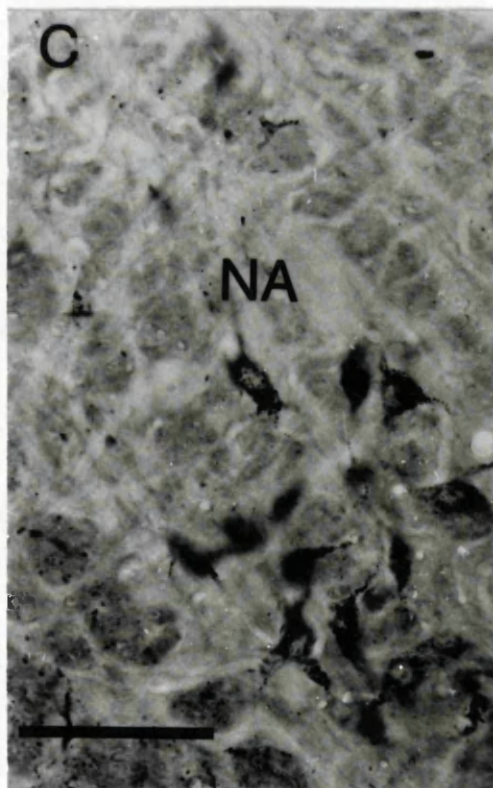
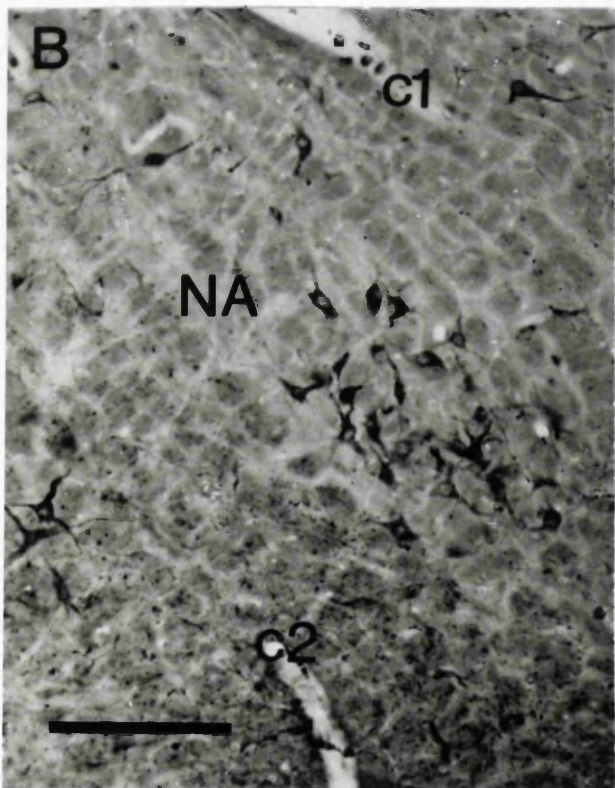
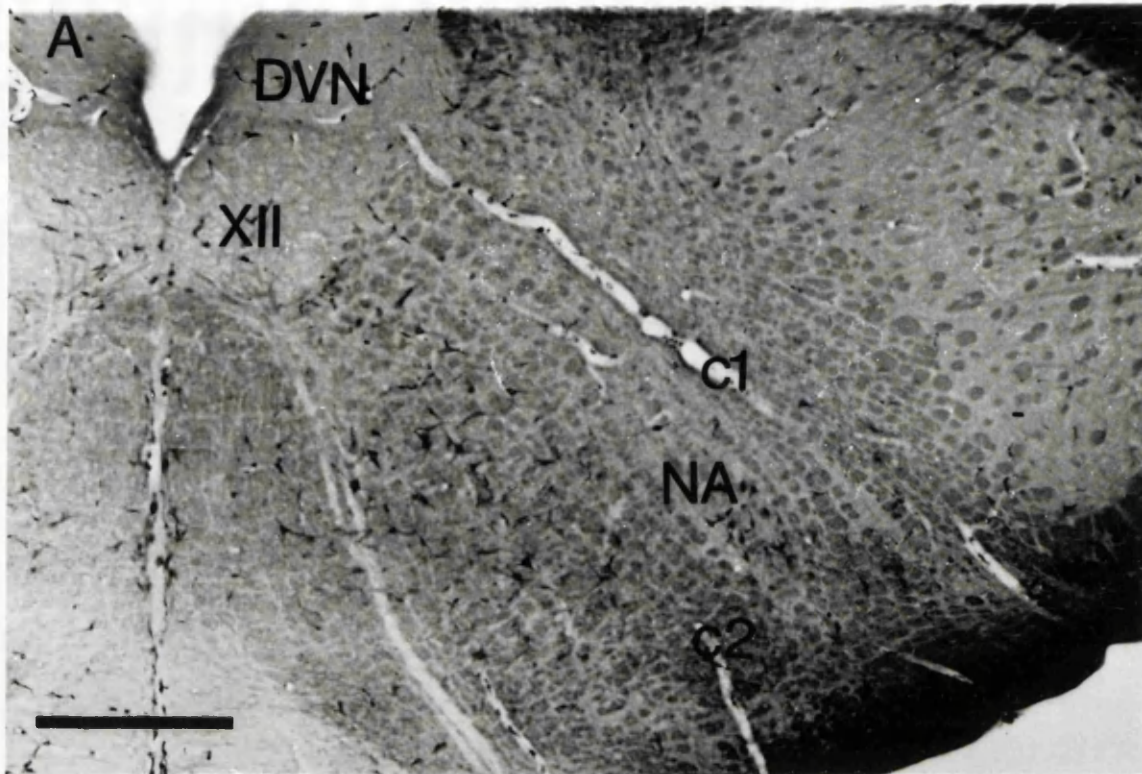


Figure 3:12. Correlated light and electron micrographs of a labelled neurone in the region of the compact nucleus ambiguus following injection of HRP into the cervical spinal cord of the rat.

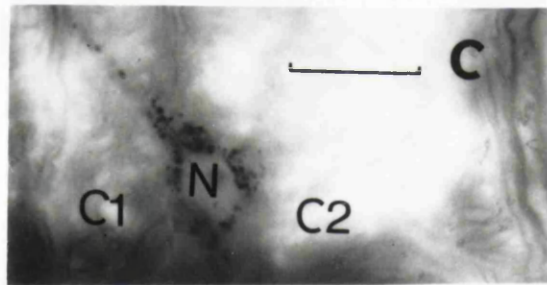
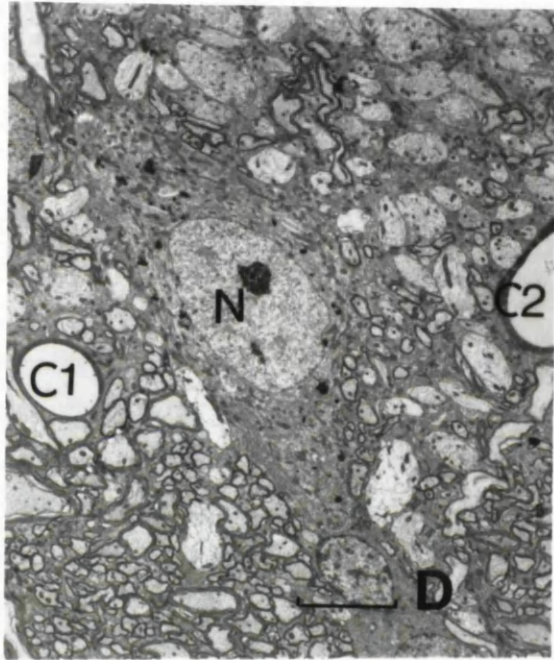
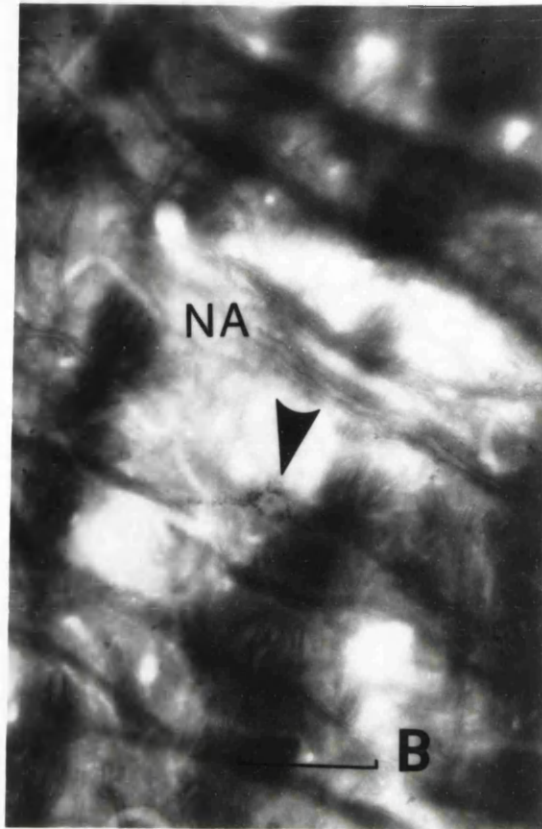
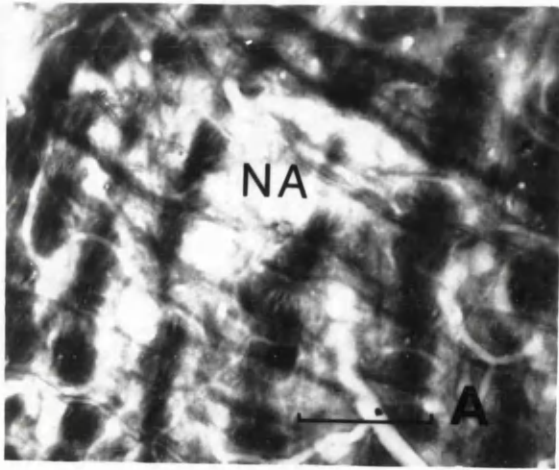
3:12.A. Low power light micrograph indicating the neuropil of the nucleus ambiguus.

3:12.B. Higher magnification of the same region indicating the presence of a labelled neurone (arrow).

3:12.C. High power light micrograph indicating structures used to correlate the labelled neurone at the ultrastructural level: N - nucleus of the labelled cell; C1 and C2 - blood vessels used to locate the neurone.

3:12.D. Electron micrograph of the labelled neurone, correlated with marked structures in C. The cell has an elongated soma, and the nucleus has small indentations in the membrane and is located in the centre of the cytoplasm. TMB reaction product is localised to the cytoplasm of the cell.

Scale Bars: A. 150 μ m B. 100 μ m C. 40 μ m D. 10 μ m.



Section 3.4. - Discussion.

The results of this study indicate that:

1. The TMB pH6 reaction is a sensitive method for light and electron microscopic localisation of retrogradely transported HRP
2. Injection of CT-HRP into the myocardium is a suitable method to retrogradely label a population of VPNs that are probably cardiac vagal preganglionic neurones.
3. Vagal preganglionic neurones in the rat are organised with respect to their projections, but despite size differences evident at the light microscopic level, they share similar ultrastructural morphological characteristics.
4. VPNs in the NA are intermingled with neurones projecting to the phrenic motor nucleus and are morphologically indistinguishable to some of these neurones.

3:4.1. Technical Considerations.

Comparison of histochemical methods used to localise HRP and CT-HRP demonstrated that the TMB pH 6 reaction was the most useful as it was more sensitive than the DAB reaction, but did not result in the loss of ultrastructural preservation observed with the more sensitive *o*-toluidine reaction. The loss of ultrastructural preservation with the *o*-toluidine reaction is contrary to that observed by others, who combined retrograde labelling with CT-HRP with the *o*-toluidine reaction to studies of sympathetic preganglionic neurones at the electron microscopic level (Bacon and Smith, 1989; Zagon and Smith, 1990). The

difference may be due to the degree of reaction product within the cell as these previous studies examined lighter labelled neurones or dendrites of neurones where reaction product is lighter than in the soma. Possible differences in the degree of labelling may be due to the concentration of retrograde tracer used, as no measure of concentration of CT-HRP was made in this study. Consequently, injections of high concentrations of CT-HRP may have resulted in retrograde transport of large amounts of tracer to the cell somata, resulting in a vigorous reaction which damaged ultrastructure. Therefore, since the *o*-tolidine reaction as applied in this study compromised ultrastructural preservation, the TMB pH 6 reaction was preferred for electron microscopy.

Cholera-toxin HRP was the most useful tracer for retrograde labelling from the heart since neurones could be labelled only by application of CT-HRP to the myocardium and not by HRP itself. This accords with reports that CT-HRP is a superior tracer to HRP itself, especially when injected into end-organs (Trojanowski *et al*, 1982; Wan *et al*, 1982). However, HRP itself has been used in previous studies to retrogradely label neurones from the myocardium (Todo *et al*, 1977; Geis, Kozelka and Wurtser, 1981; Stuesse, 1982; Plecha *et al*, 1988). The failure to do so in this study may be due to the relatively small volume injected (10 μ l) whereas others have injected greater amounts (*e.g.* 100 μ l by Geis, Kozelka and Wurster, 1981). Alternatively, it may be due to concentration of tracer used as in this study the concentration of HRP was 10 - 20%, but in a study in which neurones were labelled by injection of 3-5 μ l of HRP into the myocardium the HRP

concentration was 30% (Stuesse, 1982). Thus in this study HRP may not have been used at a sufficiently high concentration, and as no measure was made of CT-HRP concentration it is possible that this was successful as it was very concentrated. Although it may have been possible to retrogradely label neurones from the heart with HRP itself with some manipulations, it was felt that neurones were better labelled with CT-HRP as reaction product was visualised in the distal dendrites of the cells, whereas only very proximal portions of dendrites were visualised in neurones retrogradely labelled by the application of HRP to the cervical vagus nerve.

3:4.2. Location and Morphology of Labelled Neurones.

The application of HRP to the cervical vagus nerve labelled neurones in the DVN and the NA as described by numerous other studies in the rat (Contreras *et al*, 1980; Karim and Leong, 1980; Kalia and Sullivan, 1982; Ritchie *et al*, 1982; Stuesse, 1982). Despite size differences between VPNS in the separate groups, they were ultrastructurally similar, which is also the case in the cat (McLean and Hopkins, 1981, 82, 85a,b; see sections 1:2.2 and 1:3.2).

The results in this study suggest that the vINA is the main source of CVPNS, in agreement with some other studies (Rat - Nosaka *et al*, 1979a; Hopkins and Armour, 1982; Bieger and Hopkins, 1987; Miselis *et al*, 1989. Pig - Hopkins *et al*, 1984). However, labelled neurones were found in the cNA as well as the vINA (Fig. 3.7 and 3.8), which is in accordance with some previous studies (Cat -

Sugimoto *et al*, 1979; Kalia and Mesulam, 1980b; Kalia, 1981; Miura and Okada, 1981; Ciriello and Calaresu, 1982;. Rat - Stuesse, 1982). Nevertheless, the degree of retrograde labelling suggested that not all labelled neurones were specifically labelled. Neurones in the vNA which contained reaction product were labelled heavily, but those in the cNA were labelled lightly (fig. 3.8). The light labelling in neurones in the cNA may have been the result of uptake of spilled tracer by non-cardiac organs which was diluted to low concentrations by body fluids within the thorax. This appears to have been the case as a similar density of reaction product was observed in all labelled neurones following intentional spillage of CT-HRP into the thorax. This spilled tracer may have been taken up by vagal axons innervating the oesophagus as Bieger and Hopkins (1987) report labelled VPNS in the cNA following cardiac injections only if tracer was detected in the oesophagus. Furthermore, in this study labelled neurones in the cNA had a soma diameter of 30 μ m, the same as those retrogradely labelled by the intentional injection of tracers into the oesophagus (Bieger and Hopkins, 1987). Thus it is likely that labelled neurones in the cNA in this and previous studies were due to spillage of tracer onto the oesophagus (Cat - Sugimoto *et al*, 1979; Kalia and Mesulam, 1980b; Kalia, 1981; Miura and Okada, 1981; Ciriello and Calaresu, 1982;. Rat - Stuesse, 1982). This indicates that the major source of cardiac vagal innervation is the ventrolateral nucleus ambiguus, in agreement with some previous workers (Rat - Nosaka *et al*, 1979a; Hopkins and Armour, 1982; Bieger and Hopkins, 1987; Miselis *et al*, 1989. Pig - Hopkins *et al*, 1984).

In contrast to the NA, retrogradely labelled CVPNs in the dorsal vagal nucleus and the intermediate zone were few in number (Fig. 3.7). Labelled cells in the DVN were restricted to the lateral edges of the nucleus (fig. 3.7). However, some studies have reported many labelled neurones throughout the DVN, although only in some of their experimental animals (Rat - Stuesse, 1982. Cat - Bennett *et al*, 1981). It is possible retrograde labelling throughout the DVN was due to spillage of tracer onto the intra-thoracic trachea, since this portion of the trachea is innervated by VPNS throughout the DVN (Cat - Kalia and Mesulam, 1980b). It seems unlikely that neurones in the DVN in this present study were due to spillage of tracer as the few neurones in the DVN labelled in control experiments were labelled lightly, whereas following myocardial injections all neurones in the DVN were heavily labelled. In addition, the location of labelled neurones in the lateral aspects of the DVN was in agreement with many previous studies (fig. 3.7; table 3.2; Nosaka *et al*, 1979; Sugimoto *et al*, 1979; Miura and Okada, 1981; Ciriello and Calaresu, 1982; Geis and Wurster, 1980a; Hopkins and Armour, 1982; Miselis *et al*, 1989).

Comparison of retrogradely labelled neurones revealed that those in the vINA projecting to the cervical vagus nerve were morphologically identical to neurones projecting to the heart (compare figs. 3.5 and 3.9). This suggests that neurones in the vINA labelled retrogradely from the cervical vagus nerve may have been CVPNs. However, morphologically similar VPNS in the vINA have been retrogradely labelled from the lungs in the rat (Bieger and Hopkins, 1987), and in the cat neurones retrogradely

labelled by the application of HRP to branches of the vagus nerve innervating the lungs were indistinguishable on the basis of position in the NA or morphology (Bennett *et al*, 1981), and these are also likely to be retrogradely labelled from the cervical vagus nerve. Therefore, CVPNs could not be distinguished on the basis of position or morphology in a population of VPNs retrogradely labelled from the cervical vagus nerve.

Comparison of the distribution and morphology of VPNs with neurones labelled retrogradely from the phrenic motor nucleus revealed that the two groups of neurones were indistinguishable. This agrees with previous observations that neurones in the NA retrogradely labelled from the phrenic motor nucleus ranged in diameter from 20-50 μ m (Onai *et al*, 1987), and were indistinguishable from VPNs on the basis of size or morphology at the light microscopic level (Ellenberger and Feldman (1990b). The results in this study show that neurones projecting to the phrenic motor nucleus are also indistinguishable to VPNs at the ultrastructural level, although some bulbospinal neurones may have had more synaptic input onto their cell somata than VPNs. This latter observation may indicate that bulbospinal neurones in the cat and monkey are located in the NA and are indistinguishable from VPNs, since McLean and Hopkins (1985b) observed unlabelled neurones in the NA which were similar to retrogradely labelled VPNs, but which generally had more synaptic input onto their soma.

3:4.4. Functional Implications.

Morphological differences between VPNS may indicate that they have different electrophysiological properties. In a recent *in vitro* study in the NA of the guinea pig, intracellular recordings were made from neurones which may have been VPNS (Johnson and Getting, 1991). Recorded neurones exhibited different electrophysiological characteristics which were correlated to size differences. These electrophysiological properties included post-inhibitory rebound (PIR cells), delayed excitation (DE cells) or neither of these properties (Non-cells). Morphologically, DE cells were significantly smaller in size than PIR cells and Non-cells. Furthermore, the axons of all these types of cells travelled dorsomedially before their severed ends were found, which is similar to the trajectory of the axons of VPNS (Bieger and Hopkins, 1987). Thus it is possible that PIR, DE and non-cells are VPNS. DE cells may thus be CVPNS as they were the smallest of the three types of cells identified electrophysiologically, as was the case for CVPNS compared to other VPNS in this study. However, further electrophysiological studies on identified CVPNS and other VPNS are required to determine if this is the case.

The distribution of vagal preganglionic neurones and the distinct location of CVPNS in the vNA implies that VPNS are organised in a manner related to their functions. It is known that large neurones in the cNA innervate the pharynx, larynx and oesophagus, while those in the vNA are medium sized and innervate the lungs and other unidentified structures (Cat - Kalia and Mesulam,

1980a,b. Rat - Bieger and Hopkins, 1987) and in this study CVPNs were located in the vINA with other medium sized VPNs, as well as neurones projecting to the phrenic motor nucleus. This intermingling of neurones projecting to the heart, lungs and phrenic motor nucleus may be useful for integration of cardio - respiratory functions (See Richter and Spyer, 1990). As these groups of neurones were also morphologically similar, they may also have similar functions.

The extension of dendrites of CVPNs to the ventral surface of the medulla and close to the cerebrospinal fluid (CSF) may also indicate a role in cardio-respiratory integration. Increases in CSF acidity due to hypercapnia causes tachycardia which may be mediated by inhibition of CVPNs (see Daly, 1991), and may thus be due to components of CSF acting on dendrites of CVPNs.

A similar scheme for the organisation of CVPNs in the DVN is harder to define. CVPNs were located on the lateral edges of the DVN, which is also the site for VPNs innervating the caecum (see section 1:2.1). However the dendritic architecture of CVPNs in the DVN may be important in explaining why they are located in this nucleus. Their dendrites coursed through the nucleus and occasionally into the commissural and medial sub-nuclei of the NTS, and as these sub-nuclei are a main site of termination for cardiovascular afferents (see Loewy, 1990), this may indicate a short pathway for reflex alteration of CVPN output and thus cardiac functions. However, further studies are required to determine if visceral cardiovascular afferents are in direct synaptic contact with CVPNs.

Since the separation of CVPNs in the DVN and the NA may reflect the differences in their inputs, these differences may also indicate functional differentiation in the control of the heart. CVPNs in the NA may exclusively control heart rate as electrical stimulation of the NA results in decreases in heart rate and increases in contractility, although contractility changes were eliminated by cardiac pacing indicating that they were secondary to heart rate changes (Geis, Kozelka and Wurster, 1981). Conversely, CVPNs in the DVN may control ventricular contractility as electrical stimulation of the DVN in the anaesthetised cat caused no heart rate changes but a vagally mediated reduction in contractility which was not eliminated by cardiac pacing (Geis, Kozelka and Wurster, 1981). However, further work is required before this interpretation can be accepted as others have elicited a bradycardia by electrical stimulation in the DVN (Cat - Miller and Bowman, 1916; Chiurugi and Mollica, 1954; Dugin *et al*, 1976. Dog - Miller and Bowman, 1916; Gunn *et al*, 1968; Weiss and Priola, 1972. Rabbit - Schwaber and Schneiderman, 1975; Ellenberger *et al*, 1983. Rat - Nosaka *et al*, 1979b; Stuesse, 1982) and electrical stimulation is a coarse method of excitation which activates fibres of passage as well as cell bodies (see section 3:1).

Further evidence that CVPNs are separated between the NA and the DVN due to their functions is provided by studies of their electrophysiological properties. Neurones in the NA antidromically activated by stimulation of a cardiac branch of the vagus nerve have axons in the B-fibre range ($<2.5\text{mS}^{-1}$; Cat - McAllen and Spyer, 1978a. Rat - Nosaka *et al*, 1982) whereas

those in the DVN have axons in the C-fibre range (3-15mS⁻¹; Cat - Ford *et al*, 1990. Rat - Nosaka *et al*, 1982). Those in the NA were identified as CVPNs as they fired in expiration, were excited by baroreceptor input and mediated a bradycardia when excited by ionophoretic application of excitatory amino acids (Cat - McAllen and Spyer, 1978a,b), whereas the majority of those in the DVN did not have any of these properties, suggesting that they have different functions (Cat - Ford *et al*, 1990). This hints that CVPNs in the NA conduct with B-fibre axons and control heart rate and those in the DVN have C-fibre axons and control contractility of myocardial muscle. However, in the rabbit, VPNs in the DVN and NA which mediated a bradycardia upon excitation, and which were thus assumed to be CVPNs, all had axons which conducted in the B-fibre range (Jordan *et al*, 1982). It is possible that chronotropic effects may be mediated through B-fibres and contractility changes through C-fibres, regardless of the location of the CVPNs. However, stimulation of C-fibres in the peripheral end of the cut vagus nerve also elicits a bradycardia in rats (Nosaka *et al*, 1982) and in rabbits (Ford and McWilliam, 1986; Woolley *et al*, 1987; McWilliam and Woolley, 1988). Thus more studies are required to determine if the separation of VPNs in the medulla is due to functional differences and is related to their axonal conduction velocities.

3:4.5. Implications for Future Experiments.

Three main implications for future neuro-anatomical studies on VPNs arise from these experiments. The first is that CVPNs share common morphological characteristics with VPNs

innervating other target organs, and thus should be identified in future studies by retrograde labelling from the heart. Secondly, VPNS in the NA have similar ultrastructural characteristics to neurones in the NA projecting to the phrenic motor nucleus and thus must be positively identified in future studies prior to further investigations being carried out on them. Thirdly, VPNS in the rat appear to have little synaptic input onto their cell bodies and future studies aimed at investigating their inputs must use a method of identification capable of visualising their dendrites.

The intermingling of VPNS with different functions with neurons projecting to the phrenic motor nucleus also has implications for future physiological experiments. Chemical or electrical stimulation of the VLM may activate many different types of neurone, making results difficult to interpret.

3:4.6. Summary

Vagal preganglionic neurones in the rat were organised with respect to their sizes, but all displayed similar ultrastructural characteristics. A population of VPNS in the medulla that were probably cardiac vagal preganglionic neurones were labelled following injections of CT-HRP directly into the myocardium. Ultrastructural examination of CVPNS revealed that they were identical to other VPNS. Neurones retrogradely labelled from the phrenic motor nucleus were intermingled with VPNS in the NA, especially the vINA. Ultrastructural examination of neurones in all the identified groups revealed that they shared similar characteristics.

Chapter Four.

Demonstration of 5-HT immunoreactive terminals in synaptic contact with cardiac vagal preganglionic neurones in the nucleus ambiguus of the rat.

Section 4.1. - Introduction.

Pharmacological evidence suggests that the neurotransmitter 5-hydroxytryptamine (5-HT) has a role in the control of cardiac vagal preganglionic neurones (Dalton, 1986; Ramage and Fozard, 1987; Izzo *et al*, 1988). Central administration of 5-HT in conscious rats caused a profound bradycardia which was prevented by the prior administration of N-methylatropine and was thus mediated through the parasympathetic nervous system (Dalton, 1986). This bradycardia may have been mediated through actions on 5-HT_{1a} receptors since the 5-HT_{1a} receptor agonist 8-OHDPAT applied intravenously in cats caused an immediate bradycardia which was prevented by vagotomy (Ramage and Fozard, 1987). These actions may be directly on CVPNs as micro-injection of 8-OHDPAT into the nucleus ambiguus of the cat resulted in a bradycardia (Izzo *et al*, 1988). However, there is no evidence that the actions of 5-HT are directly on cardiac vagal preganglionic neurones.

Despite pharmacological evidence that 5-HT alters CVPN output, anatomical evidence that CVPNs are directly innervated by neurones utilising 5-HT as a neurotransmitter is sparse. Although 5-HT_{1A} receptors are dense in the NA (Pazos and Palacios, 1985) and 5-HT immunoreactive fibres are present in moderate densities (Steinbusch, 1981), the identity of the neurones which express these receptors and are innervated by 5-HT containing axons remains unknown.

Thus although pharmacological studies have shown that 5-HT alters the activity of cardiac vagal preganglionic neurones, there is no evidence that this is through a direct effect. Therefore, the aim of this study was to investigate if 5-HT neurones directly innervate CVPNs. This was undertaken by combining the method of retrograde labelling of CVPNs developed in chapter 3 with 5-HT immunocytochemistry and electron microscopy to determine if 5-HT containing axons innervate CVPNs.

Section 4:2. - Methods.

4:2.1. Retrograde Labelling.

CVPNs were retrogradely labelled in 8 male Sprague Dawley rats (220-280g) by the injection of CT-HRP into the myocardium as described in section 3.2. Following 24-48 hours survival the rats were perfused with a fixative containing 0.5% glutaraldehyde and 3% paraformaldehyde in 0.1M PB at 4°C for 20 minutes. The brains were removed, post-fixed for 1-3 hours, rinsed in PB and transverse medullary sections (60-80µm) cut on a vibrating microtome. In addition, some rats were perfused for control purposes, using the same fixative.

4.2.2. HRP Histochemistry

Retrogradely transported CT-HRP was localised using the TMB reaction at pH 6.0 (Marfurt *et al*, 1988) described in section 2:2.2. Some sections were directly processed for microscopic examination while most were further processed to immunocytochemically localise 5-HT.

4:2.3. Immunocytochemistry.

Immunocytochemistry was carried out using rat monoclonal antibodies directed against 5-HT (Serotec YC5/HLK). Sections were incubated in a PBS solution containing the primary antibody at a dilution of 1 in 12,000, and with 0.1-0.3% triton added. The primary antibody was detected using a rabbit anti-rat antibody conjugated

to HRP at a dilution of 1:100 in PBS. The immunocomplex was localised using the DAB reaction (section 2:2.1)

4:2.4. Specificity and Controls. (See also Appendix One: p. 288).

The specificity of the 5-HT antisera has been documented previously (Consolazione *et al*, 1981). In addition, incubation of the sections with 5-HT antibodies resulted in staining of axonal fibres, perikarya and dendrites in the medulla identical to that described in other studies (Steinbusch, 1981). In sections incubated in the same manner but with the omission of 5-HT antibody no staining occurred. Thus staining obtained using the 5-HT antibody is specific to the serum and not a product of other steps in the procedure.

In material with retrograde reaction product only, labelled structures in the NA contained a black punctate reaction product. There was no afferent labelling in the NA which could be confused with immunocytochemically labelled boutons. Thus, in material with combined retrograde labelling and immunostaining, labelled axons in the NA were 5-HT immunopositive and labelled cells were CVPNs.

4:2.5. Light and Electron Microscopy.

Following histochemical reactions to localise retrograde labelling or immunocytochemistry, sections were washed in 0.1M PB, post fixed in 1% osmium tetroxide, dehydrated and embedded in resin and mounted on slides as described in section 2.5. All sections were examined in the light microscope for immunostained fibres and

boutons and for retrogradely labelled structures in the area of the immunostained fibres. High power light microscopic examination was used to determine if immunocytochemically labelled boutons and retrogradely labelled structures were in close apposition.

Retrogradely labelled neurones which were identified as having apposing immunolabelled boutons were photographed and the photographs used to correlate the selected neurones at the light and electron microscopic levels. At the electron microscopic level the ultrathin sections were examined for correlated structures, as well as other non-correlated labelled structures.

Section 4:3. Results.

4:3.1. Immunocytochemistry.

At the light microscopic level positively stained 5-HT immunoreactive structures displayed the typical brown amorphous appearance of DAB reacted immunocytochemical staining. The distribution of 5-HT cell bodies and fibres in the medulla was identical to that described previously by Steinbusch (1981): labelled neurones were observed mainly in the caudal raphe nuclei, with a few scattered neurones extending ventrolaterally above the inferior olives. 5-HT immunoreactive fibres were observed in all regions of the medulla. The greatest density of fibre staining was in the DVN, NTS, the ventrolateral NA and the reticular formation between these areas. There were few immunoreactive fibres within the cNA.

The depth of immunostaining varied from animal to animal, in different areas in any one animal, with the glutaraldehyde concentration of the fixative and the amount of detergent added to the incubation medium. With the addition of triton to the incubation medium, penetration was almost throughout the 60-80 μ m sections, although much denser near the surface. Due to the limited penetration of staining, immunoreactive fibres could not be traced back to the cell of origin.

At the electron microscopic level labelled terminals were identified by the electron dense peroxidase reaction product associated with the vesicles and other structures within the boutons (figure 4.1). Twenty-six boutons forming synaptic specialisations with unidentified structures were observed in the NA (fig. 4.1). Twenty of these were with dendritic structures, five with spine-like processes and only one with a cell soma. In some cases these synapses could clearly be identified as asymmetric with the presence of sub-junctional bodies and round vesicles (Figure 4.1). Unlabelled boutons formed symmetric and asymmetric synapses with these structures (figure 4.1).

4:3.2. Retrograde Labelling and Immunocytochemistry.

In sections that contained both immunostained and retrogradely labelled structures, brown amorphous 5-HT immunopositive structures were sometimes apposed to retrogradely labelled structures identified by black punctate reaction product within the cytoplasm (figures 4.2). In material prepared only for light microscopy by the addition of high concentrations of triton to the antibody incubation medium, 5-HT immunoreactive fibres formed many close associations along the length of the dendrites and only occasionally with the cell bodies (figure 4.2). The number of immunoreactive boutons associated with retrogradely labelled cells were fewer in number in material prepared for electron microscopy (compare 4.2. and 4.3). As a consequence of this limited penetration of immunostaining, 5-HT containing boutons were observed in close apposition only to parts of retrogradely labelled neurones near the surface of the sections (figure 4.4).

Retrogradely labelled CVPNs were easily identified at the ultrastructural level as they contained electron dense TMB crystalline reaction product (figures 4.3 and 4.4). From three rats eight retrogradely labelled CVPNs with apposing boutons were studied at the ultrastructural level, six from the vINA and two from the intermediate zone. Correlated light and electron microscopic examination of retrogradely labelled VPNs with apposing immunoreactive boutons revealed that 5-HT boutons formed asymmetric type synaptic associations mainly with retrogradely labelled dendrites (figure 4.3). Thirty-five 5-HT immunoreactive synaptic specialisations were identified with retrogradely labelled dendritic structures, three with somatic spines and three with cell perikarya (Fig. 4.4). Some immunoreactive synaptic contacts with retrogradely labelled cells could be clearly distinguished as asymmetric with associated sub-junctional bodies and when vesicles were visible they appeared round (figure 4.3. and 4.4). Unlabelled boutons forming symmetric and asymmetric synapses were also observed in synaptic contact with labelled CVPNs (figs. 4.3 and 4.4).

Figure 4.1. Electron micrographs of 5-HT immunoreactive boutons in the NA which form synaptic contacts (arrows) of the asymmetric type with unidentified structures. Sub-junctional bodies are associated with the post-synaptic membranes in **B** and **D**. In **B** the star indicates an unlabelled bouton which also forms an asymmetric synaptic specialisation with the same structure as the immunoreactive profile.

Common scale bar in **C** = 0.5 μ m.

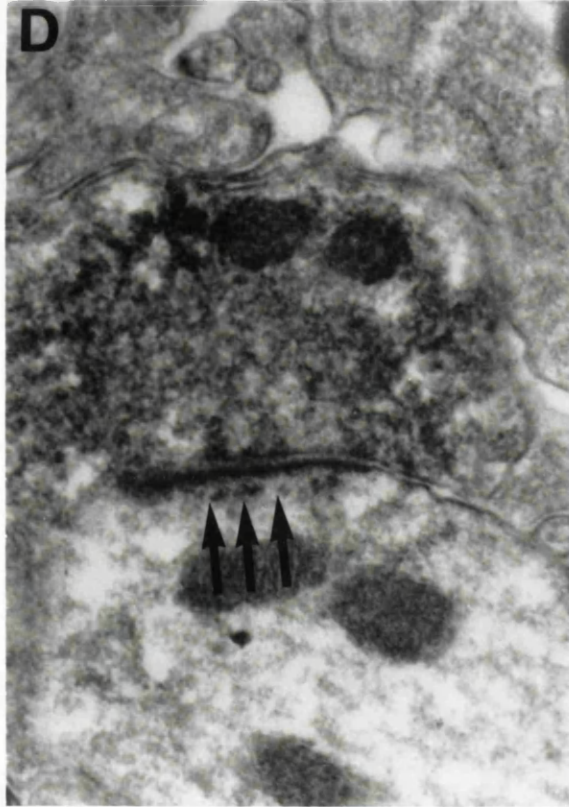
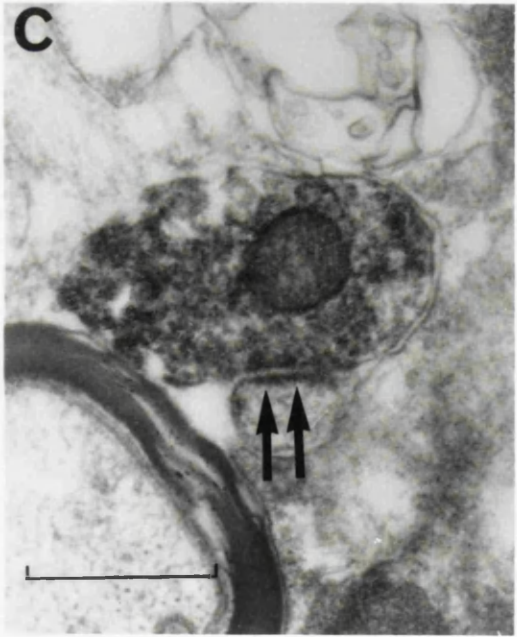
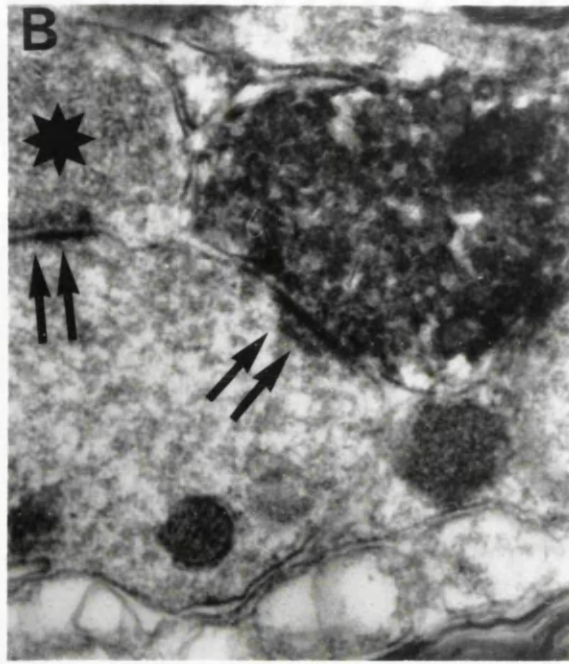


Figure 4.2. Photo-montages of light micrographs containing retrogradely labelled cardiac vagal preganglionic neurones which have numerous apposing immunoreactive terminals (arrows) apposing their membranes and which seem to run along their dendrites. The cell in **A** is from the ventral medulla and that in **B** from the intermediate zone.

Common scale bar in **A** = 25 μ m.

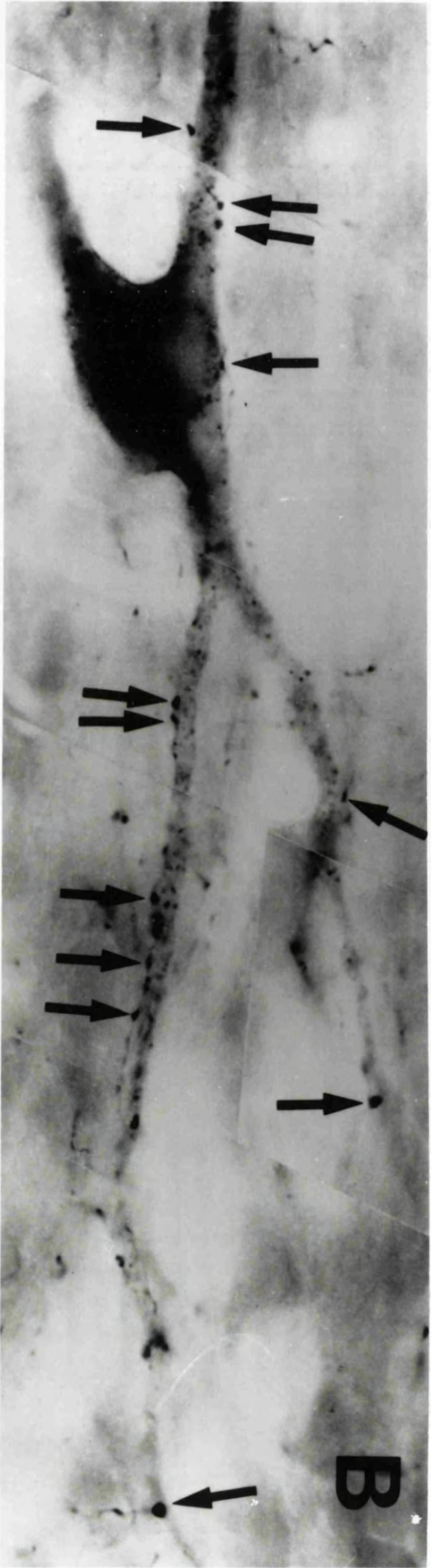
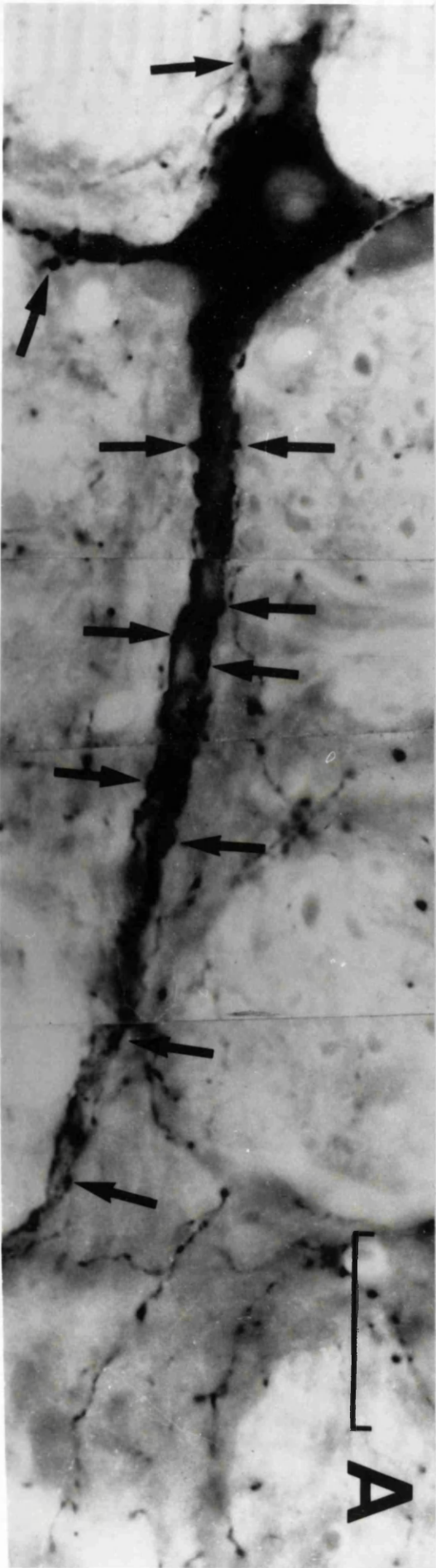


Figure 4.3. Light and electron microscopic photomicrographs of a retrogradely labelled CVPN with apposing 5-HT immunoreactive boutons.

A. A light micrograph montage of a retrogradely labelled cardiac vagal preganglionic neurone with apposing 5-HT immunoreactive boutons indicated by the arrows. B1 and B2 indicates two immunoreactive boutons shown at the ultrastructural level in later photographs. C1-C4 are blood vessels used to locate the cell in B.

B. Montage of electron micrograph of the cell in A. C1 - C4 are the same blood vessels indicated in A.

C. Higher power montage of area below C1 in B. The broad arrows point to reaction product in the cytoplasm of a piece of dendrite indicating that it is retrogradely labelled. The thin arrows point to immunoreactive B1 and B2 apposing the retrogradely labelled dendrite.

D & E. High power electron micrograph demonstrating B1 (**D**) and B2 (**E**) forming synaptic specialisations with the retrogradely labelled structure.

F. Immunoreactive and non-immunoreactive (shown by star) boutons in synaptic contact with the cell at another point which is not correlated.

Scale Bars: A. 25 μ m B. 25 μ m C. 3 μ m

D, E and F: Common scale bar in F = 0.5 μ m.

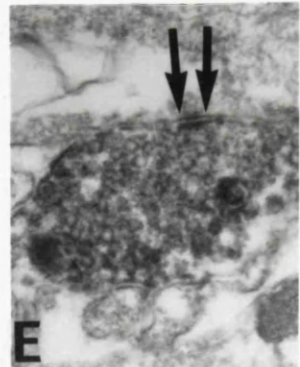
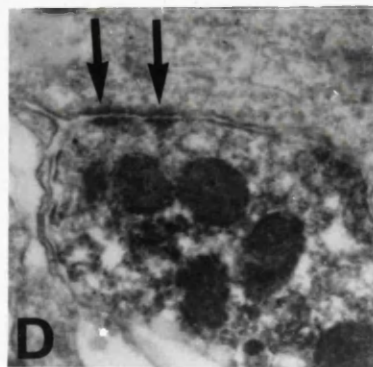
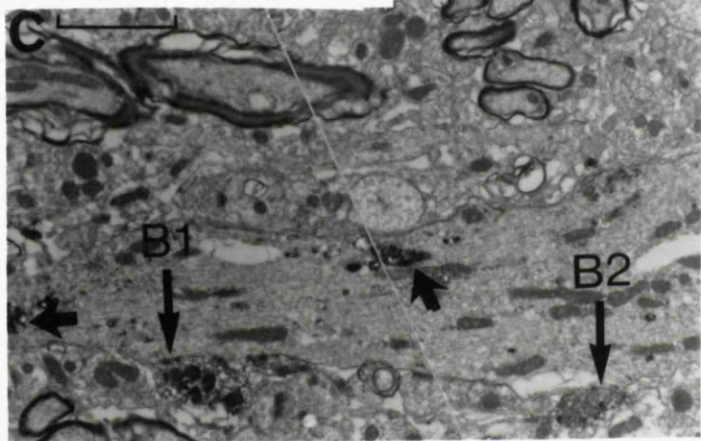
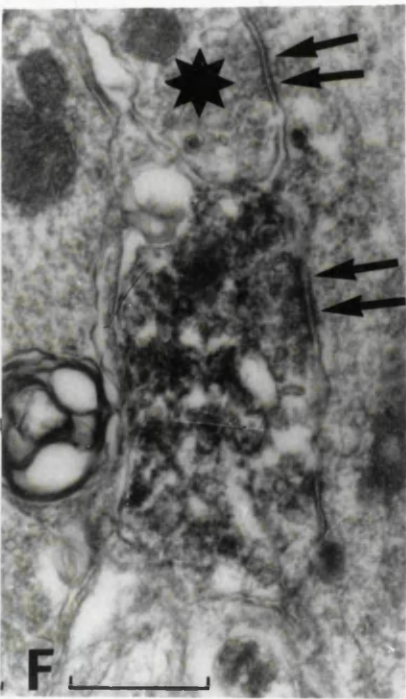
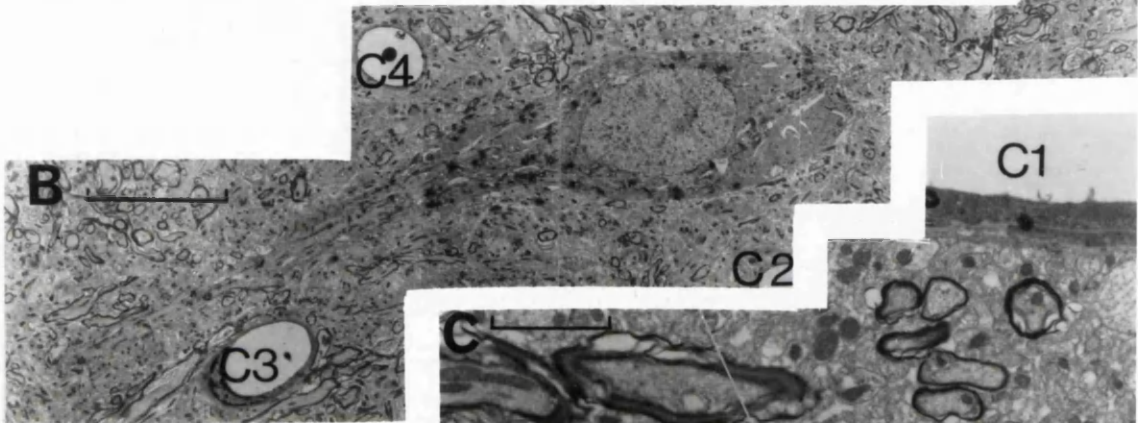
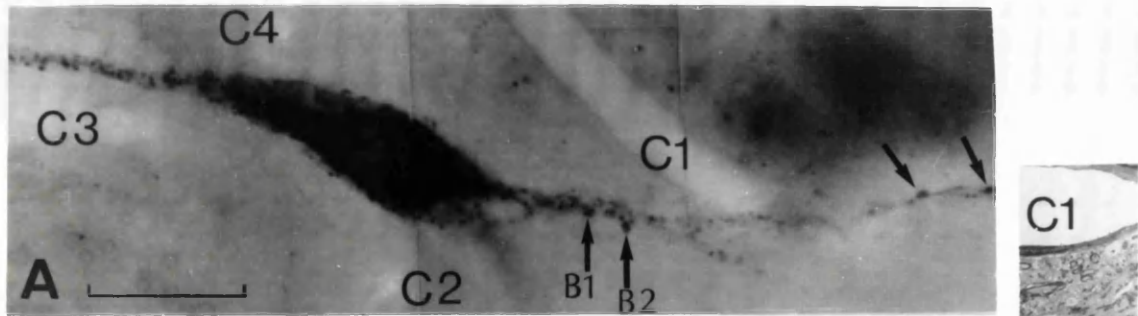
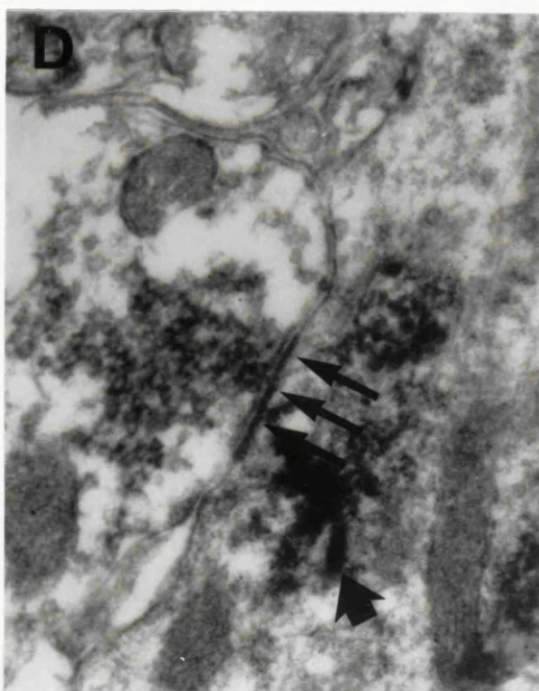
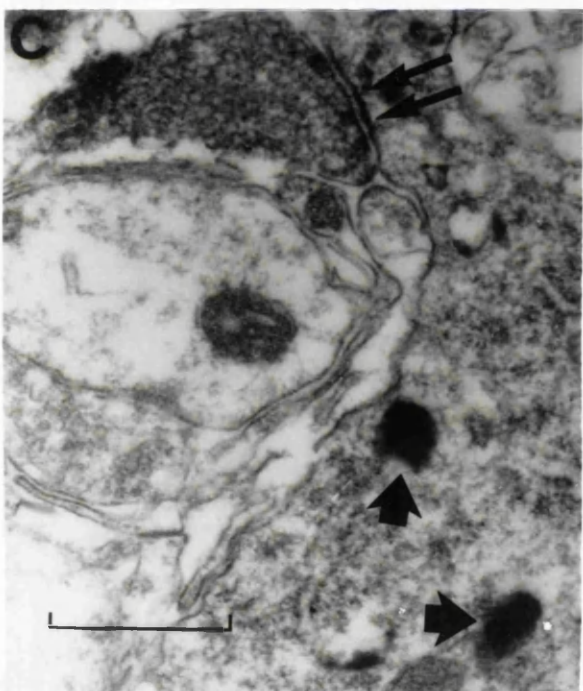
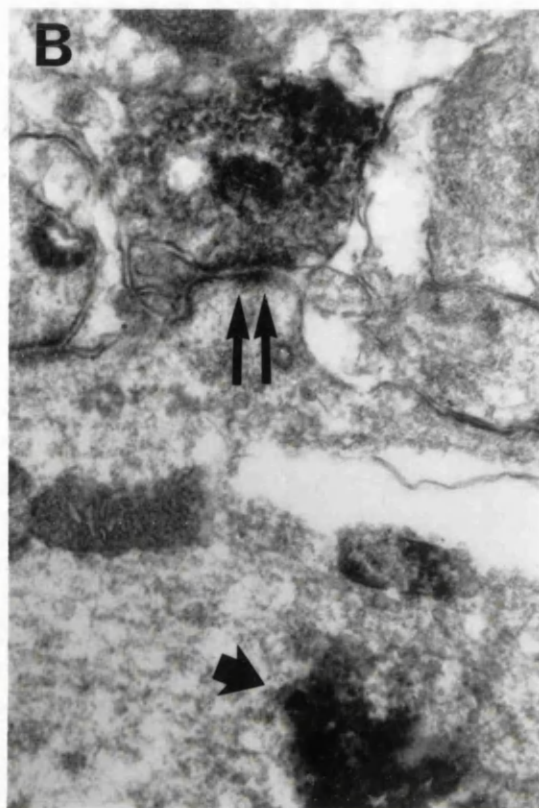


Figure 4.4. Photomicrographs of 5-HT immunoreactive boutons in synaptic contact (thin arrows) with structures in the NA identified as cardiac vagal preganglionic neurones since they contain retrograde reaction product (thick arrows) following the injection of CT-HRP into the wall of the right atrium. The boutons in **B** and **C** are in synaptic contact with somatic spines while those in **A** and **D** are in contact with dendrites. The boutons in **C** and **D** are clearly forming asymmetric connections whereas the morphology of the synapses is not so easily determined in **A** and **B**.

Common scale bar in **C** = 0.5 μ m.



Section 4:4. Discussion.

The experiments in this chapter combined the technique developed in chapter 3 to retrogradely label cardiac vagal preganglionic neurones with immunocytochemistry and electron microscopy to demonstrate that the neurotransmitter 5-HT was contained in boutons in synaptic contact with CVPNs.

The combination of immunocytochemistry and retrograde labelling to the electron microscopic level was subject to some technical limitations. Good ultrastructural preservation required high concentrations of glutaraldehyde in the fixative, and this restricted the penetration of antibodies into the tissue. Addition of the detergent triton X-100 to the incubation medium enhanced antibody penetration, but to the detriment of ultrastructural preservation. Consequently, detergent concentration was kept low (0.1%) for electron microscopic studies and false negative staining undoubtedly occurred *i.e.* unlabelled boutons may have contained the antigen but this was not detected. This is strikingly illustrated by the far greater number of 5-HT immunoreactive boutons observed in close apposition to retrogradely labelled CVPNs in material prepared with the addition of 0.3% triton to the primary antibody incubation medium (fig. 4.2) than that prepared with only 0.1% triton (fig. 4.3.A). As a consequence of this limited penetration of immunochemicals in material prepared for electron microscopy, examination was restricted to the surface of the section where morphological details were less well preserved than deeper layers. However, synaptic specialisations were visible.

The material prepared for light microscopy indicates that the number of 5-HT containing boutons innervating each CVPN neurones may be high. In addition, these sections illustrated that most of the 5-HT input to CVPNs was onto dendrites. This agrees with the location of 5-HT boutons observed in other studies which show that 5-HT containing boutons synapse mainly on dendrites and infrequently on somata in other regions of the CNS (see Maley *et al*, 1990).

Although the experiments in this study identified CVPNs as a post-synaptic target of 5-HT containing boutons, neurones in the ventrolateral medulla with other functions may also be innervated by 5-HT. Boutons containing 5-HT immunoreactivity have been shown to appose neurones in the NA which have axons in the recurrent laryngeal nerve (Holtman, 1988, 89), although no electron microscopy was carried out to establish synaptic associations. In addition, 5-HT boutons are closely associated with adrenergic neurones in the C1 region of the rostral ventrolateral medulla (Nicholas and Hancock, 1988), but again synaptic associations were not verified by electron microscopic examination. Thus more work involving electron microscopic examination is required to determine which neurones in the ventrolateral medulla are innervated by 5-HT containing boutons.

4:4.1. Possible Source of the 5-HT Innervation of CVPNs.

It was not possible to trace 5-HT immunoreactive fibres to their origin due to limited penetration of immunocytochemical reagents. Furthermore, the soma of origin may have been outside the medulla

oblongata in areas not examined. Thus the evidence for the source of the 5-HT input to CVPNs must come from indirect studies.

Retrograde tracing from the VLM has identified projections from areas which contain 5-HT neurones such as the caudal raphe nuclei (Bockstaele *et al*, 1989 in rat; Smith *et al*, 1989 in cat), the periaqueductal gray (PAG; Bockstaele *et al*, 1989 and Carrive *et al*, 1988 in rat; Lovick, 1985 in cat) and from other areas of the ipsilateral and contralateral ventral medulla (Ellenberger and Feldman, 1990). However, few double labelling studies have been carried out to determine if neurones retrogradely labelled from the NA contain 5-HT immunoreactivity. In addition, since CVPNs in the vINA are intermingled with neurones with other functions (see chapter 3), such retrograde tracing studies do not identify CVPNs as post-synaptic targets of retrogradely labelled neurones.

Evidence to date suggests that 5-HT containing neurones in the medullary raphe nuclei project to the ventrolateral medulla, and which thus may innervate CVPNs since injections of anterograde tracers into the medullary raphe nuclei has resulted in labelling in the ventral medulla, although CVPNs were not identified as post-synaptic targets (Bobillier *et al*, 1976; Nicholas and Hancock, 1990). In addition, 5-HT immunoreactive neurones in the medullary raphe were retrogradely labelled following pressure injection of rhodamine beads into the VLM (Connelly *et al*, 1989; Holtman *et al* (1988) However the possibility of labelling fibres of passage due to the physical damage caused by the pressure injections cannot be excluded in these experiments, and double labelled neurones in the raphe may be a false positive result.

4:4.2. Functional Implications.

Since 5-HT containing boutons form asymmetric type synapses with CVPNs, and asymmetric synapses have been associated with excitatory functions (Gray, 1973), the results in this study provide anatomical support for direct excitation of CVPNs by 5-HT (Dalton, 1986; Izzo *et al*, 1988). However, in other areas of the brain 5-HT has also been reported in boutons forming symmetric synapses (Maley *et al*, 1988) and it is possible that these also occur in the VLM. In this study, these may not have been evident due to the fixation parameters necessary to allow antibody penetration, which leads to deterioration in ultrastructural preservation. Alternatively, post-synaptic membrane thickenings may have been obscured by the peroxidase reaction product, which often runs along the membrane of labelled neurones when CT-HRP is used as a retrograde tracer (see chapter 3). However, if they exist, boutons forming symmetric synapses and containing 5-HT are likely to occur infrequently in the NA since only asymmetric associations were observed in the study of random boutons in this study.

If all the boutons observed apposing CVPNs in material prepared for light microscopy were synaptic boutons, then 5-HT innervation of CVPNs appears to be very dense, suggesting an important functional role for 5-HT in the control of heart rate. Furthermore, central administration of 5-HT elicits an immediate vagally mediated bradycardia (Dalton, 1986; Ramage and Fozard, 1987), suggesting that it is involved in beat-to-beat regulation of heart rate. However, further experiments are required to determine if the ionophoresis of 5-HT antagonists onto CVPNs inhibits their firing

rate, which would demonstrate that 5-HT has a tonic action on CVPNs which is active in physiological conditions.

4:4.3. Summary.

Immunocytochemistry and electron microscopy revealed that 5-HT immunoreactivity was contained in boutons in synaptic contact with neurones in the nucleus ambiguus, and combination of these techniques with retrograde labelling revealed that some of these post-synaptic targets were CVPNs.

Chapter Five.

Demonstration of substance P immunoreactivity in boutons in synaptic contact with vagal preganglionic neurones in the NA, identified by retrograde labelling from the heart or the cervical vagus nerve of the rat.

Section 5:1 Introduction.

Since the discovery of the undecapeptide substance P (von Euler *et al*, 1931) its functions have been extensively investigated and it appears to have neurotransmitter or neuromodulator effects in the central nervous system (for reviews see Nicoll *et al*, 1980 and Pernow, 1983). Autoradiographic receptor localisation studies have shown a high density of substance P receptors in the NA of the rat (Helke *et al*, 1984). These receptors have been located on vagal preganglionic neurones, since unilateral vagotomy reduced the number of substance P receptors in the NA by up to 78% when compared to the intact side (Helke *et al*, 1984, 85). In addition, substance P immunoreactive fibres are located in the NA (Cuello and Kanawaza, 1978; Ljungdahl *et al*, 1978), although the post-synaptic targets of these fibres are not known.

Recent pharmacological studies have shown that substance P may be involved in the control of vagal outflow (Haxhiu *et al*, 1989; Urbanski *et al*, 1988; Agarwal and Calaresu, 1991). Microinjection of the substance P agonist [pGlu⁵, MePhe⁸, Sor⁹]-SP(5-11) into the ventrolateral medulla of anaesthetised rats results in a depressor response associated with a decrease in heart rate which is prevented by application of intravenous atropine or vagotomy, indicating that its mediated through excitation of CVPNs (Urbanski *et al*, 1988). Furthermore, microinjection of substance P into the NA of spinalised rats elicits a bradycardia which can only be through vagally mediated effects (Agarwal and Calaresu, 1991). Substance P also increases vagal output to the airways since application to the ventral medullary surface in chloralose anaesthetised cats

increased tracheal muscle tension which was blocked by prior intravenous administration of atropine (Haxhiu *et al*, 1989).

Thus although anatomical studies have located substance P receptors on vagal preganglionic neurones in the NA of the rat, the target organs of these VPNS have not been identified. In addition, it is not clear if the pharmacological effects of substance P are via direct or indirect actions. Thus the experiments in this chapter were carried out to determine if there is an anatomical substrate for direct actions of substance P on VPNS. This was investigated by combining the methods developed in chapter 3 to retrogradely label VPNS from the heart or the cervical vagus nerve in the rat with immunocytochemistry for substance P and electron microscopy.

Section 5:2 Methods.

5:2.1. Retrograde Labelling

HRP was injected into the cervical vagus nerve in 6 male Sprague Dawley rats (220-350g) as described in section 2:1.1.A. In another 4 rats CT-HRP was injected into the myocardium as described in section 3:2.2. Following 24-72 hours recovery, animals were perfused transcardially with 750ml of fixative containing 0.1-0.4% glutaraldehyde and 3% paraformaldehyde in 0.1M PB for 20 minutes at 4°C. In addition, some rats were perfused with the same fixative for immunocytochemistry only. The brains were removed and 60-80µm transverse medullary sections cut on a vibrating microtome.

Retrogradely transported HRP and CT-HRP was localised using the TMB or 0-tolidine reactions described in sections 2:2.2 and 2:2.3. The sections were rinsed in PBS and some were dehydrated and embedded on microscope slides for control purposes, while the majority were processed for immunocytochemistry. Sections from non-injected rats were also processed for immunocytochemistry.

5:2.2. Immunocytochemistry.

Immunocytochemistry was carried out using polyclonal antibodies raised in rabbit against substance P (Eugene Tech. Int.). The sections were incubated in primary antibody (1:15,000), made up in PBS with the addition of 0.1% triton, for 12-72 hours at 4°C on a shaker. They were subsequently incubated in swine anti-rabbit antibody conjugated to HRP (1:100 in PBS; Dakopatts) for 3-6 hours.

The immunocomplex was then detected using the DAB reaction described in section 2:2.1.

5:2.3. Specificity and Controls. (See also Appendix One: p. 288).

The antisera has been shown to be specific to substance P as all staining was blocked by simultaneous incubation of rat brain tissue with substance P ($0.1\mu\text{M}$ - $10\mu\text{M}$) and antibody (information supplied by Eugene Tech Int.). In addition, the distribution of labelled fibres in the medulla obtained with incubation in substance P antibodies (see below) was similar to that described by Cuello and Kanawaza (1978) and Ljungdahl *et al* (1978). In sections incubated in the same way but with the omission of the substance P antibody no staining occurred. Substitution of substance P antibody with an antibody raised in rabbit and directed against neuropeptide Y produced a markedly different pattern of staining to that obtained with substance P. Thus the staining obtained is specific to the antiserum and stains substance P containing structures.

In sections incubated to reveal only retrogradely labelled structures there was no afferent labelling in the NA which could be confused with immunostained fibres. Retrogradely labelled cells contained punctate reaction product. As no substance P immunoreactive cells were found in sections with immunostaining only, labelled cells in sections with immunostaining and retrograde labelling were inferred to be retrogradely labelled. Thus it could be safely assumed that labelled axons in the NA were a result of immunostaining and labelled cells were VPNS.

5:2.4. Light and Electron Microscopy.

Following histochemical reactions to localise retrograde labelling or immunocytochemistry, sections were washed in 0.1M PB, post fixed in 1% osmium tetroxide, dehydrated and embedded in resin and mounted on slides as described in section 2.5. All sections were examined in the light microscope for immunostained fibres and boutons and for retrogradely labelled structures in the area of the immunostained fibres. High power light microscopic examination was used to determine if immunocytochemically labelled boutons and retrogradely labelled structures were in close apposition. Retrogradely labelled neurones which were identified as having apposing immunocytochemical boutons were photographed and the photographs used to correlate the selected neurones at the light and electron microscopic levels. At the electron microscopic level, the ultrathin sections were searched for correlated and non-correlated labelled structures.

Section 5:3 - Results.

5:3.1. Immunocytochemistry.

Substance P immunoreactivity was identified only in axonal fibres and boutons. Positively stained substance P structures displayed the typical brown amorphous characteristic of DAB reacted immunocytochemical staining. Immunoreactive varicose fibres and punctate structures were very dense in the DVN and the NTS. In the DVN fibres surrounded the medium sized perikarya. In the ventral medulla staining was dense in the loNA and scNA and very dense in the region of the vNA. Cells and dendrites in these parts of the NA appeared to be completely surrounded by immunoreactive fibres. Within the cNA, fibres and terminals were sparse. Immunoreactive staining was visible to a depth of approximately 20 μ m, although it was much denser near the surface of the material. Immunostaining was observed to a greater depth in material that was less well fixed or which was incubated with high concentrations of detergent.

At the electron microscopic level immunoreactive fibres and punctate structures observed in the NA in the light microscope were identified as vesicle containing axons and boutons. Labelled boutons contained amorphous and electron dense peroxidase reaction product which was associated with the vesicles and other structures within the bouton (fig. 5.1). In five rats, twenty seven labelled boutons were identified as forming synaptic specialisations with dendritic structures in the NA. These synapses were always asymmetric and occasionally were associated with sub-junctional bodies (Figure 5.1.).

5:3.2. Retrograde Labelling and Immunocytochemistry.

In sections that contained both immunostained and retrogradely labelled structures, substance P immunoreactive boutons were seen in association occasionally with perikarya, but more often dendrites, of retrogradely labelled neurones (figures 5.2 and 5.4). These associations were observed only at the surface of the sections due to limited penetration of immunocytochemical reagents. In material that was less well fixed or which was incubated with high concentrations of detergent there was increased penetration of immunostaining, and more substance P immunoreactive boutons were observed in apposition to retrogradely labelled structures (fig. 5.4.A).

At the electron microscopic level the reaction product for retrogradely transported HRP was identified only in cells and the immunoreaction only in boutons. Six substance P immunoreactive boutons observed at the light microscopic level in apposition to four VPNS in the NA retrogradely labelled from the cervical vagus nerve in four rats were examined at the electron microscope. These formed asymmetric synaptic specialisations with the VPNS, sometimes with associated sub-junctional bodies (figure 5.2.). Further random ultrastructural examination revealed another 14 substance P immunoreactive boutons with asymmetric type synaptic specialisations, sometimes with associated sub-junctional bodies, with eleven retrogradely labelled VPNS (figure 5.2. and 5.3.). These synapses were mainly observed on the proximal dendrites of the labelled cells but two were observed in contact with somata.

Substance P immunoreactive boutons were also observed in close apposition to neurones in the vNA which were retrogradely labelled by the injection of CT-HRP into the heart (figure 5.4). Correlated light and electron microscopic examination of three boutons from two rats revealed them to form asymmetric synaptic specialisations associated with sub-junctional bodies with two retrogradely labelled CVPNs (figure 5.4).

Figure 5.1 Electron micrographs of substance P immunoreactive boutons in synaptic contact (shown by black arrows) with dendritic structures of unidentified origin in the NA. All these synapses are of the asymmetric type and are associated with sub-junctional bodies in **B**, **C** and **D**. The synapse in **A** has no subjunctional bodies evident at any level but has an asymmetric type thickening of the post synaptic membrane. An unlabelled bouton is shown in **B** (star) in asymmetric synaptic contact with the same dendritic structure as the labelled bouton.

Common scale bar in **D** is 0.5 μ m.

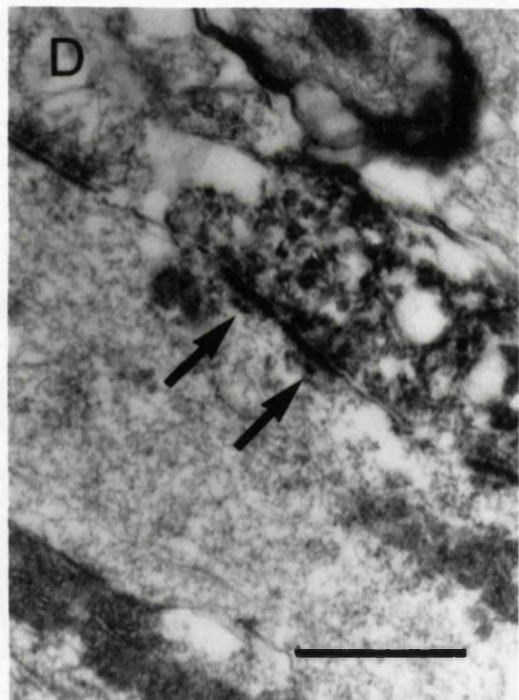
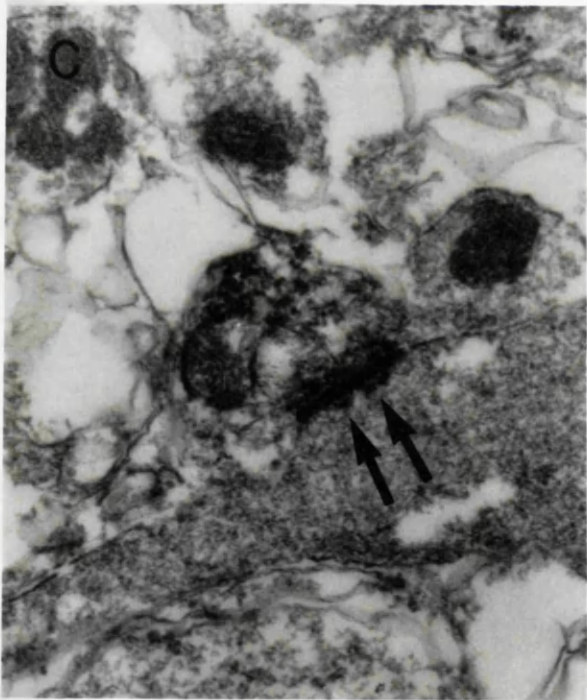
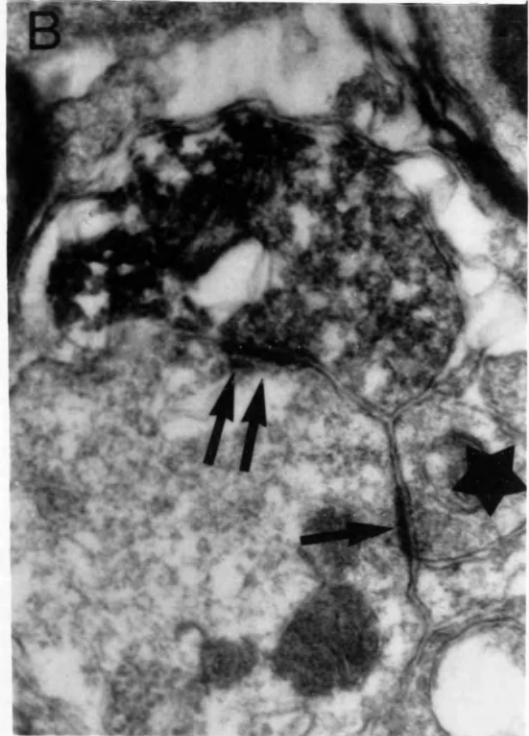
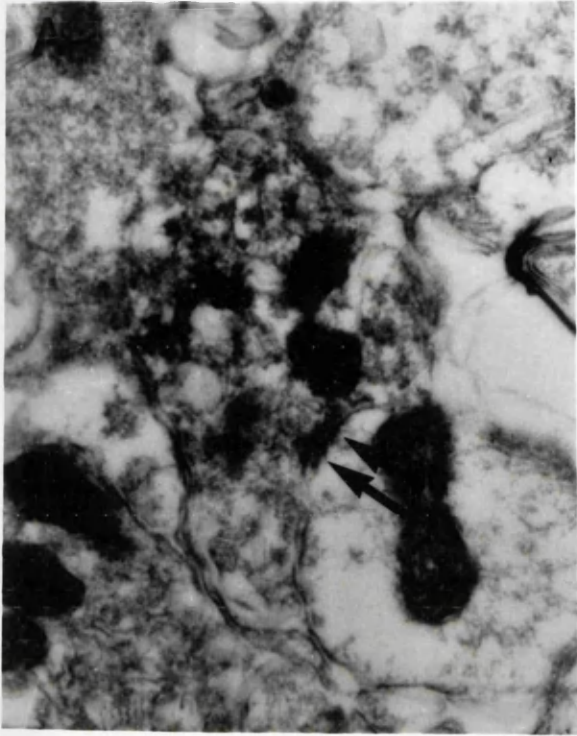


Figure 5.2. Light and electron microscopic correlation of a substance P immunoreactive bouton observed apposing a retrogradely labelled vagal preganglionic neurone in the nucleus ambiguus.

A. Low power micrograph of labelled vagal preganglionic neurones in the nucleus ambiguus. The dendrite of the neurone labelled by the arrow is shown in **B**.

B. High power light micrograph of the dendrite of the retrogradely labelled VPN shown in **A** at a different focal depth. A substance P immunoreactive bouton can be seen apposing the cell membrane (arrow). The glial cell (G) and capillary (C) are used for correlation in **C**.

C. Low power electron micrograph of the area shown in **B** where C is the same capillary and G the same glial cell. Open arrows point to TMB reaction product in the dendrite which identifies the dendrite as retrogradely labelled. M is a myelinated structure used to correlate with **D**.

D. High power electron micrograph showing a substance P immunoreactive bouton forming an asymmetric type synapse (arrows) with the dendrite of the retrogradely labelled VPN. The open arrow points to the TMB reaction product which identifies the post synaptic target as a retrogradely labelled vagal preganglionic neurone. M designates the same myelinated structure as in **C**.

Scale bars: **A.** 50 μ m **B.** 20 μ m **C.** 4 μ m **D.** 0.5 μ m

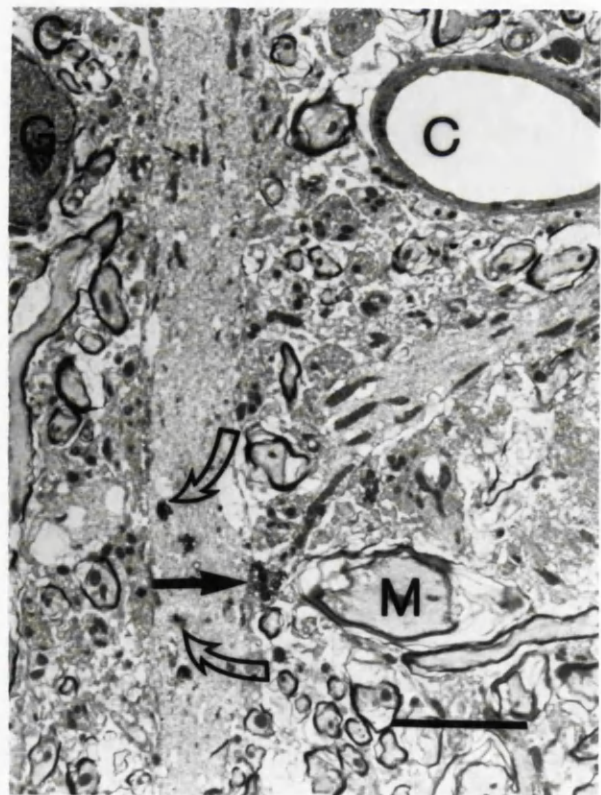
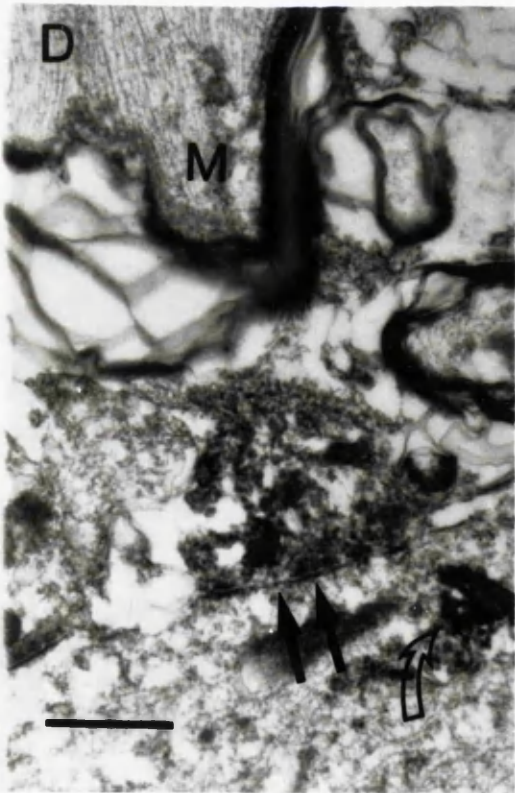
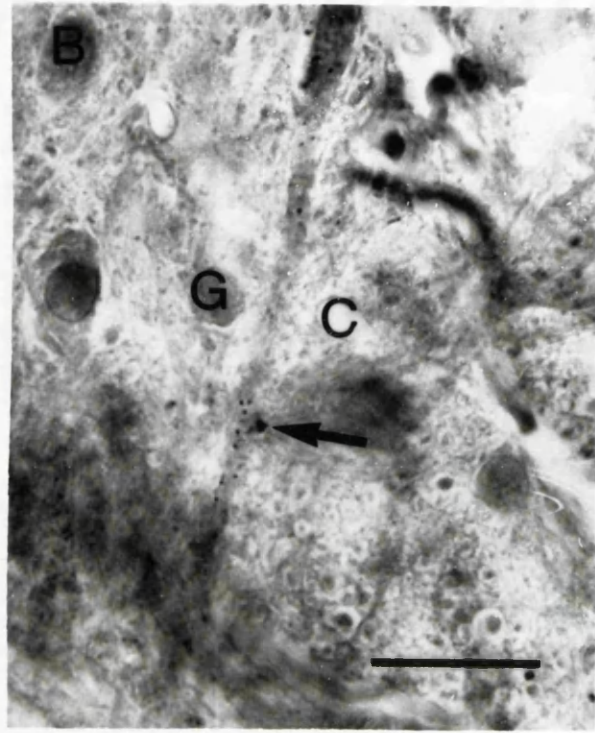
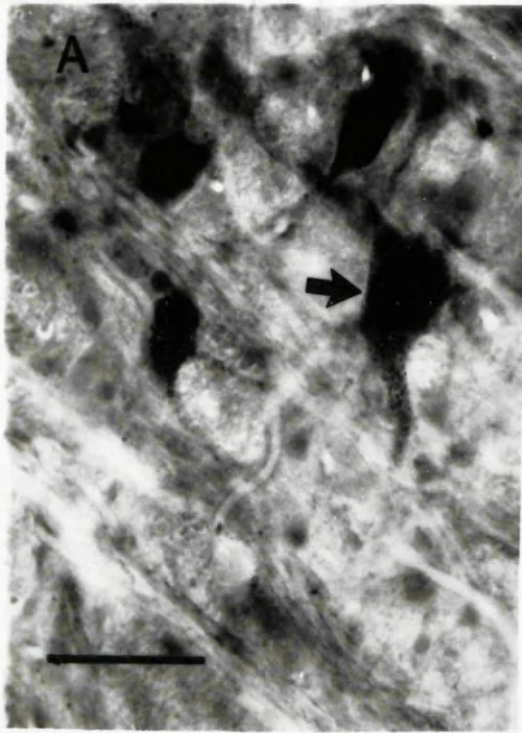


Figure 5.3. Substance P immunoreactive boutons in synaptic contact (black arrows) with VPNs, identified by TMB reaction product in the cytoplasm (open arrows) following retrogradely labelled from the cervical vagus nerve. All of the synaptic specialisations are of the asymmetric type but sub-junctional bodies are only evident in **C**.

Common scale bar in **C** = 0.5 μ m.

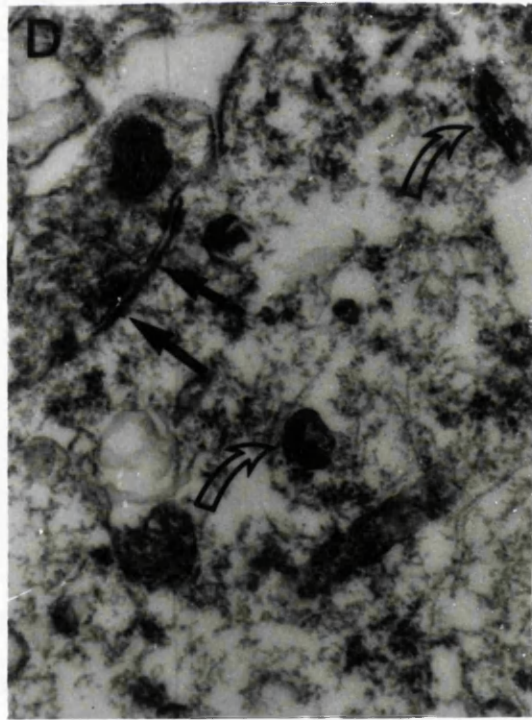
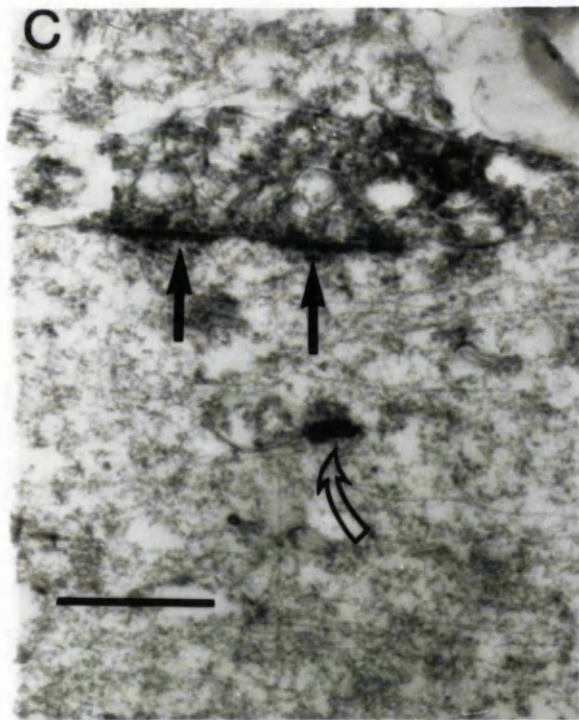
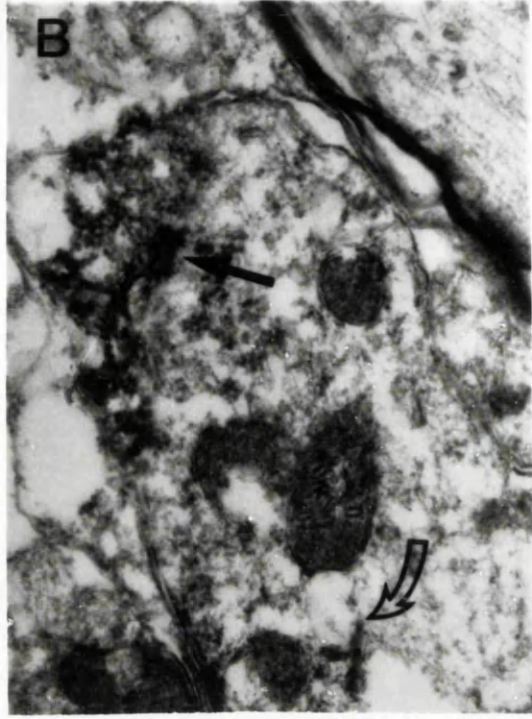
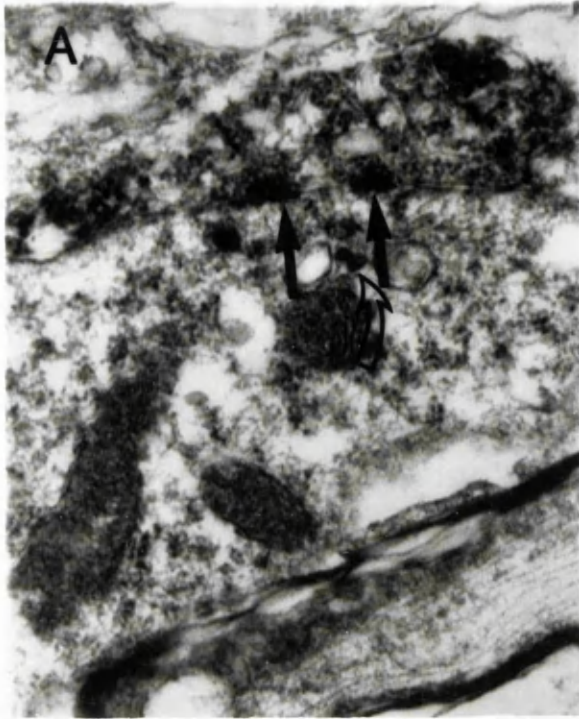


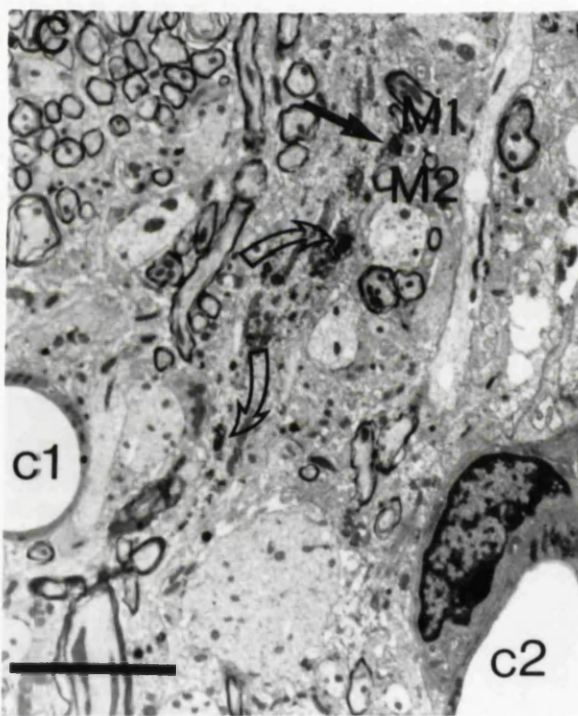
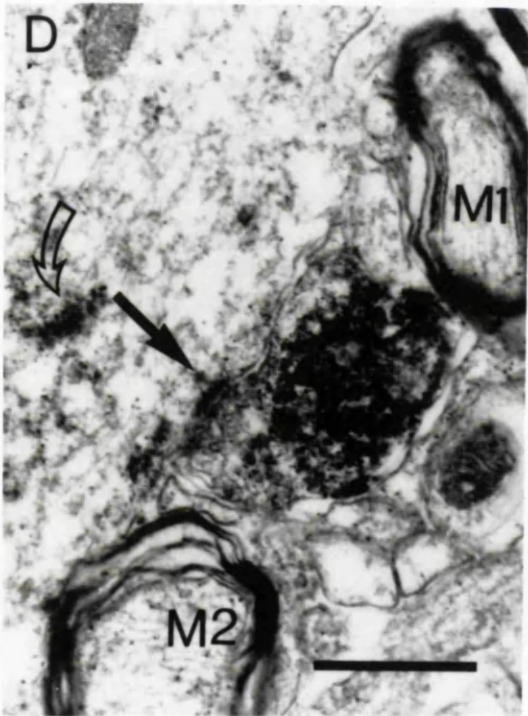
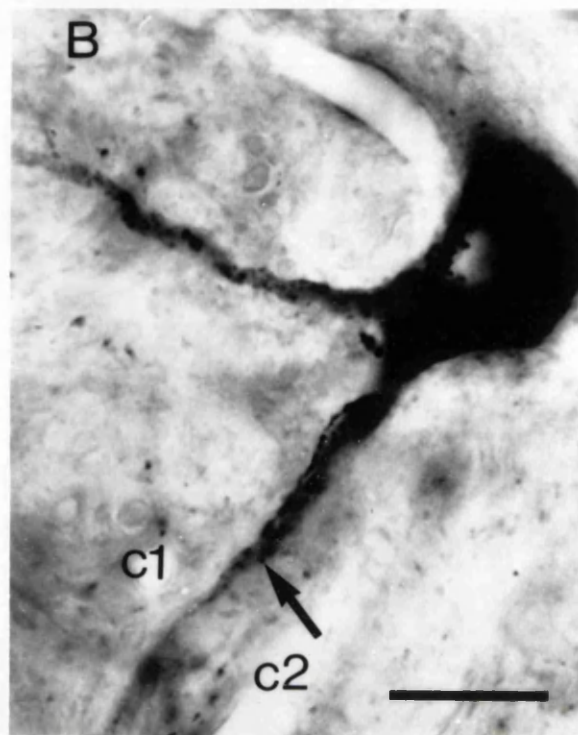
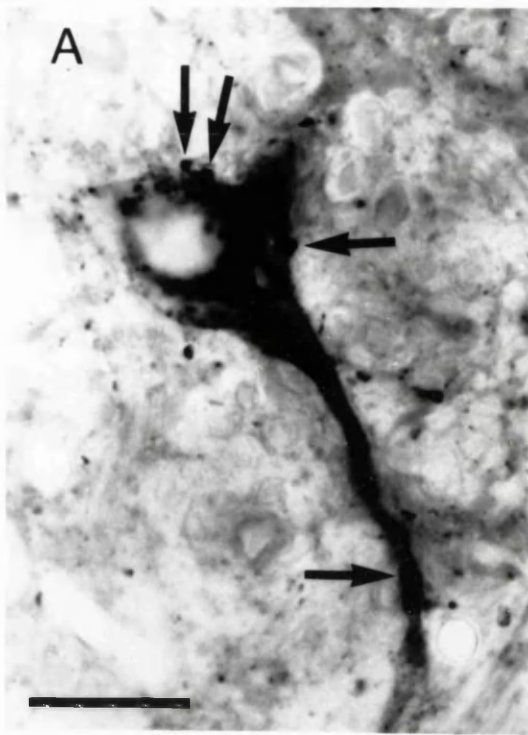
Figure 5.4.

A and B. Light micrographs of vagal preganglionic neurones in the nucleus ambiguus which were retrogradely labelled by the injection of cholera-toxin-HRP into the wall of the right atrium, and localised with the o-tolidine reaction. Immunocytochemistry to detect substance P containing structures was also carried out in this material and some substance P immunoreactive boutons (arrows) can be seen in close apposition to the retrogradely labelled neurones.

C. Low power electron micrograph of the dendrite of the cell in **B**. C1 and C2 are the same capillaries shown in **B** and the black arrow points to the apposing substance P immunoreactive bouton. The open arrows point to o-tolidine reaction product within the dendrite identifying the dendrite as a retrogradely labelled CVPN. M1 and M2 are myelinated structures used to correlate the same area at high power in **D**.

D. The filled arrow points to the correlated substance P immunoreactive bouton forming an asymmetric type synaptic contact associated with a sub-junctional body with the retrogradely labelled CVPN (open arrow points to retrograde labelling). M1 and M2 are the same myelinated structures labelled in **C**.

Scale bars: **A and B:** 20 μ m **C.** 5 μ m **D.** 0.5 μ m



Section 5:4. Discussion.

This study has employed immunocytochemistry and electron microscopy to demonstrate that substance P immunoreactive terminals form asymmetric synaptic contacts with structures in the nucleus ambiguus (fig. 5.1) and has combined these techniques with retrograde labelling to identify some of the post-synaptic targets of these terminals as vagal preganglionic neurones projecting to the heart or the cervical vagus nerve (fig. 5.2, 5.3 and 5.4).

The identification of substance P in boutons in synaptic contact with VPNs is somewhat to be expected since substance P receptors have been located on VPNs (Helke *et al*, 1984, 85). However, neurotransmitters are not always found where their receptors have been localised (Herkenham, 1987), and both pieces of information are required to determine the relationship between substance P and vagal preganglionic neurones. In addition, the methods employed in this chapter have several advantages over autoradiography as they provide information on the precise location of the retrogradely labelled cells, the distribution of the input to the labelled neurones and the synaptic morphology of the labelled inputs.

Since substance P immunoreactive boutons form synaptic connections with vagal preganglionic neurones retrogradely labelled from the heart, this may indicate that the vagally mediated bradycardia elicited by microinjection of substance P into the ventrolateral medulla (Urbanski *et al*, 1988; Agarwal and Calaresu, 1991) is due to direct actions of substance P on CVPNs. In addition, the identification of substance P in synaptic contact with VPNs in

the scNA, which is the main source of VPNS innervating muscles of the airways (Bieger and Hopkins, 1987), provides an anatomical substrate for the vagally mediated increases in airway muscle tension observed upon application of substance P to the ventral medullary surface (Haxhiu *et al*, 1989). Further experiments are required to determine if vagal preganglionic neurones with other projections are also innervated by substance P containing boutons.

The morphology associated with the substance P boutons is consistent with a direct excitatory actions of substance P on vagal preganglionic neurones. Substance P is contained in boutons forming asymmetric synapses with VPNS, and asymmetric synapses have been associated with excitatory functions (Gray, 1973). However, substance P containing terminals forming symmetric synapses may not have been identified in this study since they are harder to confirm than asymmetric specialisations, especially in material with poor ultrastructure and when the post-synaptic membrane is also labelled. However, it seems likely that if symmetric terminals containing substance P do exist they are small in number as the study of random boutons revealed only substance P containing boutons which formed asymmetric synapses.

Despite the identification of substance P inputs onto VPNS it is still possible that the pharmacologically observed effects are through indirect actions. Further experiments such as iontophoresis of substance P onto VPNS *in vivo* or application of substance P onto VPNS *in vitro* are necessary to clarify this point. In addition it is not clear if the actions of substance P on VPNS are important in the control of vagal functions in physiological conditions: this could be

studied if substance P antagonists are developed by iontophoretically administering them onto VPNs and if their firing rate decreased this would suggest that substance P has tonic excitatory actions on VPNs which are active in physiological circumstances.

In addition to vagal preganglionic neurones projecting to the heart or the cervical vagus nerve, substance P immunoreactive boutons may also innervate other neurones in the ventral medulla. Substance P immunoreactive boutons have been observed in close apposition to neurones retrogradely labelled from the recurrent laryngeal nerve (Holtman, 1988), but this was a study using fluorescent techniques and no electron microscopy was carried out to determine synaptic relationships. In addition, substance P immunoreactive boutons have been observed in synaptic contact with PNMT containing neurones in the C1 group in the rat (Milner *et al*, 1988). Other studies have shown that substance P boutons form close associations with PNMT and tyrosine hydroxylase containing neurones in the medulla which were retrogradely labelled from the diencephalon, although synaptic contacts were not established by electron microscopy (Nicholas and Hancock, 1989). However, further experiments are required to determine if neurones in the NA with other functions are innervated by substance P containing boutons.

5:4.1. Possible Source of Substance P input to VPNs.

As the animals in this study were not pretreated with colchicine, and colchicine is often necessary to detect soma containing substance P (for example, Milner *et al*, 1988), labelled fibres and

axons could not be traced back to their parent cells. Even if labelled cells were present it would be unlikely that immunoreactive fibres could have been traced back to their cell bodies due to limited penetration of immunocytochemical reagents. Thus the evidence for the source of the substance P fibres in the NA must be indirect.

Substance P has been co-localised with 5-HT in many areas of the brain, including the raphe magnus, raphe obscurus, raphe pallidus, nucleus reticularis gigantocellularis, periaqueductal gray matter and ventral lateral medulla (Hokfelt *et al*, 1978; Marson, 1989). The evidence that these areas are the sources of 5-HT, and which can thus include possible substance P projections, is presented in section 4.4.1. From this data it appears that the caudal medullary raphe nuclei may be a source of input to the ventrolateral medulla, but whether this input contains substance P and is to vagal preganglionic neurones remains to be determined.

Other areas which contain substance P immunoreactive neurones (Ljungdahl *et al*, 1978) and which have been shown to project to the ventrolateral medulla (see section 1:3.7.) include the stria terminalis, pre-optic area, suprachiasmatic area, the lateral, anterior and paraventricular areas of the hypothalamus and the nucleus tractus solitarius. However, only one of these areas has been shown to contain substance P perikarya projecting to the ventrolateral medulla: following the injection of retrograde tracer into the rostral ventrolateral medulla, some retrogradely labelled neurones in the nucleus tractus solitarius also contained immunoreactivity for substance P (Morilak *et al*, 1988). However, since the RVLM contains neurones with many diverse functions (see

section 1:3.5) it is not possible to precisely identify if VPNs are post-synaptic targets of the retrogradely labelled substance P neurones in the NTS. Similar studies have not been carried out to identify if substance P neurones in other areas of the brain are retrogradely labelled by injections of tracers into the nucleus ambiguus.

5:4.2. Summary.

Combination of retrograde labelling, immunocytochemistry and electron microscopy revealed that substance P immunoreactive boutons formed asymmetric synaptic contacts with neurones in the NA of the rat, some of which were identified as vagal preganglionic neurones by retrograde labelling from the heart or cervical vagus nerve.

Chapter Six.

Demonstration of Neuropeptide Y immunoreactivity in boutons in synaptic contact with vagal preganglionic neurones in the NA, labelled retrogradely from the cervical vagus nerve of the rat.

Section 6:1. Introduction.

Neuropeptide Y (NPY) was recently isolated in porcine brain (Tatemoto *et al*, 1982), and structurally characterised as a peptide consisting of thirty six amino acids (Tatemoto, 1982). Following its isolation, many studies have shown that NPY has neurotransmitter or neuromodulator functions both in the peripheral and central nervous systems (Allen and Bloom, 1986; Dockray, 1986; Gray and Morley, 1986; Maccarone and Jarrat, 1986; Harfstrand, 1987).

Immunocytochemical studies have shown that NPY is present in neuronal perikarya in the ventrolateral medulla, in the region of the ventrolateral nucleus ambiguus (Rat - Chronwall *et al*, 1985; Yamazoe *et al*, 1985; Harfstrand *et al*, 1987. Cat - Massari *et al*, 1990. Human - Halliday *et al*, 1988). NPY containing cell bodies in this region also contain immunoreactivity for noradrenaline and adrenaline (Rat - Hokfelt *et al*, 1983; Everitt *et al*, 1984; Rabbit - Blessing *et al*, 1986) and are thus considered to be located in the A1 and C1 cell groups of this region (see section 1:3.5.B. for description of A1 and C1 groups). Since neurones in the A1 and C1 groups have been implicated in the control of the cardiovascular system via the sympathetic nervous system (see section 1:3.5.B), NPY containing neurones in these groups have also been suggested to be involved in this control (see Blessing *et al*, 1987). This suggestion was supported by the observation that NPY containing cells in the C1 group were labelled retrogradely from the thoracic spinal cord and NPY immunoreactive boutons were observed in close apposition to retrogradely labelled sympathetic neurones in the intermediolateral cell column of the thoracic spinal cord (Blessing *et al*, 1987).

In addition to NPY containing cell bodies, immunocytochemical studies have shown that there is a dense network of NPY containing fibres in the ventrolateral medulla (Rat - Yamazoe *et al*, 1985; Harstrand *et al*, 1987. Cat - Massari *et al*, 1990). However, it is not clear whether these NPY immunoreactive fibres serve a functional role, since a plethora of autoradiographic ligand binding studies have failed to demonstrate NPY receptors in the ventrolateral medulla (Harstrand *et al*, 1986; Nakajima *et al*, 1986; Martel *et al*, 1986,90; Lynch *et al*, 1989).

Despite the apparent lack of NPY receptors in the VLM, NPY appears to have a functional role in this region. Microinjection of NPY into the rostral ventrolateral medulla of the rat has been reported to elicit an increase in blood pressure and a decrease in heart rate (Tseng *et al*, 1988), as well as having no significant cardiovascular effects (McAuley, MacRae and Reid, 1989). When micro-injected into the caudal ventrolateral medulla, NPY decreases blood pressure and heart rate (MacRae and Reid, 1988; McAuley, MacRae and Reid, 1989), and in some of these experiments the bradycardia was shown to be mediated through the vagus nerve, since it was prevented by prior administration of methylatropine (MacRae and Reid, 1988).

Since a vagally mediated bradycardia requires excitation of cardiac vagal preganglionic neurones, the pharmacological studies described above indicate that NPY has an excitatory effect on CVPNs. However, this excitatory effect of NPY on CVPNs may be through either direct or indirect actions. Consequently, the experiments in this chapter were undertaken to determine if an anatomical substrate existed for direct actions of NPY on CVPNs. This was

carried out by localising NPY containing structures with immunocytochemistry, which was combined with retrograde labelling from the cervical vagus nerve and electron microscopy to identify if NPY was contained in boutons in synaptic contact with retrogradely labelled VPNs in the NA of the rat. To determine if NPY could be acting on CVPNs, specific examination was directed at VPNs in the ventrolateral nucleus ambiguus, since this was shown to be the main site of origin of cardiac vagal preganglionic neurones in Chapter Three.

Section 6:2. Methods.

6:2.1. Retrograde Labelling.

Vagal preganglionic neurones were labelled retrogradely in 7 male Sprague Dawley rats (200-350g) by the injection of HRP into the cervical vagus nerve as described in section 2.1.A.1. Following 36-72 hours recovery the animals were perfused with 0.2-0.5% glutaraldehyde and 3% paraformaldehyde. The medulla was blocked and 60-80 μ m transverse sections cut on a vibrating microtome. The sections were washed in 0.1M pH6 PB for twenty minutes before the histochemical localisation of HRP using the TMB pH 6 method (section 2.2.B). In addition, two rats were perfused with the same fixative for control purposes.

Sections were rinsed three times in PBS and some processed for immunocytochemistry while others were dehydrated and embedded on microscope slides for control purposes. Sections from non-injected rats were transferred directly to primary antibody.

6:2.2. Immunocytochemistry.

Immunocytochemistry was carried out using polyclonal antibodies raised in rabbit against Neuropeptide Y (Gift from Dr. R. Corder). The sections were washed in PBS and then incubated in primary antibody (1:12,000) and 0.1% triton in PBS for 12-72 hours at 4°C on a shaker. The tissue was again washed in PBS and incubated in swine anti-rabbit antibody conjugated to HRP (1:100 in PBS; Dakopatts) for 3-6 hours. The immunocomplex was then detected using the TMB

reaction in section 2.1.A.1.

Following the immunoreaction the sections were post fixed in 1% osmium tetroxide for 30 minutes, dehydrated, embedded in resin and mounted on microscope slides (See section 2.3).

6:2.3. Specificity and Controls. (See also Appendix One: p. 288).

The specificity of the neuropeptide Y antisera has been determined previously (tested by radio-immunoassay - personal communication by Dr. R. Corder). In addition the specificity of staining was determined by comparison of the pattern of immunoreactivity with previous reports of NPY immunostaining in the medulla oblongata, and was identical to these descriptions (see below; Chronwall *et al*, 1985; Yamazoe *et al*, 1985). In sections incubated with a primary antibody also raised in rabbit but directed to substance P, the pattern of staining was markedly different. Thus the antibody is specific for NPY. In addition in sections incubated in the same way but with the omission of the NPY antibody no staining occurred, which indicates that positively immunostained structures are specific to the primary antibody and are not a product of other steps in the procedure.

In sections with retrograde labelling only, there was no afferent labelling in the NA which could be confused with immunoreactive boutons. Thus labelled boutons in the NA were likely to be NPY containing axons and labelled cells with black punctate reaction product vagal preganglionic neurones.

6:2.4. Light and Electron Microscopy.

Retrogradely labelled VPNs from the vINA were examined as this was shown in chapter three to contain the majority of cardiac vagal preganglionic neurones. Retrogradely labelled neurones with apposing immunoreactive boutons were photographed and these photographs used to correlate the structures at light and electron microscopic levels. At the electron microscope ultrathin sections were examined for correlated and non-correlated retrogradely labelled and immunoreactive structures.

Section 6:3. Results.

6:3.1. Immunocytochemistry.

Positively stained NPY fibres and boutons appeared amorphous and reddish-brown and could be identified on the surface of the section when 0.1% triton was added to the incubation medium (figure 6.1). In sections with greater concentrations of triton, immunoreactivity could be detected throughout the section. There was a dense concentration of labelled fibres and terminals in the DVN and the NTS. In the ventrolateral medulla fibres were arranged in an extremely dense lattice-like network in the region of the vINA. Fibre labelling was only slightly less dense within the loNA and scNA, but labelled fibres were scarce in the cNA.

NPY immunoreactive cell bodies had a reddish brown amorphous labelling in the cytoplasm of the cells, but not over the nuclei (see figure 6:1). Labelled neurones in the ventrolateral medulla were found in the A1 and C1 regions, ventrolateral to the cNA. Immunoreactive soma were also located in the region of the C2 group in dorsomedial portions of the NTS, and occasionally in rostral medial parts of the dorsal vagal nucleus. A few labelled neurones were also located rostrally in the raphe obscurus.

At the electron microscopic level immunoreactive boutons contained electron dense peroxidase reaction product associated with the vesicles and other structures (figure 6:2.). All 20 immunoreactive boutons studied were identified as forming asymmetric synaptic associations with dendritic structures in the NA (figure 6.2), and

some of these synapses were characterised by the presence of sub-junctional bodies associated with the post-synaptic membrane (figure 6.2). Also examined were non-immunoreactive boutons, which formed both symmetric and asymmetric synapses onto the same structures as labelled boutons (6.2.B.).

6.3.2. Retrograde Transport and Immunocytochemistry.

In sections that contained both retrogradely labelled VPNs and immunoreactive NPY structures, VPNs and NPY neurones were often intermingled in the ventrolateral nucleus ambiguus (figure 6.1). Retrogradely labelled VPNs, containing crystalline TMB reaction product, were easily distinguished from immunoreactive structures which contained amorphous reddish-brown reaction product (figure 6.1). At the light microscopic level, amorphous immunoreactive boutons were occasionally observed in close apposition to retrogradely labelled black punctate structures (figure 6.1 and 6.3.A). At the ultrastructural level three retrogradely labelled VPNs in the vINA were each revealed by correlated light and electron microscopy to be post-synaptic to a NPY immunoreactive bouton, which formed an asymmetric synapse with the VPN (figure 6.3). Serial ultrastructural examination of a further seven VPNs in the vINA revealed another 10 immunoreactive boutons which also formed asymmetric synaptic contacts, and sub-junctional bodies were associated with the post synaptic membrane in six of these synapses (6.3.E and 6.4.B &D). Non-immunoreactive boutons were also in synaptic contact with VPNs, forming both symmetric and asymmetric synaptic contacts.

Figure 6.1. - Light micrographs indicating that the distribution of VPNs and NPY immunoreactive neurones overlaps in the vINA.

A and B. High magnification light micrographs of an area of the ventrolateral medulla containing vagal preganglionic neurones (VPN) labelled retrogradely from the cervical vagus nerve and neuropeptide Y immunoreactive neurones (NPY-IR). VPNs contained punctate reaction product of the TMB method used to localise retrogradely transported horseradish peroxidase, whereas NPY-IR neurones contain amorphous reaction product typical of that of immunostaining localised with the DAB histochemical reaction.

The thin black arrows in **A** point to NPY immunoreactive boutons in the region of the VPN, the uppermost of which is close to the dendrite of the VPN. In **B** the arrows orientate both photographs: M is towards the midline of the section and V towards the ventral surface.

Scale Bars: 25µm.

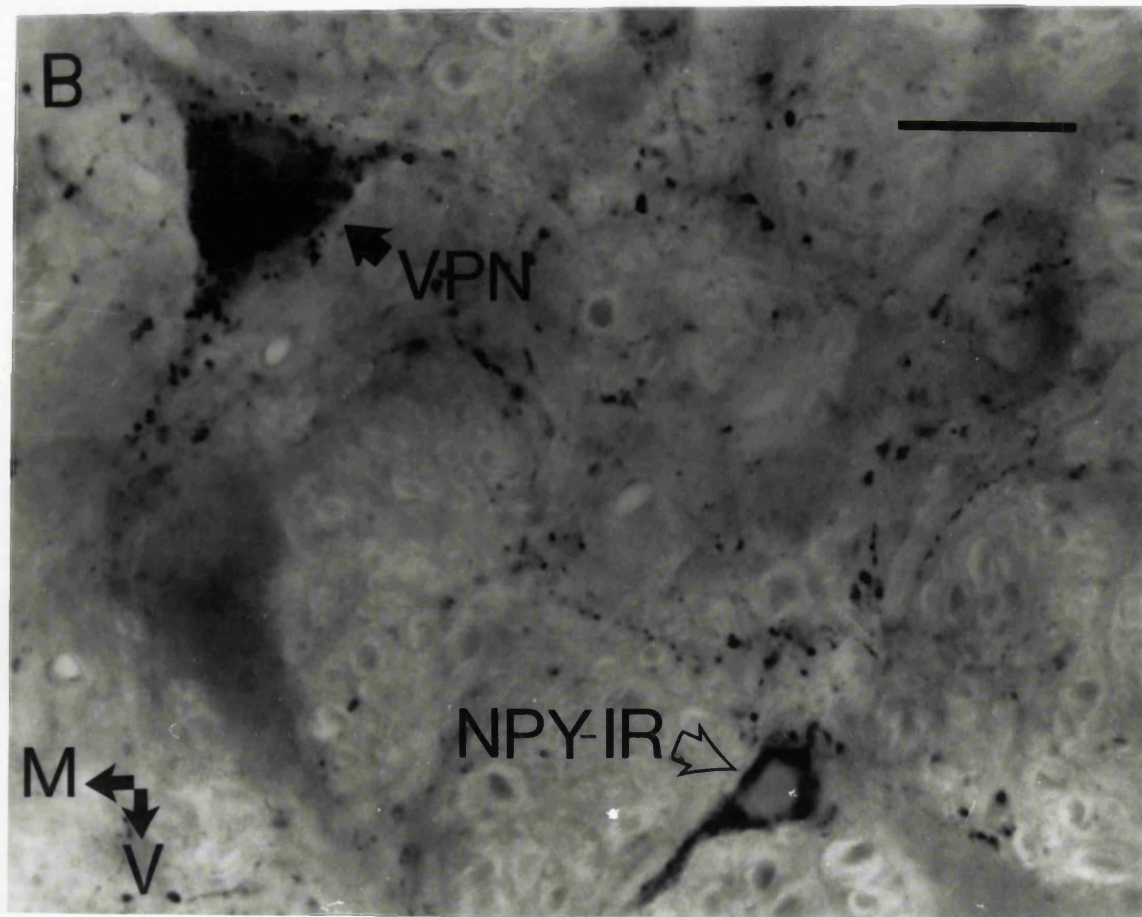
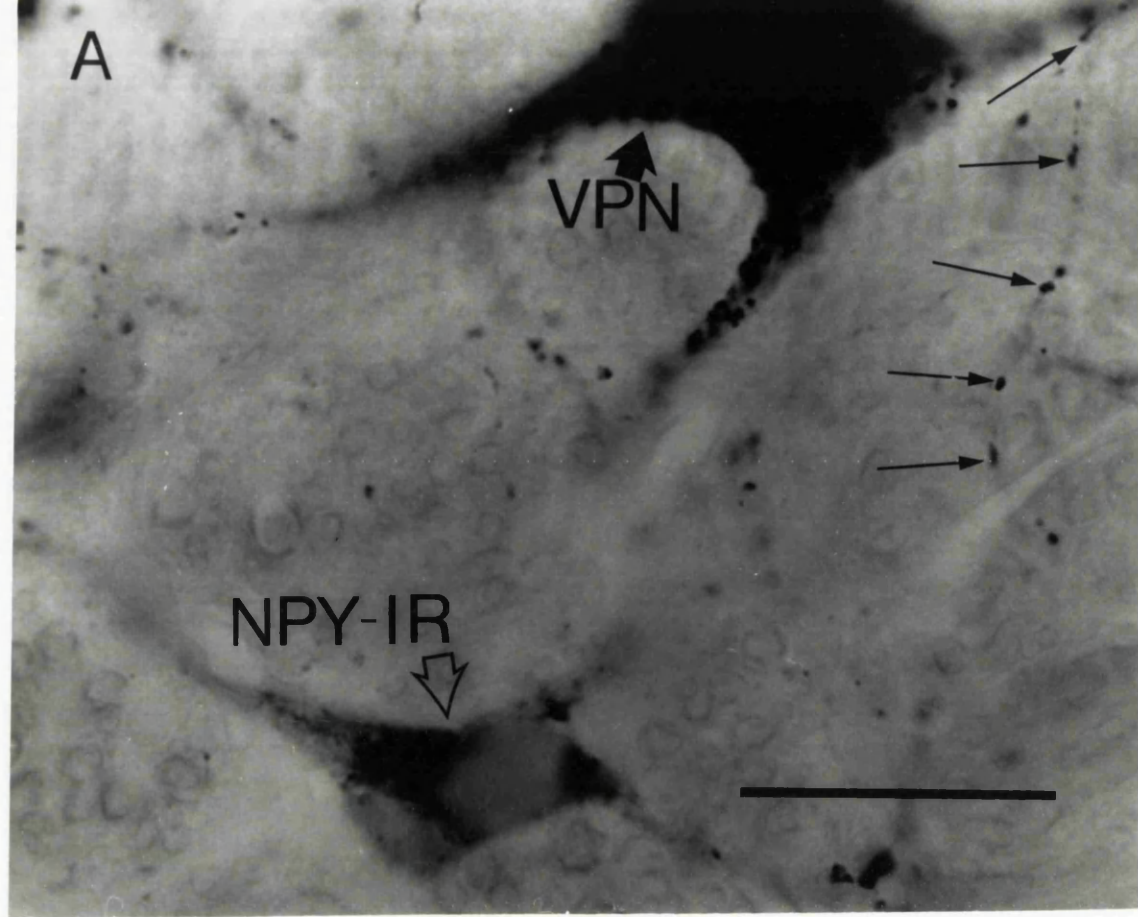


Figure 6.2. Electron micrographs of Neuropeptide Y immunoreactive boutons forming asymmetric synaptic contacts (arrows) with dendritic structures in the nucleus ambiguus. Each synapse has sub-junctional bodies associated with the post synaptic membrane. Unlabelled boutons in **B** form asymmetric (eight pointed star) and symmetric (five pointed star) synapses onto the same dendritic structures as the labelled bouton.

Common scale bar in **C**: 0.5 μ m.

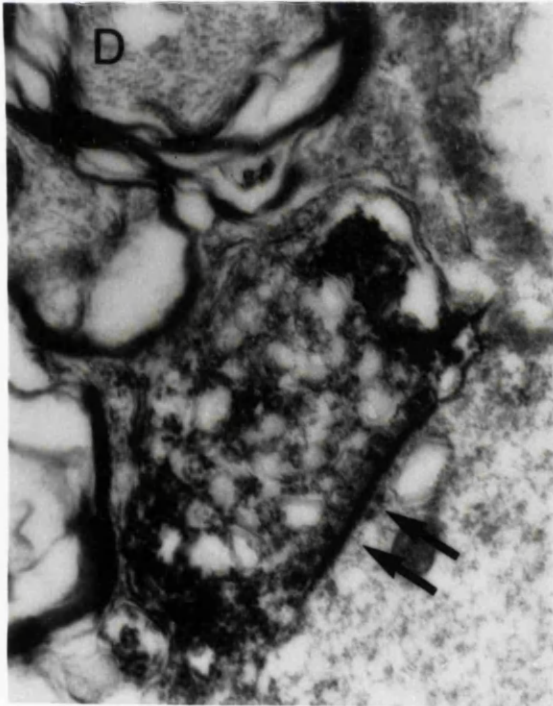
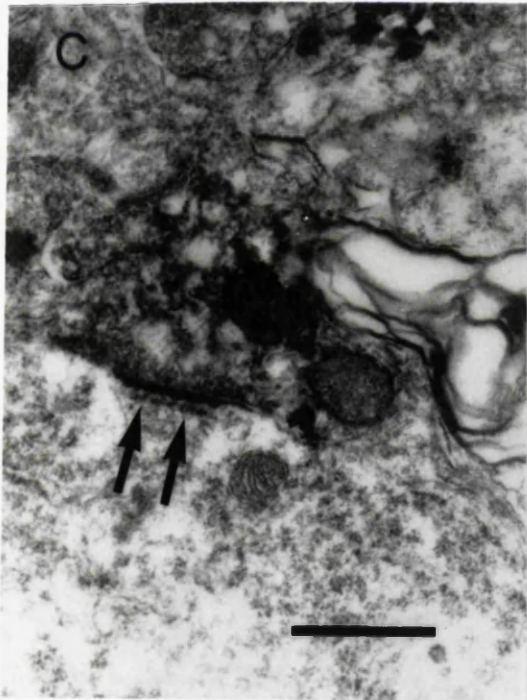
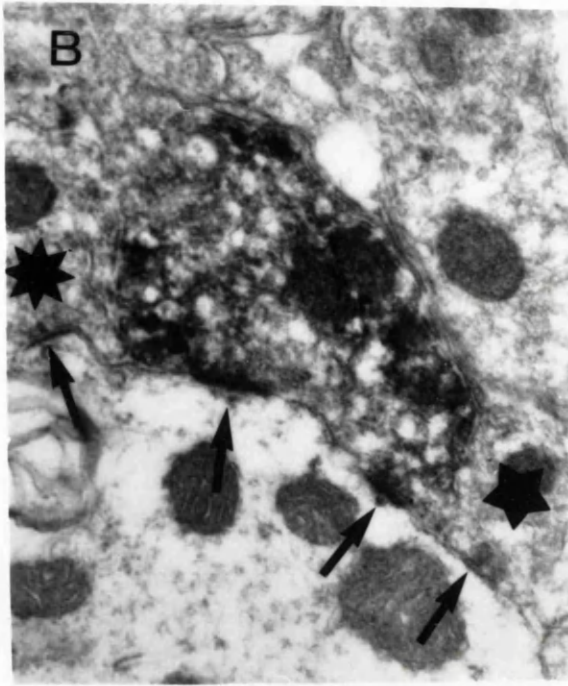
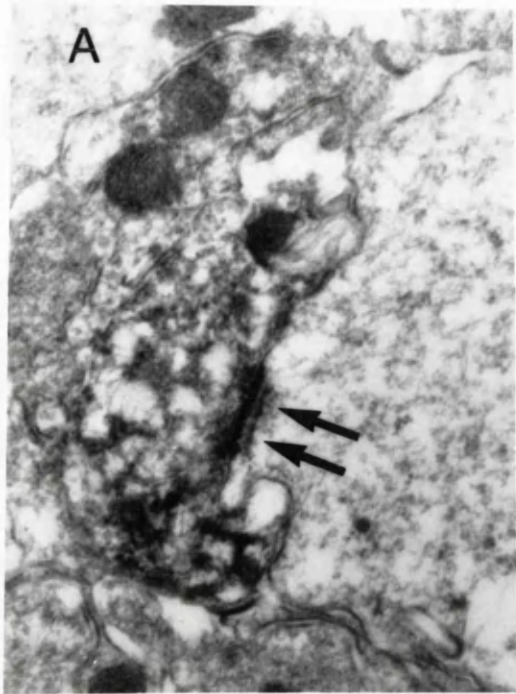


Figure 6.3. - Correlated light and electron micrographs of a retrogradely labelled VPN in the vNA with an apposing NPY immunoreactive bouton.

A. High power light micrograph of a retrogradely labelled VPN in the NA. The arrow points to a NPY immunoreactive bouton apposing the dendrite of the VPN. C1 and C2 are capillaries used for correlation in **B**.

B. Low power electron micrograph of the dendrite of the labelled VPN in **A**. C1 and C2 are the same capillaries shown in **A**. Open arrows point to retrograde reaction product within the cytoplasm of the cell, and the black arrow to the bouton apposing the membrane. G is the glial cell shown in **C**.

C. Higher power of area in **B**. C1 and G are same elements shown in **B**. Open arrows point to TMB reaction product in cytoplasm and black arrow to apposing NPY immunoreactive bouton. M is a myelinated structure shown in **D**.

D. High power E.M. of the NPY immunoreactive bouton in synaptic contact (black arrow) with the retrogradely labelled vagal preganglionic neurone (open arrow points to retrograde labelling).

M is the same myelinated structure shown in **C**.

E. High power E.M. demonstrating the NPY immunoreactive bouton forms an asymmetric synapse which is associated with a sub-junctional body (arrow), with the VPN.

Scale bars: **A.** 15 μ m **B.** 6 μ m **C.** 4 μ m **D.** 1 μ m **E.** 0.25 μ m

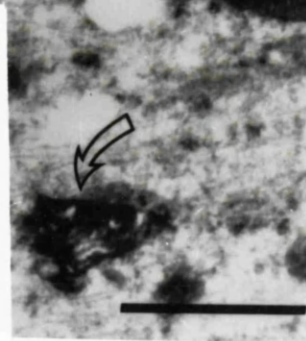
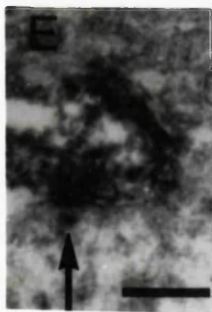
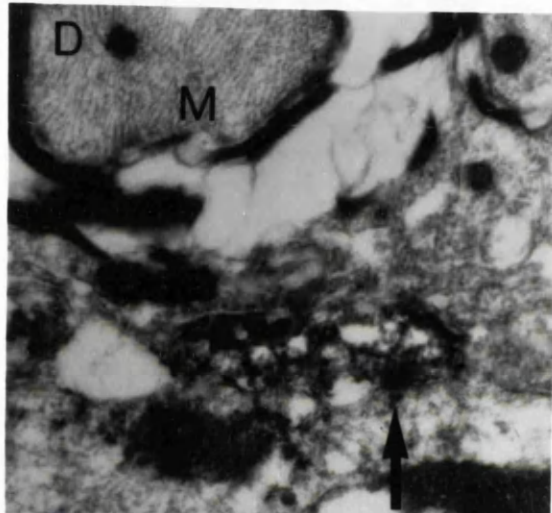
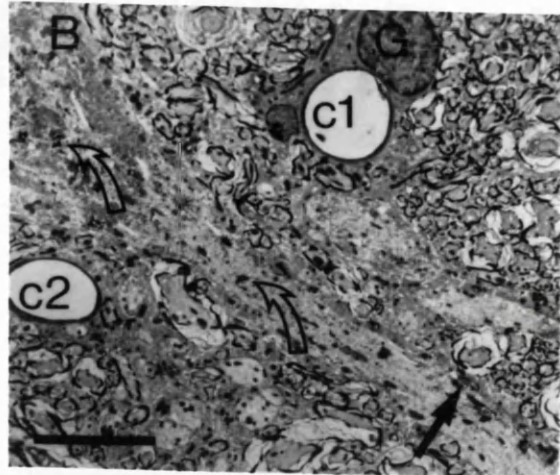
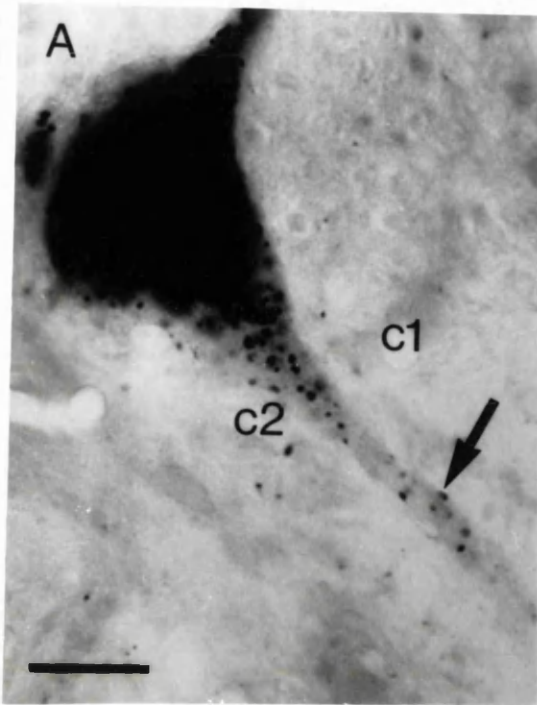
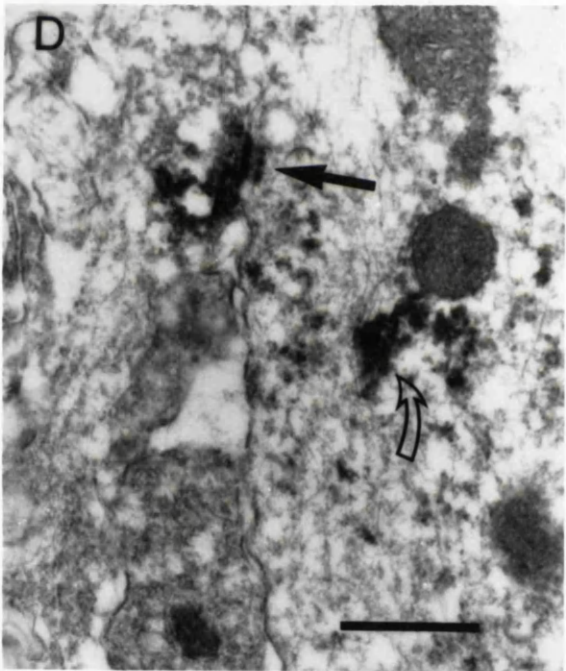
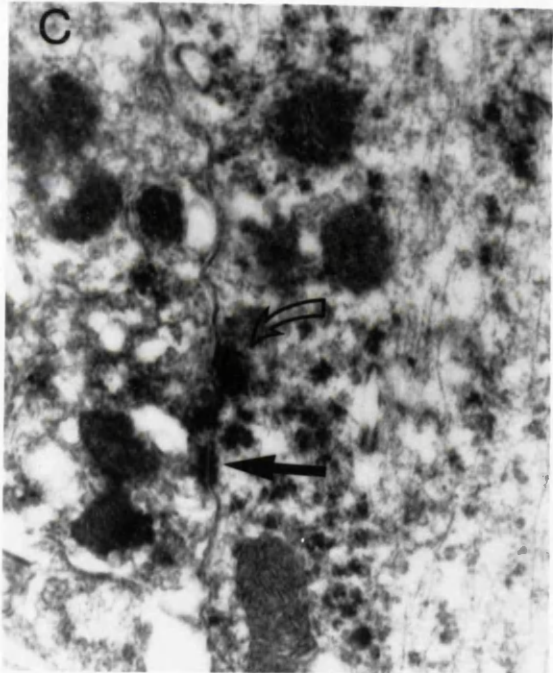
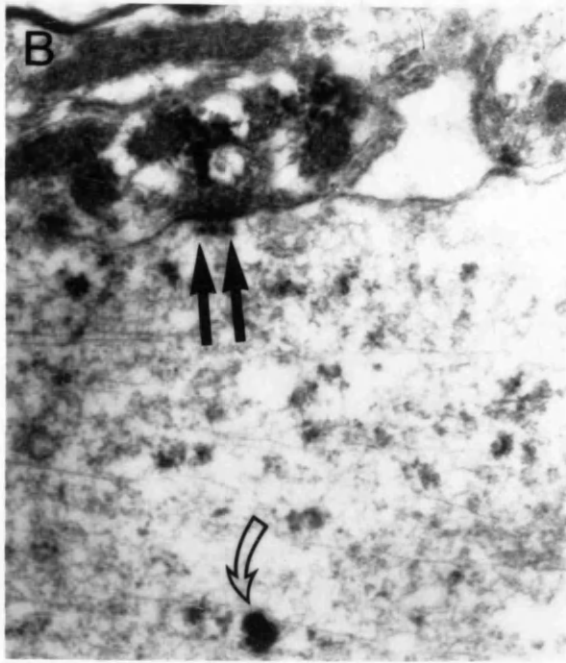
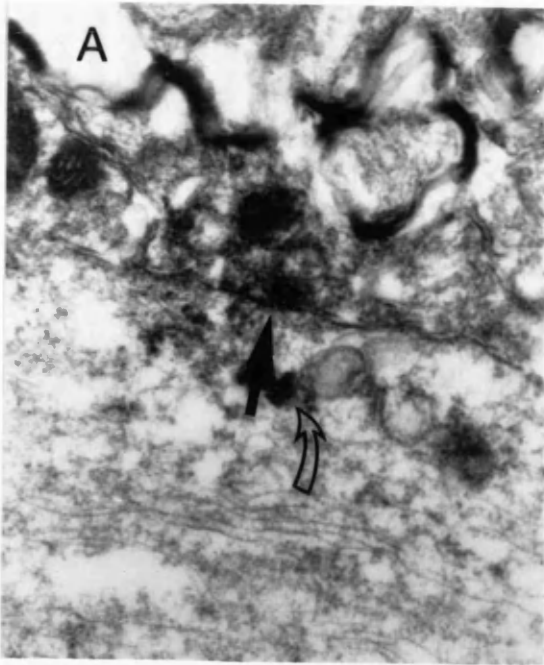


Figure 6.4. High powered electron micrographs of Neuropeptide Y immunoreactive boutons forming asymmetric synaptic contacts (black arrows) with structures in the vINA identified as retrogradely labelled vagal preganglionic neurones by the TMB reaction product in their cytoplasm (open arrows). The synapses in **B.** and **D.** have sub-junctional bodies associated with the post-synaptic membrane.

Common scale bar in **D.** = 0.5 μ m.



Section 6:4. Discussion.

This study has demonstrated that Neuropeptide Y immunoreactive neurones are intermingled with vagal preganglionic neurones in the ventrolateral nucleus ambiguus of the rat (figure 6.1); that NPY immunoreactive boutons in the vINA form asymmetric synapses in the vINA (figure 6.2) and that some of the post-synaptic targets of these boutons are vagal preganglionic neurones projecting to the cervical vagus nerve (fig. 6.3. and 6.4).

The intermingling of VPNs and NPY neurones in the ventrolateral NA further emphasizes the heterogeneity of the neurones in this region demonstrated in Chapter Three. Thus this region of the medulla oblongata contains vagal preganglionic neurones, neurones involved in respiratory control (as shown in chapter 3) and NPY immunoreactive neurones, which may be involved in the sympathetic nervous system control of the cardiovascular system (see section 6:1). This collection of neurones subserving different functions illustrates the necessity of identifying post-synaptic targets in studies of input to neurones in this region.

In this study vagal preganglionic neurones in the ventrolateral nucleus ambiguus have been identified as a post-synaptic target of NPY immunoreactive boutons. Although the vagal preganglionic neurones were identified only as projecting to the cervical vagus nerve, some of them may innervate the heart, since examination was made of VPNs in the vINA, where most of the cardiac vagal preganglionic neurones were located in the experiments in Chapter 3.

However, it has been shown by other workers that vagal preganglionic neurones in the vINA also project to the lungs and unidentified structures innervated by the superior laryngeal nerves (Bieger and Hopkins, 1987). Nevertheless, VPNs in the present study were labelled retrogradely by the injection of HRP into the cervical vagus nerve, below the entry of the superior laryngeal nerve to the vagal nerve trunk, and thus labelled cells in the vINA may innervate only the lungs or the heart. Consequently, some of the VPNs identified as receiving synaptic input from NPY containing boutons were likely to be cardiac vagal preganglionic neurones. However, further studies are required to precisely determine the projections of VPNs innervated by NPY containing boutons, as well as to determine if other neurones in the region are post-synaptic to NPY containing boutons.

6:4.1. Possible Sources of NPY Input to VPNs.

Likely sources of the NPY fibres observed in the ventrolateral medulla are areas where NPY cell bodies have been observed (Chronwall *et al*, 1985 and Yamazoe *et al*, 1985 in rat; Halliday *et al*, 1983, human; Bons *et al*, 1990, primate; Massari *et al*, 1990, cat), and which contain cells projecting to the ventrolateral medulla (see section 1:3.5.). Such overlap occurs in the lateral periaqueductal gray, lateral and anterior hypothalamic areas, raphe obscurus, the ventrolateral medulla and the NTS.

Few studies have combined retrograde tracing from the ventrolateral medulla with immunocytochemical detection of NPY to determine which groups of NPY containing neurones innervate the

VLM. A possibility for the source of NPY input to VPNs in the NA are the NPY neurones in the VLM, but this is difficult to demonstrate with combined retrograde labelling and immunocytochemical studies since these groups of neurones overlap as shown in this study (figure 6.1). NPY neurones in both the contralateral VLM (Llewellyn-Smith *et al*, 1990) and the NTS (Morilak *et al*, 1989) do not appear to innervate the VLM since no NPY containing neurones in these regions were reported to be labelled retrogradely by the injection of tracers into the ventrolateral medulla. Similar investigations are required to determine which regions are sources of the neuropeptide Y input to the ventrolateral medulla, and further studies to identify which neurones in the VLM are innervated by NPY containing neurones projecting to this region.

6:4.2. Functional Implications.

The identification of NPY immunoreactive boutons forming asymmetric type synapses with retrogradely labelled VPNs in the vINA provides anatomical evidence that NPY microinjected into the ventrolateral medulla directly excites CVPNs, since asymmetric synapses have been associated with excitatory functions (Gray, 1973). However, such microinjections of NPY may also decrease heart rate by other mechanisms. For example, the bradycardia elicited by microinjections of NPY into the rostral ventrolateral medulla by Tseng *et al* (1988) appears not be due to a direct action of NPY on CVPNs but through the baroreflex, since the bradycardia occurred some time after blood pressure had risen. This seems likely as McAuley *et al* (1989) did not evoke an increase in blood pressure by injection of NPY into the RVLM, and no bradycardia was

evident. In contrast, the decrease in heart rate reported by MacRae and Reid (1988) upon microinjections of NPY into the caudal ventrolateral medulla was accompanied by a decrease in blood pressure and thus cannot be associated with the baroreceptor reflex. However, it does not appear to be wholly due to excitation of CVPNs since it was only partially blocked by prior administration of atropine. The remainder of the response may be due to excitation of the sympathoinhibitory neurones in the region of the A1 group, since these are intermingled with VPNs, as was demonstrated by the intermingling of NPY immunoreactive neurones of the A1 group with VPNs observed in this study (fig. 6.1). However, none of these pharmacological studies show histological verification of injection sites and it is difficult to ascertain which neurones are being activated. Thus further experiments are required to elucidate the functional role of NPY in the ventrolateral medulla.

6:4.3. Summary.

Neuropeptide Y immunoreactive perikarya were intermingled with retrogradely labelled vagal preganglionic neurones in the ventrolateral medulla. NPY was identified in boutons forming asymmetric synaptic contacts with structures in the NA. Vagal preganglionic neurones were identified as one of these post-synaptic targets, and selection of VPNs on the basis of distribution suggested that some may be CVPNs.

Chapter Seven.

**Identification of the nucleus tractus solitarius as a
source of input to vagal preganglionic neurones in the
nucleus ambiguus of the cat.**

7:1. Introduction.

The nucleus tractus solitarius (NTS) lies in the dorsomedial medulla close to the floor of the fourth ventricle (see fig. 7.1; Loewy, 1990). It contains visceral sensory relay cells arranged in a number of different cytoarchitectonic sub-nuclei. These sub-nuclei are named on the basis of their position relative to the tractus solitarius and are similar in cat (Loewy and Burton, 1978; see figure 7.1.) and rat (Kalia and Sullivan, 1982). Anatomical and physiological studies have shown that NTS sub-nuclei receive visceral sensory afferent information, which is viscerotopically organised (see figure 7.1; Loewy, 1990). The parvocellular sub-nucleus of the NTS is innervated predominantly by afferents from the gastrointestinal tract (Leslie, Gwyn and Hopkins, 1982; Altschuler *et al*, 1989), the interstitial NTS is innervated by respiratory tract afferents (Donoghue *et al*, 1984; Claps and Torrealba, 1988), the ventral and ventrolateral NTS by pulmonary afferents and the medial and dorsolateral NTS receive cardiovascular afferent input from cardiovascular receptors (Berger, 1979; Kalia and Welles, 1980; Panneton and Loewy, 1980; Wallach and Loewy, 1980; Ciriello, Hrycyshyn and Calaresu, 1981a; Davies and Kalia, 1981; Nomura and Mizuno, 1982; Ciriello, 1983; Donoghue *et al*, 1984; Chernicky *et al*, 1987; Housley *et al*, 1987; Claps and Torrealba, 1988; Mifflin, Spyer and Withington-Wray, 1988; for review see Loewy, 1990 and figure 7.1).

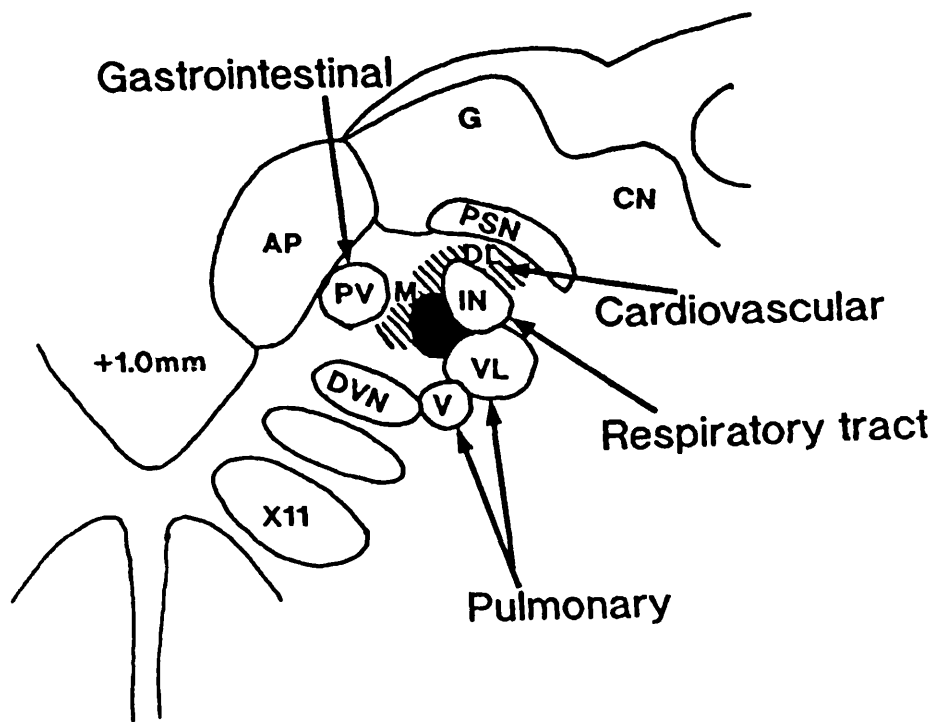


Figure 7.1. Drawing of the NTS of the cat at an intermediate level, showing the various sub-nuclei and the viscerotopic pattern of innervation of these nuclei. The hatched area represents the main regions of termination of cardiovascular afferents. Abbreviations: AP - area postrema; DVN - dorsal vagal motor nucleus; X11 - hypoglossal motor nucleus; G - gracile nucleus; CN - cuneate nucleus; PSN - parasolitary nucleus. The other letters represent sub-nuclei of the NTS - PV - parvocellular; M - medial; IN - interstitial; DL - dorsolateral; V - ventral; VL - ventrolateral.

Adapted from A.D. Loewy (1990) in "Central Regulation of Autonomic Functions."

Since neurones in the NTS are innervated by sensory visceral afferents, the projections of these neurones are important as they may be involved in reflex pathways controlling autonomic variables (see Spyer, 1990). In particular, projections from neurones in the NTS to vagal preganglionic neurones in the nucleus ambiguus would indicate a direct pathway for reflex changes in the activity of VPNS innervating structures above the diaphragm (for example, the baroreceptor reflex described in Spyer, 1990).

Neurones in the NTS have been shown to project to the NA by injection of retrograde tracers into the NA, which labels cells in many sub-nuclei of the NTS, but principally in the medial and ventrolateral sub-nuclei (Stuesse, 1984; terHorst *et al*, 1984; Dampney *et al*, 1987; Smith *et al*, 1989; Bockstaele *et al*, 1990; Nunez-Abades, Portillo and Pasaro, 1990; Takayama *et al*, 1990). However, since the NA contains many neurones other than vagal preganglionic neurones (see 1:3.5, 3:4 and 6:4), these techniques cannot determine if labelled neurones in the NTS innervate VPNS or other neurones in the region.

Early anterograde tracing techniques also suggested that there may be monosynaptic connection from the NTS to the NA, but also failed to identify the nature of the post-synaptic targets in the NA (Cat - Morest, 1967; Cottle and Calaresu, 1975; Loewy and Burton, 1978. Rat - Norgren, 1978. Monkey - Beckstead, Morse and Norgren, 1980). Lesions of the caudal NTS resulted in degenerated fibres in the NA (Morest, 1967; Cottle and Calaresu, 1975, both in cat), but the origin of the cell bodies contributing to degenerated fibres is difficult to identify since lesions may also affect fibres of passage.

In other studies, injections of tritiated amino acids into the caudal NTS resulted in a moderate to high density of silver grains over the NA (Loewy and Burton, 1978, in cat; Norgren, 1978, in rat; Beckstead, Morse and Norgren, 1980, in monkey). However, due to the low resolution of autoradiographic detection it was not possible to determine if anterograde labelling was due to terminals or fibres of passage, and consequently the post-synaptic targets of these fibres could not be identified.

The use of plant lectins as anterograde tracers has provided better resolution than provided by degeneration or silver grain techniques as they can be distinguished in boutons as well as fibres of passage (Gerfen and Sawchenko, 1984). Following the injection of phaseolus vulgaris leucoagglutinin (PHA-L) or wheat germ agglutinin conjugated to HRP (WGA-HRP) into various nuclei of the caudal NTS in rats, terminal-like labelling was observed in the region of retrogradely labelled VPNs in the NA (Ross *et al*, 1985; Cunningham and Sawchenko, 1989a). However, these studies did not identify close associations or synaptic relationships between anterogradely labelled boutons and retrogradely labelled VPNs.

The experiments in this present study were thus carried out to determine if vagal preganglionic neurones in the NA were a post-synaptic target of neurones in the NTS. Initial investigations sought to determine the location of neurones in the NTS projecting to the NA by retrograde labelling from an area of the NA electrophysiologically identified as containing VPNs. Subsequently, the projections from the NTS to the NA were identified by the injection of the anterograde tracer biocytin into the NTS, and VPNs

in the NA identified by retrograde tracing from the cervical vagus nerve. Correlated light and electron microscopic examination of the material was undertaken to determine if anterogradely labelled boutons were in synaptic contact with retrogradely labelled VPNs. These experiments were carried out in cats to allow injections of tracers to be limited to specific nuclei.

Section 7:2. Methods.

7:2.1. - Retrograde Tracing from the Nucleus Ambiguus.

Two female cats (2.2 and 2.4kg) were anaesthetised with an intraperitoneal injection of sodium pentobarbitone. The femoral vein was cannulated for maintenance of anaesthesia and the right cervical vagus nerve isolated via a dorsal midline incision. The animals were placed in a stereotaxic head frame (Kopf Instruments). The vagus was cut and the central end placed on a bipolar stimulating electrode. Temperature was maintained at 37°C by a heating blanket controlled via a rectal thermometer. The medulla was exposed through a dorsal midline incision extending from the lambdoidal suture ridge to the first or second cervical segment of the spinal cord, the skin reflected and the nuchal muscles separated and retracted. The occipital bone was carefully removed using a small pair of rongeurs taking care not to damage the medulla. The dura was incised and the cerebellum retracted to expose the surface of the medulla. Recordings were made through a glass microelectrode (tip diameter approximately 20µm; filled with 10% HRP in Tris 0.2M KCl) which was advanced into the medulla approximately 3mm rostral to the obex, 2mm lateral on the right hand side and 6mm deep. When antidromic potentials were recorded to stimulation of the cervical vagus nerve (frequency 10HZ, intensity 100mv to 1volt, width 0.1ms; see figure 7:2.A and B) HRP was ejected from the microelectrode by the application of 5µA of positive current for 15 minutes from an ionophoretic unit (Neurophore). The animals were allowed to recover for 48 hours prior to perfusion with 2% glutaraldehyde and 2% paraformaldehyde.

The brains were removed and the medulla and pons blocked for sectioning at 50-80 μ m on a vibrating microtome. Injected and transported HRP was subsequently localised in these sections using the DAB, TMB or *o*-toluidine reactions (see section 2:2) and the sections then prepared for light and electron microscopy as described in section 2:4.

7:2.2. Retrograde Labelling of VPNS and Anterograde Labelling.

In two female cats, 10 μ l of cholera toxin-HRP were injected into the myocardium using the same procedure described in section 3.2 for rats. In another five cats HRP was injected into the cervical vagus nerve as detailed in section 2:1.1.A.

After a recovery period of 36-48 hours cats were re-anaesthetised with an intraperitoneal injection of 40mg/kg Sagatal and prepared for injection of biocytin into the NTS in a similar manner to that described in section 7:2.1. In three cats the vagus or carotid sinus nerves were placed on bipolar stimulating electrodes and stimulated at frequency 10HZ, intensity 100mv - 1volt, width 0.1ms. A glass microelectrode was filled with 5% biocytin in 2.0M sodium methyl sulphate and advanced into the brainstem at sites from the level of obex to 2mm rostral and 0.5-1mm lateral to the midline. At areas where synaptic potentials were evoked to stimulation of the cervical vagus or carotid sinus nerves (see figure 7.4.A and B), biocytin was ionophoresed by application of 5 μ A of positive current for 15 minutes. In the remaining two animals biocytin was ionophoresed into the NTS without the aid of electrophysiological identification of the injection site. Anaesthesia was maintained for

6-12 hours prior to perfusion with 0.5%-1% glutaraldehyde and 3% paraformaldehyde.

Injected and transported HRP was localised using the TMB or DAB reactions described in section 2.2. Biocytin was subsequently localised by washing the sections in PBS and incubated for 2-4 hours in streptavidin-HRP (Amersham International) in PBS (1:100 or 1:50) with added triton (0.1-0.6%) at room temperature and the peroxidase activity revealed by the DAB method (section 2:2.1) or the Hanker - Yates method (section 2:2.4). Section were then prepared for light and electron microscopy as described in section 2:4.

7:2.2.1. Controls.

After perfusion and sectioning some sections were reacted only to localise retrogradely transported HRP to determine if labelled axons in the NA could be due to application of HRP to the cervical vagus nerve. In addition, to ascertain if labelled cells in the NA could be due to injection of biocytin into the NTS, other sections were incubated only to localise biocytin

Section 7:3. Results.

7:3.1. Injection of HRP into the NA.

HRP was ionophoresed into regions of the NA where extracellular antidromic potentials were evoked to stimulation of the cervical vagus nerve (figure 7.2.A and B). Recorded activity was classified as antidromic on the basis of the following criteria described by Lipski (1981) : the evoked potential had a constant short latency, the response was an all-or-none response and activated at a stable threshold current and followed rates of stimulation up to 100Hz. The most convincing test for antidromic activation, the collision test, was not carried out as none of the evoked cells were spontaneously active. However, the location of injection sites was verified as within the NA in both animals upon detection of HRP (fig. 7.2.C), and one injection (fig. 7.2.C.) was larger than the other. The centre of the injection sites were characterised by a dense deposit of *o*-toluidine reacted HRP reaction product in the NA, around the edges of which were labelled fibres and cells (fig. 7.2.C.).

Retrogradely labelled neurones were found in the same areas in both cats, although the number of labelled neurones was clearly fewer in the cat with the smaller size of HRP deposit. In the NTS labelled neurones were situated bilaterally, but with ipsilateral predominance. Most labelled neurones were found at the same rostrocaudal level as the injection site mainly in the medial NTS, just dorsal to the tractus (fig. 7.2.D. and fig. 7.3), although fewer numbers were also found in the ventrolateral, commissural, interstitial and ventral sub-nuclei (see figure 7.3). Labelled cells

were also found in the medullary raphe nuclei, the reticular formation between the NA and the NTS, and the contralateral NA, as well as the ipsilateral NA distant from the injection site. In the pons only a few labelled cells were seen in the medial parabrachial nucleus and the Kolliker-Fuse nucleus.

7:3.2. Anterograde Labelling and Retrograde Tracing of VPNs.

7:3.2.A. Appearance of Anterograde Labelling.

Biocytin was ionophoresed into areas of the NTS in two cats without the aid of electrophysiological identification of the injection site, and in the remaining three animals into areas where evoked synaptic potentials were recorded to stimulation of the cervical vagus or carotid sinus nerves (figure 7.4.A. and B). Potentials were classified as synaptic using the following criteria: the latency of recorded potential varied considerably and the evoked potential did not follow high rates of stimulation (100Hz).

Injection sites in different animals were centred on the commissural, medial, ventral and ventrolateral parts of the NTS (figure 7.4.C). The centre of the injection was characterised by a core of labelled neurones that appeared to be completely filled by reaction product (figure 7.4.C). Some cells were labelled at a distance from the injection site (up to 500 μ m) but the dendritic fields of these cells usually overlapped the injection site. Biocytin was also localised in axonal fibres which were most dense at the injection site and which could be followed in serial sections usually into fibre tracts coursing away from the injection site. When

injected in and around the tractus biocytin was not detected in the tractus itself, indicating that it was not taken up by fibres of passage (figure 7.4.C).

Six to twelve hours following an injection anterogradely transported biocytin was localised in axonal fibres and varicose boutons which appeared similar to immunopositive structures localised with peroxidase detection systems (fig. 7.4.D., 7.7. and 7.8). Varicosities were often observed to branch from thick and fine calibre labelled axons (fig. 7.4.D, 7.7. and 7.8.). Anterogradely labelled fibres were detected bilaterally in the medulla, but there was more labelling ipsilateral to the injection site (figure 7.5). In the rostrocaudal plane labelled fibres were most dense at the level of the injection site (figure 7.5). Labelled boutons were most dense in the NA and ventrolateral medulla where some of the labelled boutons were observed in close apposition to cell perikarya (figure 7.4.D). Biocytin labelled boutons were also evident in the DVN, raphe nuclei, hypoglossal nuclei and the contralateral NTS (figure 7.5).

Electron microscopic examination revealed that boutons in the NA which contained biocytin were synaptic boutons (fig. 7.6). These labelled boutons were easily distinguished from unlabelled boutons as they contained electron dense amorphous peroxidase reaction product in association with vesicles and other structures (fig. 7.6). The reaction product was so dense in many boutons that it obscured the morphology of the vesicles as well as that of the associated synapse (fig. 7.6). Twenty five labelled boutons in the NA were identified at the ultrastructural level forming synaptic contacts with unidentified structures (fig. 7.6). Eight of these boutons formed

symmetric synaptic associations and 12 formed asymmetric synaptic associations, two of which had subjunctional bodies associated with the post-synaptic membrane (figure 7.6), while the morphology of the remaining five synapses could not be determined. These unidentified structures also received symmetric and asymmetric input from unlabelled boutons (figure 7.6). In addition to boutons in the NA, peroxidase reaction product was also localised in myelinated and unmyelinated axons (figure 7.6).

7:3.2.B. Retrograde and Anterograde Labelling.

Controls - In material with HRP applied to the cervical vagus nerve only there was no terminal labelling in the NA. In material with anterograde labelling only there were occasional labelled cells in the NA. However, these cells contained an amorphous reaction product easily distinguishable from the punctate reaction product found in retrogradely labelled neurones. Thus labelled cells in the NA with punctate reaction product in the cytoplasm were VPNS and labelled axons contained anterogradely transported biocytin.

Retrogradely labelled neurones were found in the medulla after injection of HRP into the cervical vagus nerve but not following injection of CT-HRP into the wall of the right atrium. Neurones labelled retrogradely by the injection of HRP into the cervical vagus nerve were visualised by the TMB reaction which resulted in a black punctate reaction product within cell soma and dendritic cytoplasm (figure 7.7 and 7.8.A.). There appeared to be fewer labelled cells than were found with the same procedures in the rat in Chapter 3. Labelled cells in the DVN were arranged in a tightly packed group

similar to that in the rat (see chapter 3). Retrogradely labelled neurones in the NA were loosely arranged and no clear sub-divisions of the NA could be discerned.

Light microscopic examination revealed that the soma and dendrites of retrogradely labelled VPNs in the NA often had numerous apposing anterogradely labelled boutons, which sometimes branched from labelled axons coursing through the region (fig. 7.7.). Correlated light and electron microscopy revealed twelve anterogradely labelled boutons forming synaptic associations with four retrogradely VPNs, with up to six onto one cell (figure 7.8). Further ultrastructural examination revealed another 5 anterogradely labelled boutons in synaptic contact with 2 retrogradely labelled VPNs (figure 7.9). Of the total of 17 anterogradely labelled boutons observed in synaptic contact with VPNs, 5 were associated with symmetric synaptic morphology and 7 formed asymmetric type synapses, with one of these asymmetric synapses associated with a sub-junctional body, and the morphology of five synaptic contacts could not be determined.

Figure 7.2. A. Single sweep of potentials in the NA evoked to electrical stimulation of the cervical vagus nerve. The arrow points to the stimulus artefact and the star is underneath the evoked response.

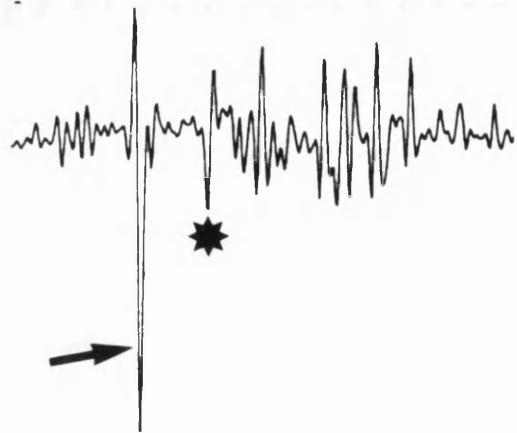
B. 5 superimposed sweeps of activity evoked in the NA to high frequency stimulation of the cervical vagus nerve. The response indicated by the star follows the stimulus at a constant latency, indicating that it is an antidromic response (the arrow points to the stimulus artefact).

C. Photomicrograph of half a section of the medulla oblongata of the cat indicating the position and size of a large ionophoretic injection of HRP in a region of the NA where the above antidromic responses were recorded to stimulation of the cervical vagus nerve. The HRP was localised using the *o*-toluidine reaction.

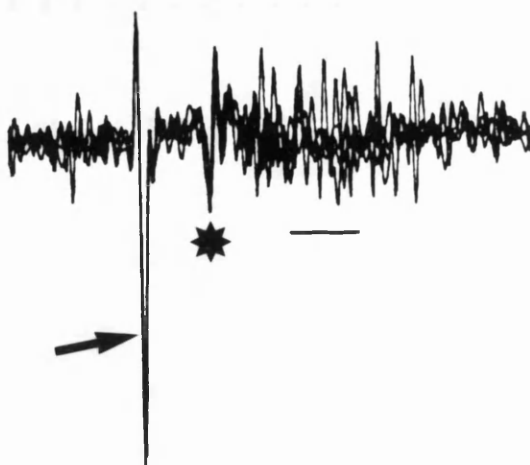
D. A photomicrograph of labelled cells (arrows) in the medial NTS labelled retrogradely by the injection of HRP into the NA shown in **C** and localised using the *o*-toluidine reaction.

Scale bars: **A and B.** 5 milliseconds. **C.** 1mm. **D.** 200 μ m.

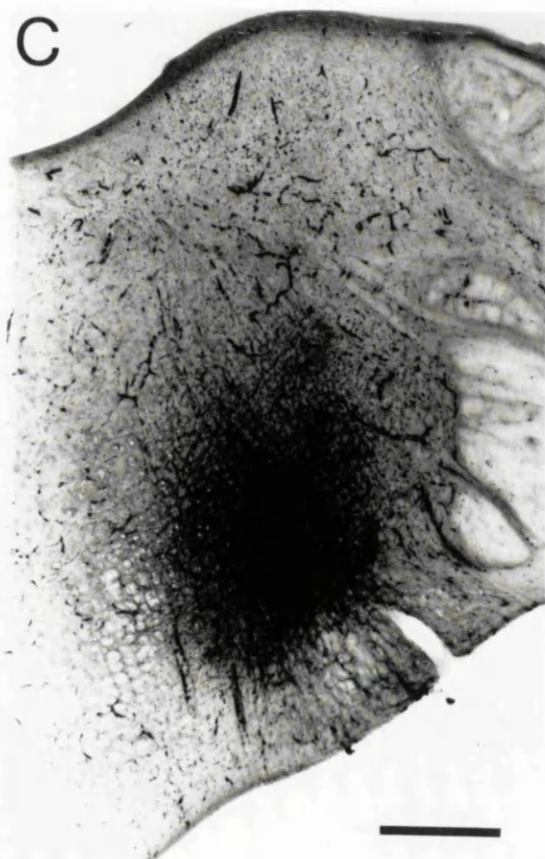
A



B



C



D

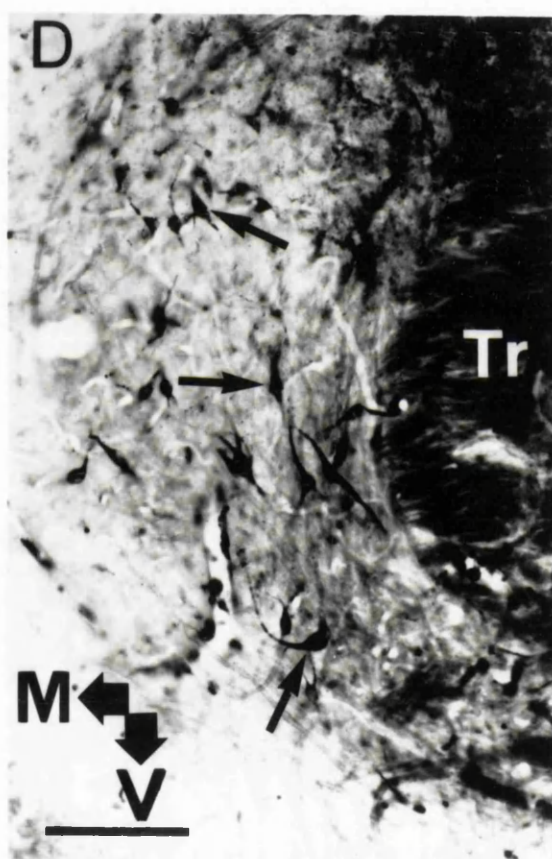


Figure 7.3. Diagrammatic representation of the NTS of the cat at various levels with respect to obex. The positions of retrogradely labelled neurones (black asterisks) ipsilateral to the injection of HRP into the NA shown in figure 7.1.C., are taken from three sections around the level of each diagram. Labelled neurones were found both bilaterally in the NTS, but the ipsilateral projection was heavier. The sub-nuclei are the same as those marked in figure 7.1).

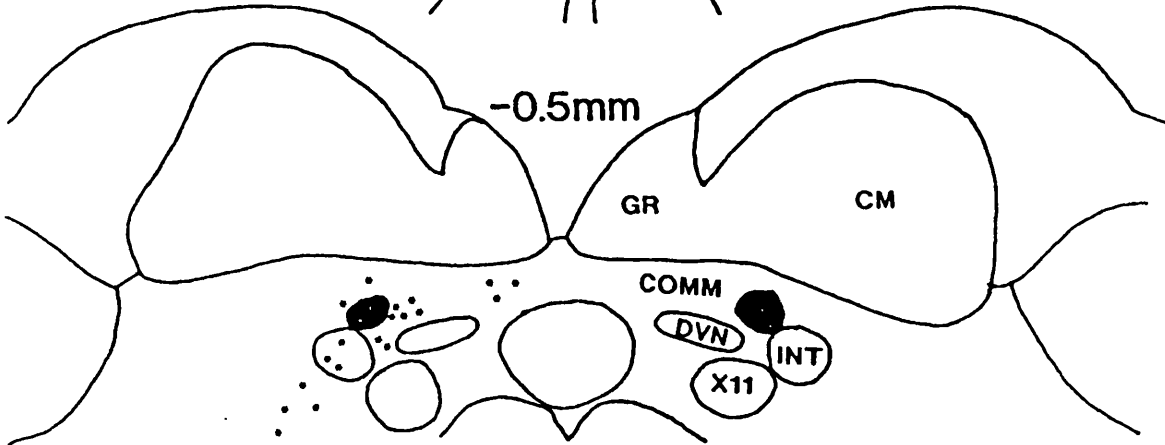
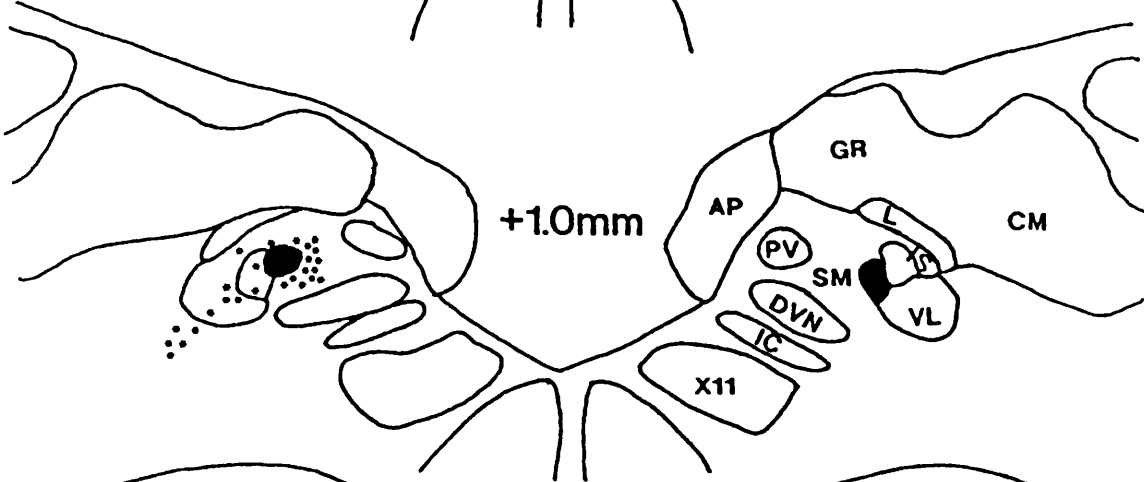
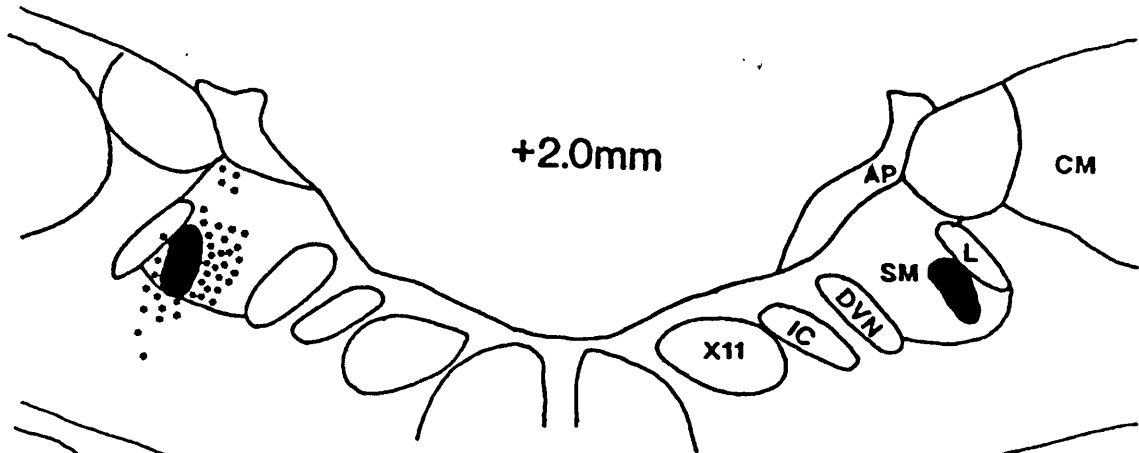


Figure 7.4.

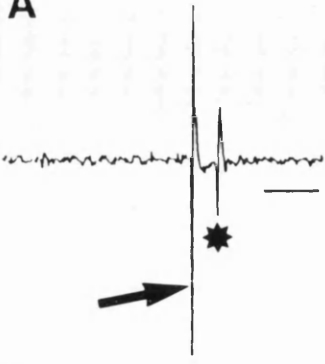
A and B. Recordings of activity in the NTS evoked to stimulation of the carotid sinus nerve. The arrow points to stimulus artefact and the dot is beneath evoked activity in both diagrams. **A** is a single sweep and **B** is two super-imposed sweeps showing that the evoked activity has a variable latency and is thus synaptic.

C. A photomicrograph of a biocytin ionophoretic injection in the commissural region of the NTS in the medulla of a cat. Biocytin is taken up by cells at the injection site (dense core of reaction product) and not by axons of passage as indicated by the lack of labelling in the tractus itself (**Tr**). Occasionally neurones are labelled at a distance from the injection site, but these usually have dendrites which run through the centre of the injection (arrow). The letter **L** is the lateral aspect of the medulla and **V** the ventral aspect.

D. A photomicrograph of anterogradely labelled terminals in the NA (arrows) in close apposition to unidentified cells following an injection of biocytin into the NTS. Biocytin was also detected in thick (open arrow) and fine calibre axons (curved open arrow).

Scale Bars: **A. and B.** 5 milliseconds. **C.** 100 μ m **D.** 40 μ m

A



B

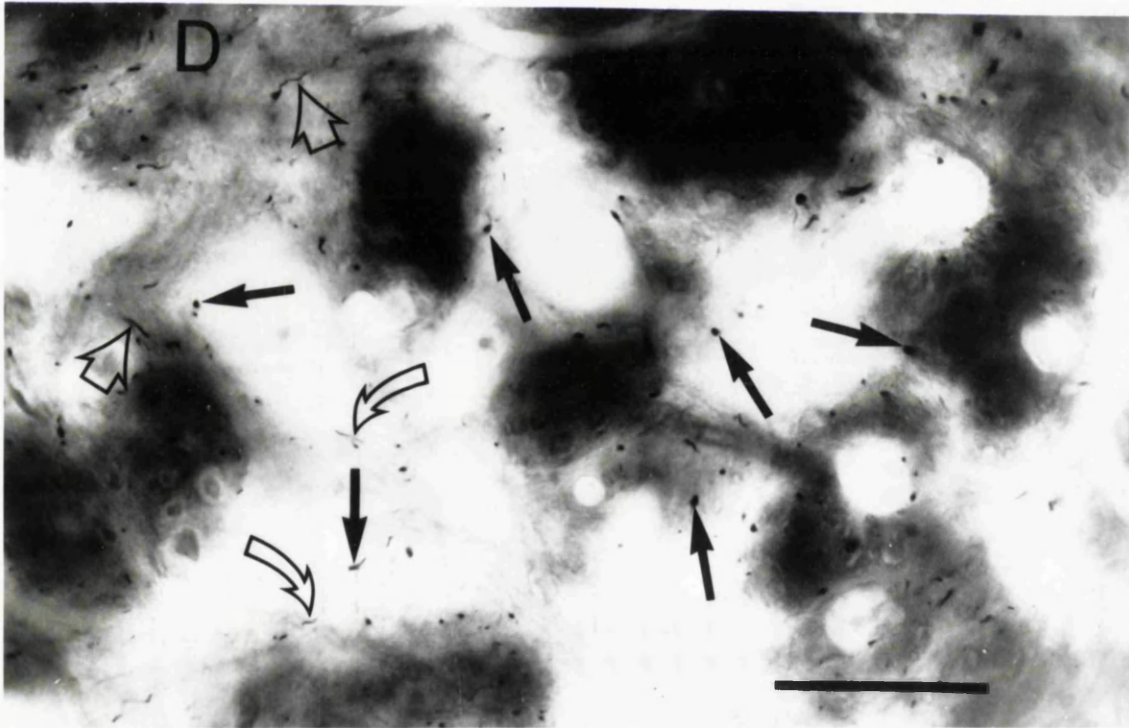
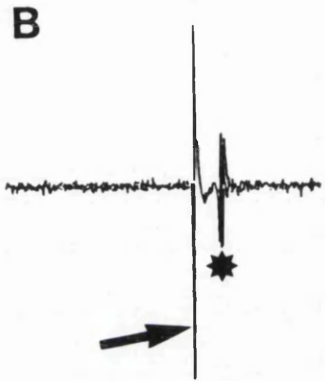


Figure 7.4.

A and B. Recordings of activity in the NTS evoked to stimulation of the carotid sinus nerve. The arrow points to stimulus artefact and the dot is beneath evoked activity in both diagrams. **A** is a single sweep and **B** is two super-imposed sweeps showing that the evoked activity has a variable latency and is thus synaptic.

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Scale Bars: **A. and B.** 5 milliseconds. **C.** 100 μ m **D.** 40 μ m

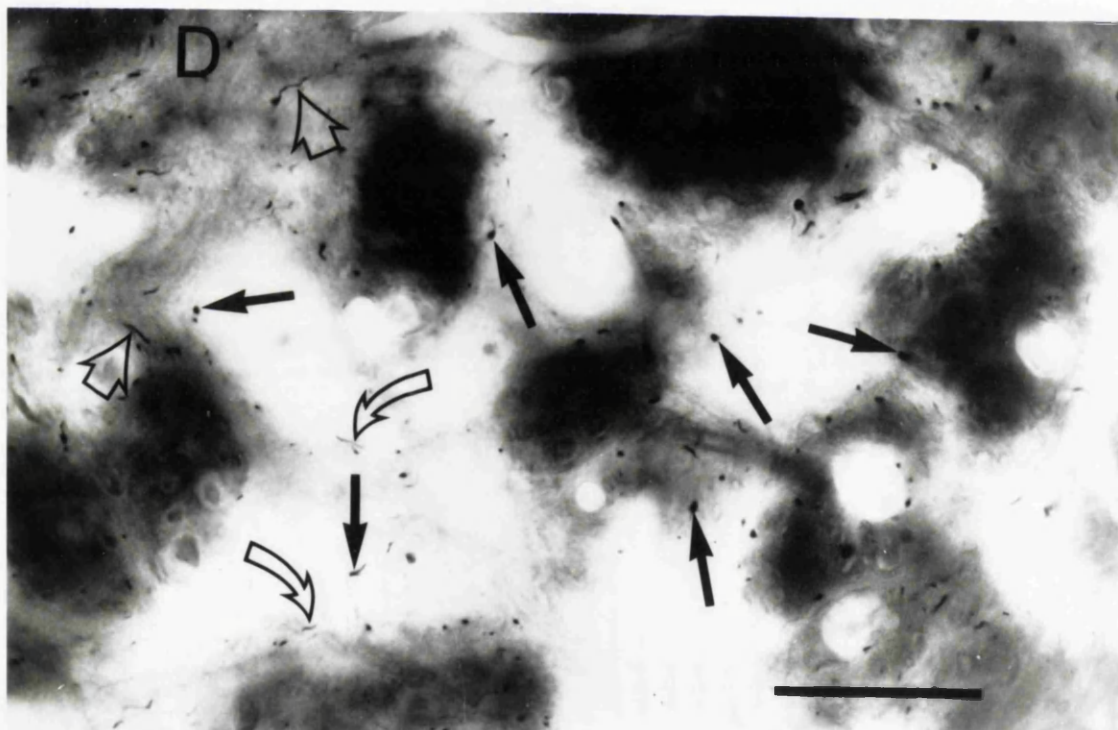
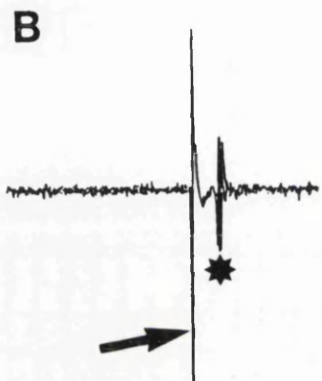
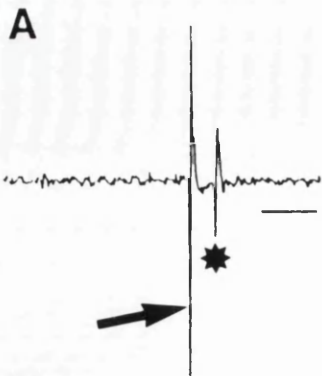


Figure 7.5. Diagrams of sections of the medulla oblongata of the cat illustrating the distribution of labelling of terminals (black dots) and fibres (lines) following an injection of biocytin into the commissural/medial NTS (black area). Labelled terminals are found mainly in a band radiating from the NTS to the VLM, although some are also found in the midline raphe nuclei, the olivary complex, the DVN and occasionally the hypoglossal nucleus.

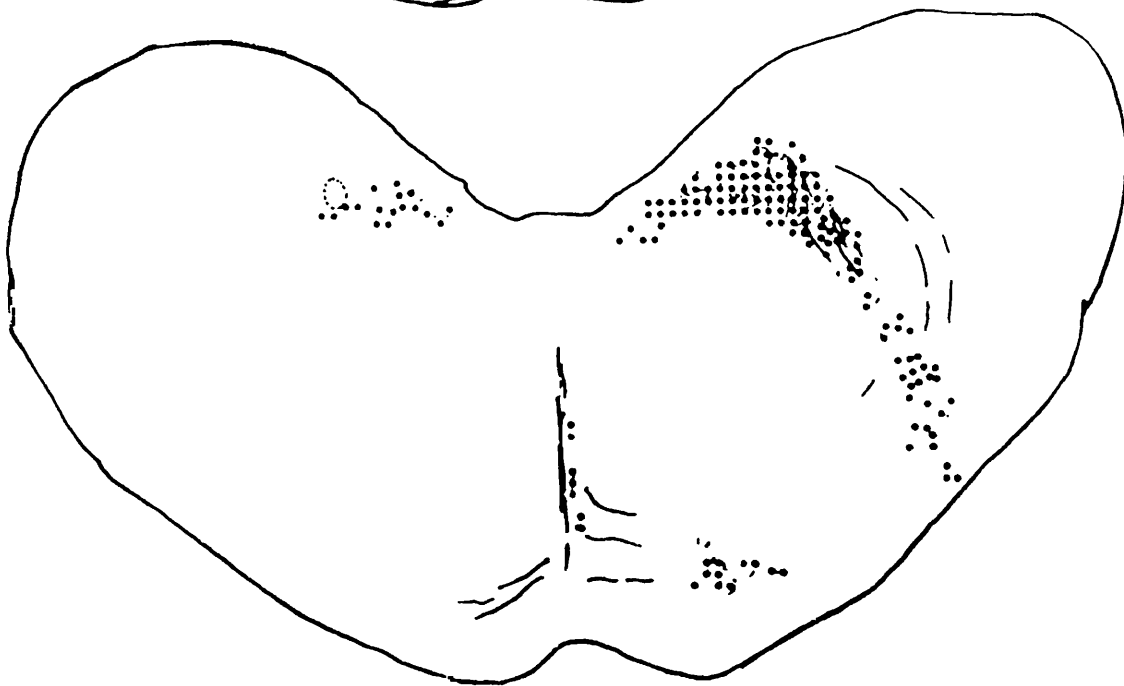
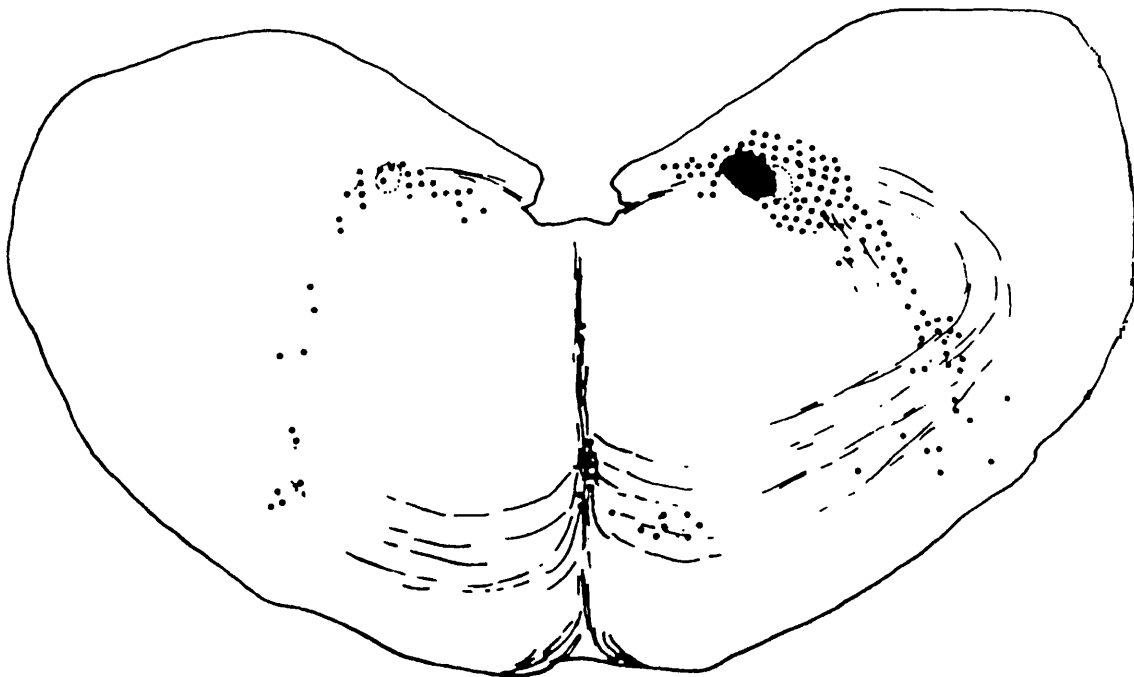
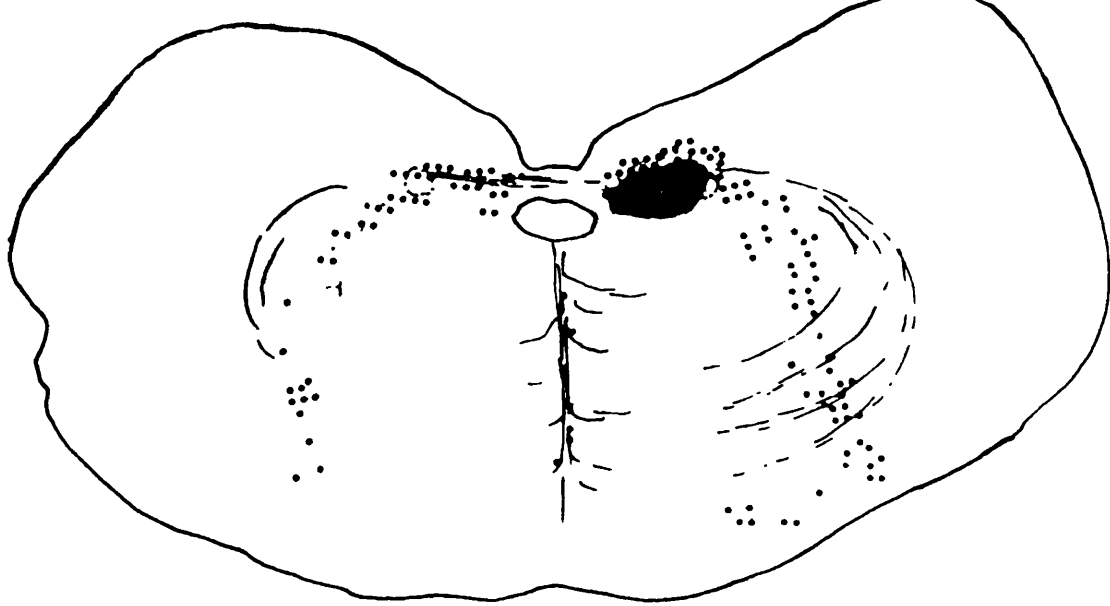


Figure 7.6. - Electron micrographs of boutons in the NA anterogradely labelled by the injection of biocytin into the NTS.

A. Labelled bouton forming an asymmetric type synapse with a dendritic shaft (large arrow). Also shown is unlabelled bouton (star), which forms a symmetric synapse (small arrows) with the dendritic shaft.

B. Labelled bouton in symmetric synaptic contact (large arrows) with unidentified dendritic structure. An unlabelled bouton (star) forms an asymmetric synapse (small arrows) with the same structure.

C. Labelled bouton in asymmetric contact (arrow) with dendritic structure.

D. Myelinated axon (star) containing peroxidase reaction product.

E. Labelled bouton in asymmetric contact with an unidentified structure (large arrows). An unlabelled bouton (star) forms a symmetric synapse with the same structure.

Common Scale bar in **B.** = 0.5 μ m.

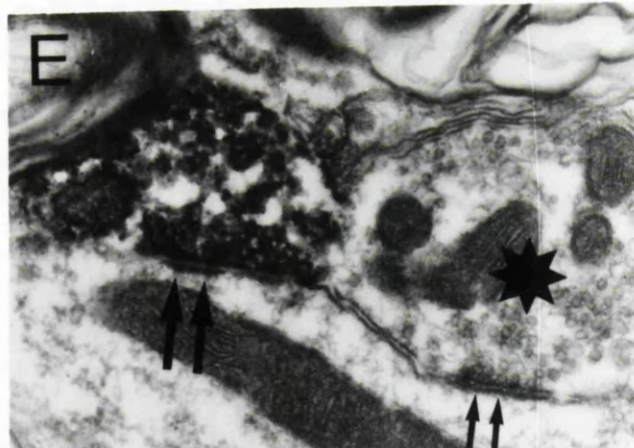
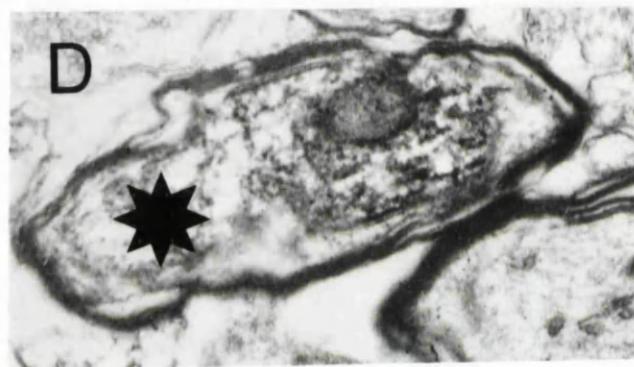
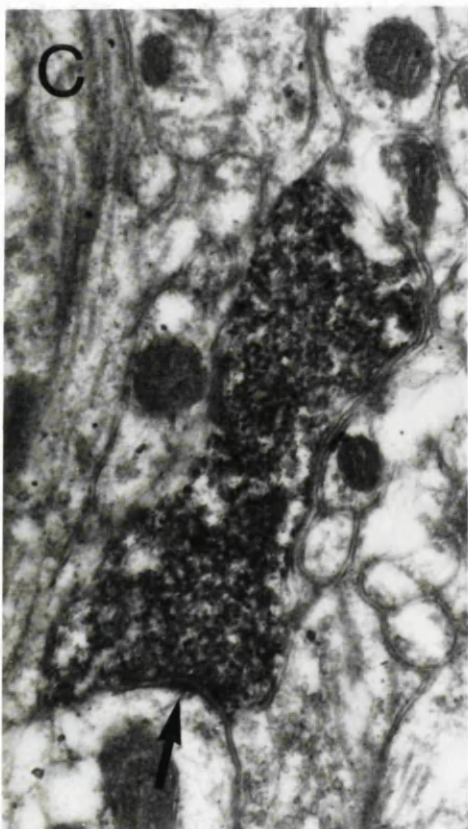
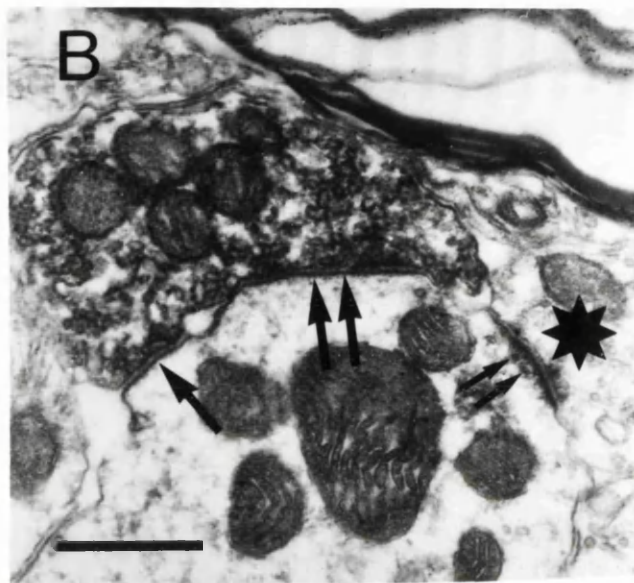
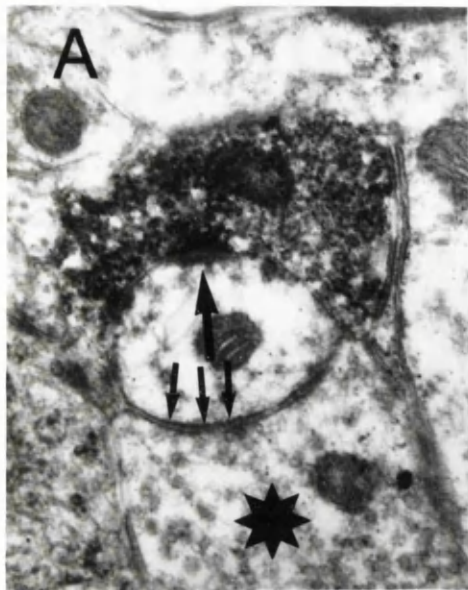


Figure 7.7. - A and B. Light micrographs of retrogradely labelled vagal preganglionic neurones in the nucleus ambiguus of the cat, with apposing anterogradely labelled boutons (large arrows) following an injection of biocytin into the NTS. Also in the vicinity of the VPNs are labelled axons (arrowheads), with boutons occasionally branching off along their lengths.

Common scale bar in **B.** 20 μ m.

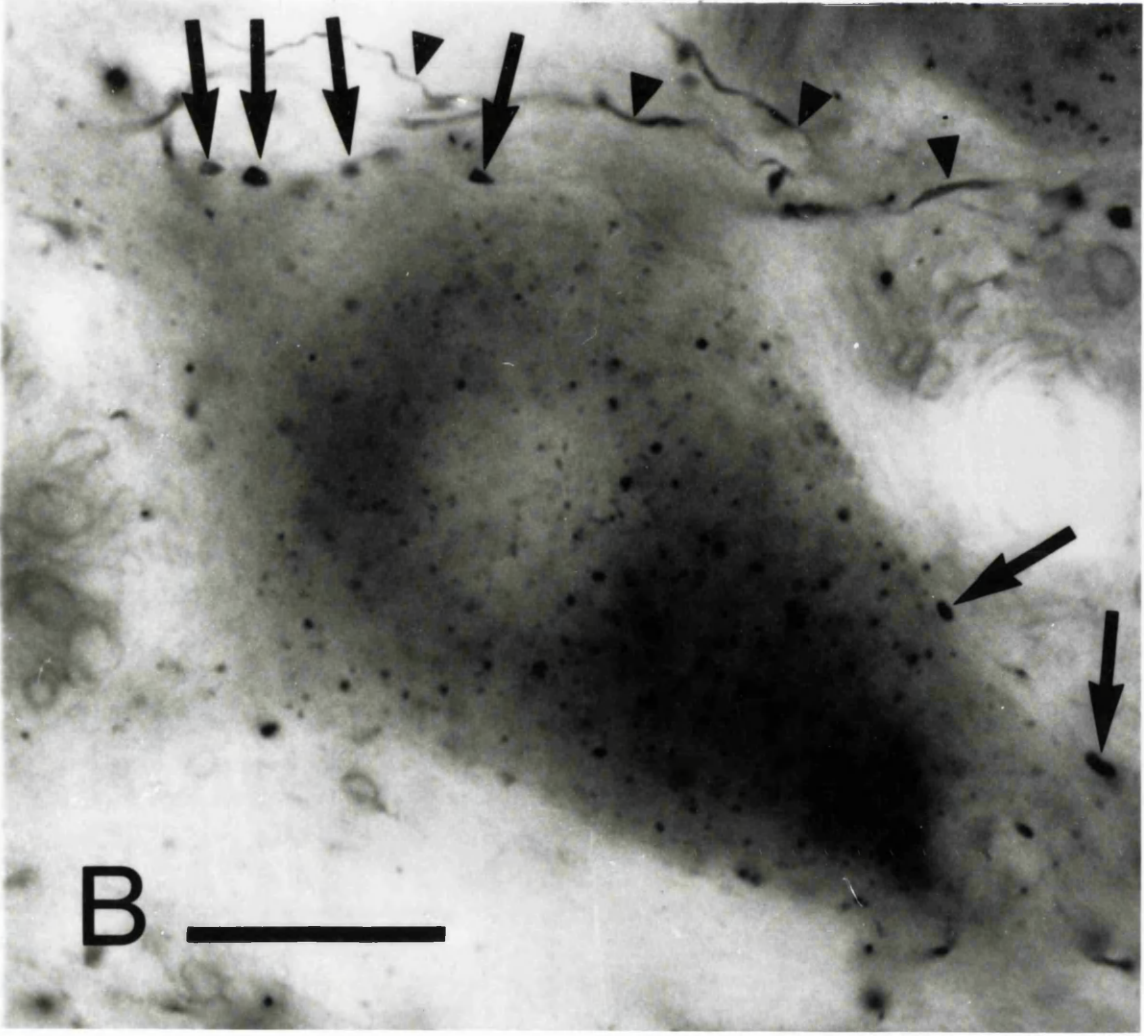
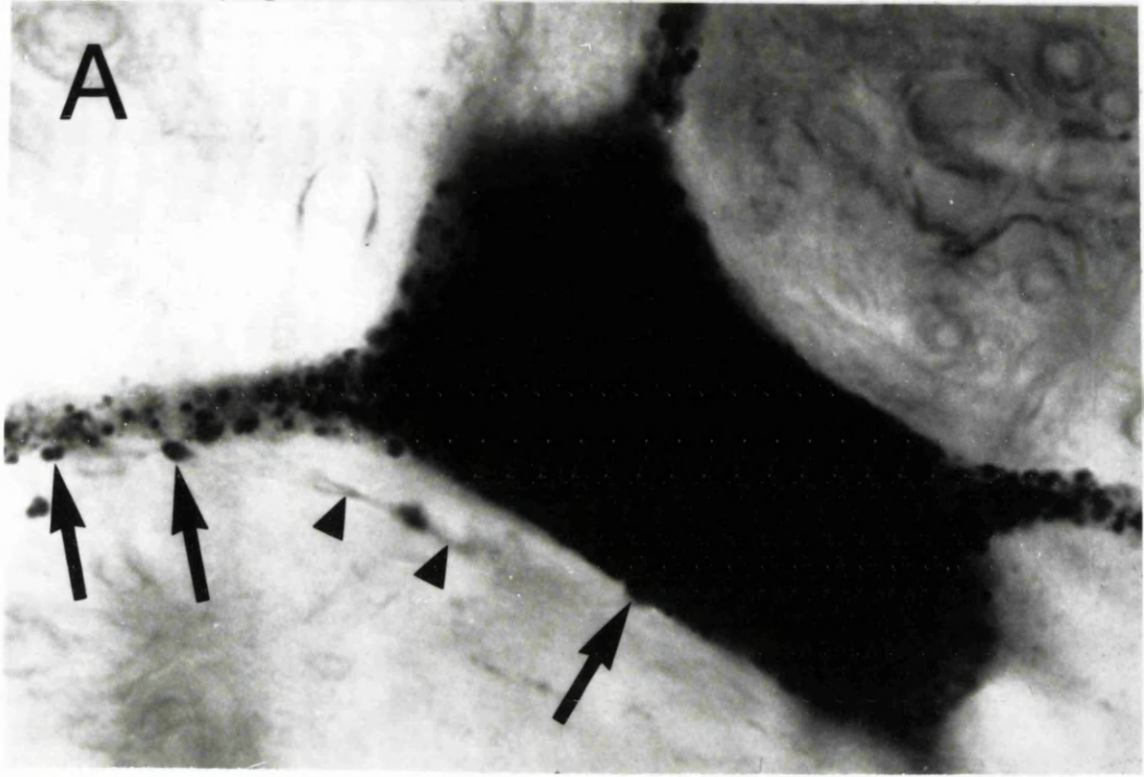


Figure 7.8. - Correlated light and electron micrographs of a bouton labelled by the injection of biocytin into the NTS, in synaptic contact with a retrogradely labelled VPN in the NA.

A. Light micrograph of a VPN containing HRP reaction product (curved open arrows) with apposing biocytin labelled boutons (small black arrows). The boutons appear to branch from a labelled axon (arrowheads) which passes through the region via a myelinated fibre tract (star). The broad open arrow points to a myelinated structure in close proximity to a bouton which apposes the VPN (large black arrow).

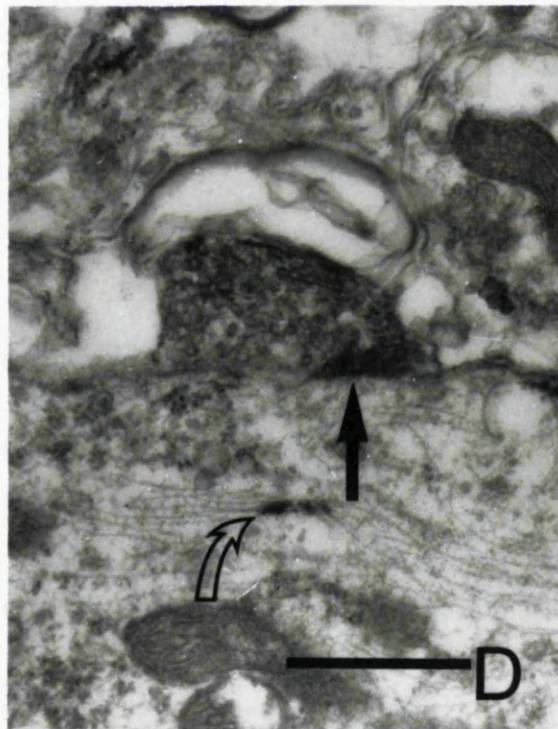
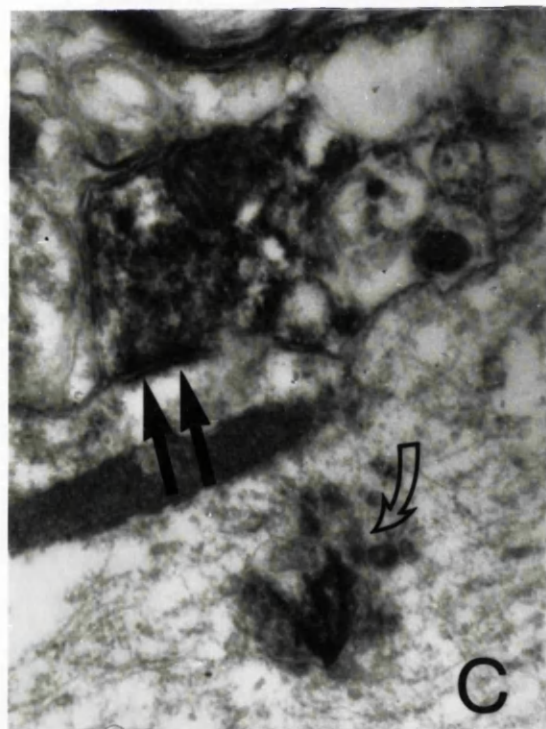
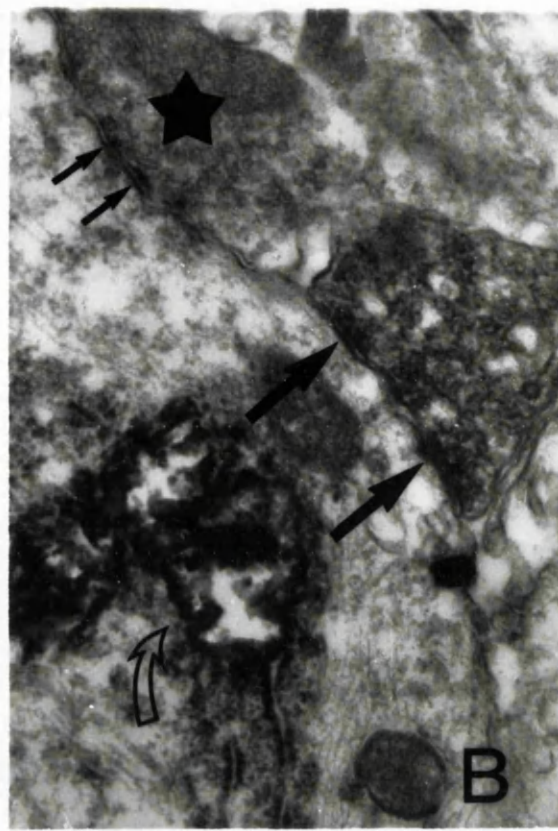
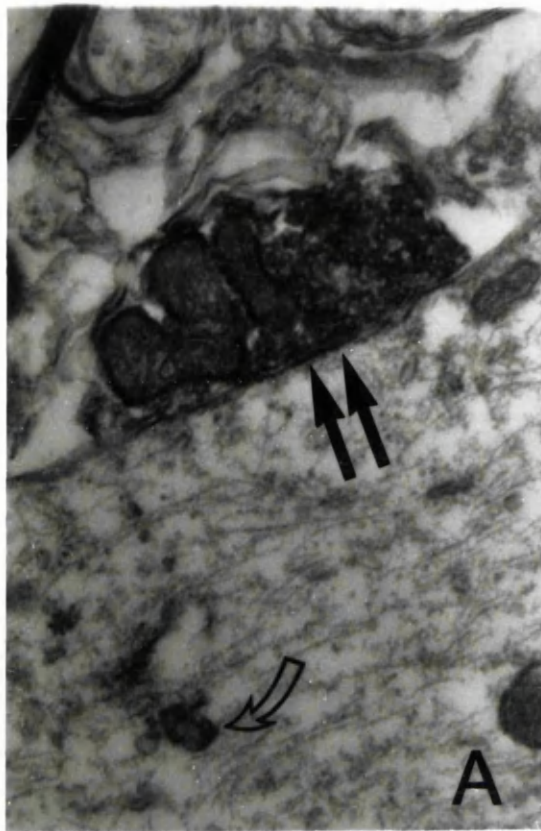
B. Electron micrograph of a region containing the myelinated structure (M) indicated by the broad open arrow in **A**. The adjacent anterogradely labelled bouton (shown in **A** by an arrow) can be seen apposing the membrane of the VPN, (arrow) which contains retrograde reaction product in the cytoplasm (curved open arrow).

C. A high power electron micrograph of the bouton in **B** indicating that it forms a synaptic contact (large arrow), which appears to be of the symmetric type, with the retrogradely labelled VPN. An unlabelled bouton (star) also forms a synaptic contact with the VPN (small arrows).

Scale Bars: **A.** 15 μ m **B.** 2 μ m **B.** 0.5 μ m

Figure 7.9. - Electron micrographs of boutons anterogradely labelled from cells in the NTS, in synaptic contact (black arrows) with VPNs in the NA containing retrograde reaction product (curved open arrows). An unlabelled bouton is shown in **B** (star), which also forms a synapse (small arrow) with the labelled VPN. Due to the very dense HRP reaction product the morphology of the vesicles within the bouton and the synapses is difficult to determine.

Common scale bar in **D** = 0.5 μ m.



7:4. Discussion.

In this study, neurones in the nucleus tractus solitarius were shown to be a source of input to an area of the nucleus ambiguus electrophysiologically identified as containing vagal preganglionic neurones (fig. 7.2 and 7.3). Furthermore, VPNS in the NA projecting to the cervical vagus nerve were identified as post-synaptic targets of boutons anterogradely labelled by the injection of biocytin into various sub-nuclei of the NTS (fig. 7.8. and 7.9.).

7:4.1. Technical Considerations.

7:4.1.A. Use of Biocytin as Anterograde Tracer.

Biocytin was used as an anterograde tracer for these studies for a variety of reasons. It has been shown that when ionophoretically injected into the central nervous system biocytin is taken up by cell bodies ^{AND DENDRITES} at the injection site and transported towards their axon terminals, but is not taken up by fibres of passage (King *et al*, 1989; Izzo, 1991). This was confirmed when injections were made in the region of the tractus solitarius as no labelling was found to occur in the fibres of passage in the tractus (fig. 7.4.C). In addition, the main advantage of biocytin over other anterograde tracers is that it has a fast transport time (approx. 3mm/hour; King *et al*, 1989; Izzo, 1991) and thus only acute surgical procedures were required, which enabled elaborate electrophysiological identification of injection sites. Furthermore, biocytin is suitable for electron microscopy since the detection system for biocytin is not immunological and a high glutaraldehyde concentration was used to

preserve ultrastructure without unduly hindering detection of biocytin (Izzo, 1991).

7:4.1.B. Retrograde Labelling.

Although VPNS could be labelled retrogradely from the cervical vagus nerve there were no labelled neurones following the injection of CT-HRP into the wall of the right atrium. The failure to retrogradely label CVPNS may be due to many reasons. It is unlikely that the CT-HRP was at fault since the CT-HRP used in these experiments was from the same batch as used successfully to retrogradely label CVPNS in some rats in chapter 3. It is also unlikely that the recovery period of 48 hours was insufficient to allow retrograde transport of CT-HRP to the cell bodies of CVPNS as HRP applied to a cardiac branch of the vagus nerve can be detected in CVPNS after a similar time period (*e.g.* Izzo *et al*, 1988). One possible reason is that the injections of CT-HRP were not placed in the sino-atrial node, since the atria of the cat are somewhat larger than in the rat.

7:4.2. Specificity of Connections.

Following injection of HRP into a region of the nucleus ambiguus where antidromic potentials were recorded to stimulation of the cervical vagus nerve retrogradely labelled neurones were observed in the medial, commissural, ventral and ventrolateral sub-nuclei of the nucleus tractus solitarius (figs. 7.2 and 7.3.). Previous studies have described similar patterns of labelling following injections of retrograde tracer into areas of the NA defined as cardioinhibitory by

electrical stimulation (Stuesse and Fish, 1984) or areas containing neurones with respiratory related activity (Smith *et al*, 1989). However, since the NA contains neurones with many functions (see sections 1:3.5; as demonstrated in Chapters Three and Six) this type of study cannot determine the post-synaptic targets of labelled neurones. This is illustrated by the injection sites of this study being almost identical with those into the ventral respiratory group of another study (Smith *et al*, 1989), despite the different functional identification of the injection sites. Thus these experiments describe the distribution of neurones in the NTS which innervate the nucleus ambiguus, but give no information regarding the post-synaptic targets of the labelled neurones.

In this study some of the post-synaptic targets of NTS neurones were identified by combining anterograde labelling from the NTS with retrograde labelling from the cervical vagus nerve and electron microscopy (Fig. 7.8 and 7.9). With these techniques VPNs in the NA were identified as post-synaptic targets of anterogradely labelled boutons (Fig. 7.8 and 7.9). This improves on previous studies which, although they have identified terminal like labelling in the region of VPNs following injections of anterograde tracers into the NTS, have not established close associations or synaptic relationships by electron microscopy (Ross *et al*, 1985; Cunningham and Sawchenko, 1989).

Although VPNs in the NA were identified as post-synaptic targets of biocytin labelled boutons, the projections of these VPNs is difficult to distinguish since they were labelled retrogradely from the cervical vagus nerve, which contains axons innervating many organs.

There was no obvious organisation of VPNS in the NA similar to that observed in the rat (See chapter 3) to aid identification of functions of VPNS, perhaps because there were fewer retrogradely labelled cells. Further experiments are thus required to determine the target organs of VPNS innervated by neurones in the NTS.

In addition to vagal preganglionic neurones projections from the NTS may also innervate some of the other neurones in the NA. Other studies have shown that injection of the anterograde tracer PHA-L into the medial NTS anterogradely labels fibres in close apposition to PNMT immunoreactive neurones of the C1 group (Hancock, 1988), which may be important in the baroreflex as these neurones have also been implicated in cardiovascular control (see section 1:3.5.B). Furthermore, boutons labelled by injections into the NTS may also innervate neurones in other parts of the medulla since in this present study anterogradely labelled terminals were observed in other nuclei with diverse functions such as other NTS nuclei, hypoglossal nuclei, dorsal vagal nuclei and raphe nuclei (fig 7.5), although the post-synaptic targets of these boutons were not identified. Thus more experiments are required to establish the identity of neurones in the medulla innervated by neurones in the NTS.

7:4.3. Organisation of Projections From the NTS to the NA.

The pattern of labelling in this study suggests that there may be some organisation of projections from the NTS to the NA. Following injection of HRP into the NA the majority of labelled cells in the NTS were at the same level as the injection site and anterogradely

labelled fibres in the NA were also at the same level as the injection sites of biocytin into the NTS (Fig. 7.5). Since there is a rostrocaudal separation of VPNs in the NA in relation to their projections (see section 1:3.1), this implies that there is an organisation of their inputs from the NTS. However, it is possible that the observed patterns of projections from the NTS to the NA are not a reflection of organisation but are an experimental artefact, perhaps due to limited transport of tracers, but this seems unlikely since the same organisation of projections were observed in both retrograde tracing from the NA and anterograde tracing from the NTS.

The pattern of labelling from the NTS to the NA may reflect a relationship between the input to neurones in the NTS and the organisation of their efferent connections. However, this does not appear to be the case for visceral afferent inputs to the NTS as they are not organised rostro-caudally (Kalia and Mesulam, 1980b), which may indicate that visceral afferent input with one function (*e.g.* from the baroreceptors) can affect VPNs in the NA with diverse functions. Since the NTS also receives input from other regions of the CNS (see Loewy, 1990) the projections of NTS neurones may reflect their input from these regions. However, further experimental work is required to determine if this is the case.

7:4.4. Possible Transmitters in NTS neurones innervating VPNs.

Studies involving dual labelling with immunofluorescence techniques have demonstrated that the injection of PHA-L into the NTS anterogradely labelled fibres in the vicinity of VPNs in the NA

that had been labelled retrogradely from the oesophagus, and these fibres also contained immunoreactivity for somatostatin (Cunningham and Sawchenko, 1989a) and enkephalin (Cunningham *et al*, 1989b). However, the synaptic relationship between labelled boutons and retrogradely labelled VPNs was not determined by electron microscopy and it is not conclusive that these transmitters are present in neurones in the NTS which innervate VPNs.

Other studies which suggest the neurotransmitters in neurones projecting from the NTS to the VLM involve immunocytochemical detection of neurotransmitters in retrogradely labelled neurones in the NTS following injections of retrograde tracers into the VLM (Morilak *et al*, 1989; Somogyi *et al*, 1989; Blessing, 1990). In this way, separate groups of neurones in the NTS which project to the VLM have been shown to contain substance P and enkephalin (Morilak *et al*, 1988), tyrosine hydroxylase but not PNMT and which are noradrenergic or dopaminergic (Somogyi *et al*, 1989) and glutamate decarboxylase, an enzyme involved in the synthesis of GABA (Blessing, 1990). In addition, neurones in the NTS containing excitatory amino acids have been identified as projecting to the VLM by the injection of tritiated D-aspartate into the RVLM (Somogyi *et al*, 1989). However, the identity of the postsynaptic targets of these labelled NTS neurones was not ascertained in this study.

7:4.5. Functional Implications.

Since the NTS is the main site of termination of visceral afferents (see figure 7.1), projections from neurones in this region to VPNs in the NA identifies a direct pathway for reflex control of vagal output. However, as VPNs were labelled retrogradely from the cervical vagus nerve it is difficult to ascribe functions to this pathway. Nevertheless, some functions may be postulated by considering the functions of the injection sites, but some caution must be applied as although sub-nuclei of the NTS predominantly receive afferent input with specific functions, some overlap exists (see fig. 7.1) and neurones in any one sub-nucleus receive converging information (Jordan and Spyer, 1987).

Despite the problems of ascribing functions to the connection from the NTS to VPNs in the NA, the possibility that the pathway may subserve many functions is illustrated by connections from the commissural NTS to VPNs, as this sub nucleus of the NTS receives diverse afferent input from cardiovascular, respiratory and gastrointestinal systems (see figure 7.1; Loewy, 1990).

Since some biocytin injection sites were physiologically identified as receiving carotid sinus nerve input (Fig. 7.4), which conveys information to the NTS from chemo- or baro-receptors situated in the carotid sinus (see Spyer, 1990), connections from neurones in the NTS which receive this input to VPNs provides an anatomical substrate for a direct route for reflex control of vagal output involved in regulation of blood pressure. However, physiological experiments suggest that the baroreceptor input to CVPNs is

mediated through two pathways, since excitation of CVPNs occurred 20-60msec or 70-110ms after natural stimulation of the carotid sinus baroreceptors (McAllen and Spyer, 1978b). The conduction delay for both of these pathways is representative of multisynaptic pathways and is thus in variance with the direct route suggested by this study. This discrepancy is difficult to reconcile, but it is possible that there is both direct and indirect pathways for the conveyance of baroreceptor input to CVPNs, and electrophysiological studies have noticed only the long latency pathways due to recordings being made from only a small number of neurones (N=23, McAllen and Spyer, 1978b; n=24, Gilbey *et al*, 1984). Alternatively, the direct pathways for relay of afferent input to CVPNs may be more common and have not been identified due to intrinsic properties of CVPNs. Recently, a group of neurones has been identified *in vitro* in the NA of the guinea pig which exhibit delayed excitation (Johnson and Getting, 1991). A positive stimulus applied to these neurones following a hypolarising current results in an action potential after a delay of 50-450ms. These cells may be VPNs as they were filled intracellularly with Lucifer Yellow and their axons travelled in a dorsomedial direction similar to that of VPNs (Bieger and Hopkins, 1987). Furthermore, these cells may be CVPNs as their somata were significantly smaller in size than other neurones in the region with different properties, and CVPNs were found to be significantly smaller than other VPNs in the NA in the study in Chapter Three. Thus the baroreceptor input to CVPNs may have a long-latency due to the fact that CVPNs have delayed excitation properties and not because it is relayed through a multi-synaptic pathway.

Projections from the NTS to VPNs in the NA may be important in controlling bronchomotor tone. VPNs in the NA are known to maintain smooth muscle tone in the airways (Widdicombe, 1966) and stimulation of the baroreceptors or peripheral chemoreceptors causes increases in the activity of branches of the vagus innervating the lungs (Nadel and Widdicombe, 1962; Mitchell *et al*, 1985). Since some injections of anterograde tracers were made into regions of the NTS which receive these afferents, the pathway shown here provides an anatomical substrate for a direct route for these changes. However, further studies are required to identify VPNs in the NA as projecting to the NA.

Monosynaptic connections from the NTS to VPNs may also mediate oesophageal reflexes. In this study injection of HRP in the region of the cNA, which has been previously shown to contain neurones innervating the oesophagus (Kalia and Mesulam, 1980b), retrogradely labelled neurones in the medial NTS (fig. 7.2; and see - Cunningham and Sawchenko, 1989a in the rat). Furthermore, injection of PHA-L into the central subnucleus of the NTS, which is a part of the medial NTS and which receives afferent input from oesophageal afferents (Altshchuler *et al*, 1989), anterogradely labels boutons in the region of VPNs labelled retrogradely from the oesophagus (Cunningham and Sawchenko, 1989a,b).

The morphology of the anterogradely labelled boutons in synaptic contact with VPNs may be indicative of different roles for neurones in the NTS in the control of VPNs. Boutons forming both symmetric and asymmetric type synapses were identified in contact with VPNs, and these have been associated with inhibitory and excitatory

functions respectively (Gray, 1973). For example, symmetrical type connections made by boutons labelled from the NTS may be involved in the post-inspiratory excitation observed in cardiac vagal preganglionic neurones, whereas symmetric type boutons may play a role in the inhibition of CVPNs in inspiration (Gilbey *et al*, 1984). Similar models may explain rhythmic activity in VPNs with other projections (see section 1:3.5).

The possibility that neurones in the NTS may innervate neurones in the VLM with different functions may have some important functional implications. Axons from neurones in the NTS which innervate VPNs may also be pre-synaptic to other neurones, and a single NTS neurone could theoretically innervate both CVPNs and depressor neurones. Thus neurones in the NTS excited by increases in blood pressure input which in turn excite CVPNs to increase parasympathetic outflow may also excite depressor neurones to simultaneously inhibit sympathetic outflow, thus eliciting a depressor response. If such connections occur, these NTS neurones may contain excitatory amino acids as the injection of tritiated amino acids into the CVLM retrogradely labels neurones in the NTS (Somogyi *et al*, 1989). Conversely, inhibition of CVPNs and the VLDNs may be part of a response to low blood pressure and thus elicit a pressor response. Neurones in the NTS inhibitory to CVPNs and VLDNs may contain GABA as injection of retrograde tracer into the CVLM retrogradely labels neurons in the NTS which are also immunoreactive for glutamic acid decarboxylase, an enzyme involved in the synthesis of GABA (Blessing, 1990). In addition microinjection of GABA agonists into the CVLM elicits a pressor response and a tachycardia, and the tachycardia is due to

withdrawal of vagal inhibition of heart rate since it was not abolished by pre-treatment with phentolamine (Willette *et al*, 1983b).

7:4.6. Experimental Implications.

Projections from the NTS to the NA were approximately at the same level as the injection site, which could enable recordings to be made from connected neurones in the NTS and NA in *in vitro* brain slice preparations. If combined with intracellular filling of paired neurones and electron microscopy this could yield important information about synaptic function and associated morphology.

7:4.7. Summary.

Vagal preganglionic neurones in the NA of the cat are monosynaptically innervated by neurones in regions of the nucleus tractus solitarius which receive visceral sensory afferent input from the vagus or carotid sinus nerves.

Chapter Eight.

General Discussion.

The experiments in this thesis utilised retrograde labelling techniques to investigate the organisation and morphology of vagal preganglionic neurones in the medulla oblongata of the rat, with specific reference to cardiac vagal preganglionic neurones. With these methods it was observed that CVPNs were located mainly in the nucleus ambiguus and subsequent studies concentrated on retrogradely labelled neurones in this region. In order to identify the chemical nature of some of the synaptic inputs to VPNs in the NA, retrograde labelling techniques were combined with immunocytochemistry and electron microscopy. These techniques identified serotonin, substance P and neuropeptide Y in boutons in synaptic contact with CVPNs. In addition, retrograde labelling was also combined with electron microscopy and anterograde tracing methods to establish that neurones in the NTS were a source of input to VPNs in the NA of the cat.

8:1. Organisation of CVPNs.

The distribution of CVPNs in the medulla was studied by injection of retrograde tracers into the myocardium. However, spillage of this tracer was thought to occur due to the difficulties of injecting the small surface area of the fast beating myocardium, resulting in labelled neurones that were not CVPNs. Nevertheless, labelled CVPNs could be distinguished from neurones labelled due to spillage as they were heavier labelled (see section 3.4). Using this criteria to identify CVPNs it was

observed that they were located mainly in the vINA, outside the main group of VPNS which lie dorsally in the compact and semi-compact regions of the NA.

The study of organisation of VPNS in these experiments was limited by the fact that the retrograde labelling techniques applied were unlikely to localise all of these neurones. This was highlighted by the fact that different numbers of CVPNS were labelled in different animals. Thus the degree of organisation of VPNS in the NA is probably greater than that observed in these studies. This view is supported by a recent immunocytochemical study of acetylcholine synthesising neurones in the ventrolateral medulla of the rat (Ruggerio *et al*, 1990), which are probably VPNS since these are the main group of cholinergic neurones in this region (Palkovits and Jacobowitz, 1974; Armstrong *et al*, 1983; Houser *et al*, 1983; Butcher and Woolf, 1984). In their investigation, Ruggerio *et al* (1990) identified a distinct group of choline acetyl transferase (CHAT) containing neurones in the vINA which consisted of neurones with a soma diameter 16-30 μ m (Ruggerio *et al*, 1990), similar in size to CVPNS in the vINA (Diameter 20-28 μ m - table 3.2.). Furthermore, these CHAT neurones were in greater numbers and located in a more discrete group than retrogradely labelled CVPNS described in chapter three. Thus no attempts were made to quantify the results of retrograde labelling in these studies.

Results from a number of previous studies also provide information on the location of CVPNS (Rat - Nosaka *et al*, 1979a; Hopkins and Armour, 1982; Bieger and Hopkins, 1987; Miselis *et*

al, 1989. Pig - Hopkins *et al*, 1984). These studies report that CVPNs were located mainly in the vINA, segregated from other VPns in the NA. Interestingly, CVPNs in the pig formed a distinct nucleus of cells in the vINA (Hopkins *et al*, 1984). This may indicate that the organisation of CVPNs discernible in rat increases with species complexity, and future experiments may determine if this is the case in other higher species, including man.

8:1.1. The Size of CVPNs may be Related to Their Organisation.

CVPNs in the vINA were found to be significantly smaller in size than other VPns in the NA (table 3.2). This difference may indicate that they are located with other neurones of similar size. Comparison of the morphology of CVPNs with PNMT neurones in the VLM (some of which may innervate sympathetic preganglionic neurones in the intermediolateral cell column - Ross *et al*, 1984), reveals some similar characteristics - dendritic arborisations mainly within the VLM, soma diameters in the range 16-24 μ m and an abundant cytoplasm containing an eccentrically located nucleus with few membrane invaginations (Milner *et al*, 1987).

The smaller soma diameter of CVPNs and PNMT immunoreactive neurones in comparison to other VPns in the NA (*e.g.* see table 3.1), may be a basis for functional specialisation in the VLM. *In vitro* intracellular recordings from neurones in the NA of the guinea pig which were subsequently filled with fluorescent dye revealed that size of neurones was related to their

electrophysiological characteristics (Johnson and Getting 1991). The smallest neurones exhibited a delay in the onset of action potential firing when cells were hyperpolarised prior to a depolarising current, a property known as delayed excitation (DE cells). The larger cells could be divided into two groups. One of these groups displayed post-inhibitory rebound (PIR), which was a transient depolarisation that produced at least one action potential when the cell was released from hyperpolarisation. The other group of larger cells displayed neither DE or PIR. However, none of the cells were identified as VPNS. Nevertheless, it may be reasonable to presume that the size of VPNS is related to their electrophysiological characteristics. However, further experiments are required on identified VPNS before they are ascribed specific electrophysiological properties. These would require retrograde labelling of VPNS prior to *in vitro* recordings, perhaps with a fluorescent dye, and subsequent recording from these cells.

8:1.2. The Location of CVPNS may be Related to their Functions.

Vagal preganglionic neurones in the medulla can be divided broadly into two groups - those in the DVN generally innervate sub-diaphragmatic structures (Kalia and Mesulam, 1980b) and those in the NA innervate mainly supra-diaphragmatic structures (Kalia and Mesulam, 1980b; Bieger and Hopkins, 1987). The observations in this thesis are consistent with a topographical basis for the location of CVPNS in the NA. However, a few CVPNS were also found in the DVN, as also described in other neuroanatomical (Cat - Sugimoto *et al*, 1979; Geis and Wurster,

1980a; Kalia and Mesulam, 1980; Bennett *et al*, 1981; Geis *et al*, 1981; Kalia, 1981; Miura and Okada, 1981; Ciriello and Calaresu, 1982. Dog - Hopkins and Armour, 1982, 84; Plecha *et al*, 1988. Rat - Nosaka *et al*, 1979a; Miselis *et al*, 1989. Pig - Hopkins *et al*, 1984) and electrophysiological studies (Jordan *et al*, 1982), and it remains to be determined if there are functional differences between CVPNs in both nuclei (discussed further in section 3:4).

The organisation of CVPNs raises the possibility that the location of CVPNs in the medulla is related to their functions. There is evidence to suggest that CVPNs may be located mainly in the vNA due to their involvement in the control of the cardiovascular and respiratory systems. This hypothesis is supported by the fact that other neurones in this region are also involved in the control of these systems (see below).

The firing patterns of CVPNs indicate that they may be located with other neurones involved in the control of the respiratory system. CVPNs in the NA have been shown to discharge with respiratory related rhythms as they fire action potentials predominantly in post-inspiration (McAllen and Spyer, 1978a; Jordan *et al*, 1982; Gilbey *et al*, 1984). Such a pattern of activity is common to other neurones in the VLM since this region contains the functionally defined Ventral Respiratory Group (see section 1:3.5.A; Feldman, 1986), which contains neurones involved in the central control of respiration. These include neurones in the VLM which project to motoneurones in the spinal cord (which in turn innervate respiratory muscles - see

Ellenberger and Feldman, 1990a, b, c), the NTS (Kalia et al, 1979; Ellenberger and Feldman, 1990a) and other parts of the contralateral and ipsilateral VRG (Ellenberger and Feldman, 1990a).

The discharge patterns of other VPNS in the NA also supports a grouping of CVPNS on the basis of a role in the control of respiration. Respiratory related discharge patterns are common to most of the VPNS in the NA as VPNS innervating the laryngeal muscles (Barillot *et al*, 1984,90), the trachea (Mitchell *et al*, 1985) and the lungs (McAllen and Spyer, 1978a) have respiratory related firing patterns, while the firing patterns of VPNS innervating the oesophagus are unknown. Indeed, recent experiments suggest that the contribution of VPNS to the VRG has been hitherto underestimated, at least in the rat (Zheng *et al*, 1991). Intracellular recordings were made from neurones in the VRG which could not be antidromically activated by stimulation of the cervical vagus nerve, but which may have been VPNS as they were found to have axons leaving the medulla in the vagal nerve tract upon dye filling (Zheng *et al*, 1991).

The location of CVPNS in the vNA may be indicative of a degree of organisation of neurones in the VLM further to that based on respiratory related activity. VPNS in the vNA overlap with the caudal depressor region and the rostral pressor region (see Guyenet, 1990; section 1:3.5.B). This has been demonstrated anatomically since catecholamine containing neurones, (which are often presumed to have pressor and depressor roles), have been shown to be intermingled with VPNS in this region (Ritchie

et al, 1982). These neurones also discharge with patterns that can be related to respiration (see Gebber, 1990) and may be located in the VLM on this basis. However, these neurones and CVPNs have a direct role in the control of the heart and the cardiovascular system. This role may be reflected by their distinct location in the VLM, ventral to the dorsal group of VPNs and the VRG. Thus neurones in the vINA may constitute a sub-set of cells in the VLM which display respiratory related activity but which have specialised roles in the control of the cardiovascular system.

The reason for the location of VPNs in the NA with other neurones involved in the control of the respiratory and cardiovascular systems may be that they receive common inputs. A physiological basis for this assertion exists since some experiments indicate that VPNs receive the same central respiratory drive as other neurones. Mitchell *et al* (1985) recorded pressure changes in the lungs and airways which peaked simultaneously with phrenic nerve activity. The pressure changes were vagally mediated since they were reduced in preparations pre-treated with atropine and there was an augmentation of activity recorded in pulmonary branches of the vagus nerve concomitant with increases in pressure. The simultaneous changes in airway pressure and phrenic nerve activity were taken to indicate that vagal preganglionic neurones innervating the airways received the same central respiratory drive as phrenic motoneurones (Mitchell *et al*, 1985).

Other studies suggest that neurones in the VLM share common

inputs. In the present experiments, 5-HT, substance P and NPY were localised in boutons in synaptic contact with CVPNs, but all three neurotransmitters have also been shown to affect other neurones in the VLM involved in the control of the cardiovascular and respiratory systems. Injections of 5-HT_{1a} agonists inhibit pressor neurones (Dalton, 1986; Ramage, 1990) while 5-HT₂ receptor agonists excite pressor neurones (King and Holtman, 1989; Clement and McCall, 1990). In addition, 5-HT_{1B} and 5-HT₂ receptor agonists decrease the activity of the recurrent laryngeal and phrenic nerves (King and Holtman, 1989). Likewise, a substance P agonist appears to excite neurones in the pressor region (Urbanski *et al*, 1989) and application of substance P to the ventral medullary surface increases tracheal muscle tone (Haxhiu *et al*, 1989). Neuropeptide Y appears to increase blood pressure through excitation of pressor neurones (Tseng *et al*, 1988), but a role for NPY in the control of respiration remains to be established. However, further neuroanatomical studies are needed to identify the post-synaptic targets of neurones containing these transmitters to determine if their effects are through direct or indirect actions on neurones with these functions.

Anatomical experiments indicate that neurones in the VLM with divergent functions share the same input. Injection of anterograde tracers into the medullary raphe nuclei labelled boutons in the VLM which were observed in close contact with catecholaminergic neurones (Nicholas and Hancock, 1990) or neurones retrogradely labelled from the superior laryngeal nerve (Holtman, Marion and Speck, 1990). Similarly, injections of

anterograde tracer into the NTS labelled boutons in close apposition to C1 neurones (Hancock, 1988) and, in this thesis, boutons in synaptic contact with VPNs in the NA. In addition, 5-HT and substance P were observed in boutons in synaptic contact with CVPNs and other VPNs in these studies, and both these transmitters have also been identified in boutons in close apposition to catecholaminergic neurones in the VLM (Nicholas and Hancock, 1988).

Thus CVPNs lie mainly in the vINA, amid premotor neurones involved in the control of the respiratory and cardiovascular systems, and appear to share some inputs with these neurones. These factors may indicate that the location of CVPNs is related to their functions. Support for this argument can be found in the recent proposals that the VLM is a site of cardiorespiratory integration (Millhorn and Eldridge, 1986; Richter and Spyer, 1990). Indeed, Richter and Spyer suggested that neurones in the VLM form a cardiorespiratory network and that CVPNs are part of a group of post-inspiratory neurones in the VLM which form a subset in this network. The results of this thesis are consistent with such a role for CVPNs, but are not proof that such networks exist. Further experiments on the connections within the VLM are required before these networks can be proved to exist.

8:2. Synaptic Inputs onto CVPNs.

8:2.1. Chemistry of Inputs to CVPNs.

The experiments in these studies combined the technique of retrograde labelling of VPNs with immunocytochemistry or anterograde tracing to identify the chemistry and origin of some of the synaptic inputs onto VPNs in the NA, some of which were CVPNs. Central to the accuracy of these studies was the fact that VPNs were identified by retrograde labelling prior to subsequent techniques. This is essential in the nucleus ambiguus as it is a diffuse nucleus (see section 1:3.5), containing neurones with diverse functions. The diversity of neurones in the NA was illustrated in these studies by the observations that VPNs were intermingled with neurones labelled retrogradely from the phrenic motonucleus or with neuropeptide Y immunoreactive neurones.

5-HT, substance P and neuropeptide Y were localised in boutons innervating CVPNs or VPNs in the vINA which were likely to be CVPNs. As these transmitters had previously been shown to excite cardiac vagal preganglionic neurones in the NA to cause a bradycardia (5-HT- Izzo et al, 1988; Substance P - Urbanski *et al*, 1988; NPY - MacRae and Reid, 1988), the morphology of their synapses agrees with the view that asymmetric synapses are excitatory in function (Gray, 1973). In contrast, GABA is contained in boutons which form symmetric type synapses with

CVPNs in the cat (Maqbool *et al*, 1991) and inhibits CVPNs when applied ionophoretically (Gilbey *et al*, 1984). The question of whether other neurochemicals are contained in boutons in synaptic contact with CVPNs and their synaptic morphology remains to be established.

Pharmacological studies may indicate which neurotransmitters are contained in boutons in synaptic contact with CVPNs. Those which elicit a vagally mediated bradycardia on micro-injection into the NA such as opioid peptides (Laubie *et al*, 1979), excitatory amino acids (Willette *et al*, 1983a) and Atrial Natriuretic Factor (Ermirio *et al*, 1991) may be contained in boutons which form asymmetric synapses with CVPNs. In contrast, micro-injection of oxytocin and vasopressin (Kiriakopolous and Caverson, 1991) into the NA elicits a tachycardia which is due to removal of vagal inhibition on the heart, and these may be contained in boutons which form symmetric synapses with CVPNs. However, a comparison of the results from two pharmacological studies emphasises the importance of anatomical studies in identifying the nature of inputs to VPMs. In one study, acetylcholine was applied ionophoretically to antidromically identified CVPNs and inhibited these neurones (Gilbey *et al*, 1984). However, when microinjected into the VLM, acetylcholine elicited a vagally mediated bradycardia, suggesting that it excited CVPNs (Agarwal and Calaresu, 1991). These divergent results can clearly be explained by the presence of inhibitory interneurons which are either excited or inhibited by acetylcholine and cause the opposite effects in CVPNs. Thus the mechanism of how

acetylcholine acts on CVPNs could be better understood by investigating the existence and morphology of synapses onto CVPNs which contain acetylcholine.

Further investigations on the identity of neurotransmitters affecting CVPNs may be facilitated by recently developed anatomical techniques. Receptors can be localised at light and electron microscopic levels using specific antibodies (see Wenthold *et al*, 1990). Staining with these antibodies combined with retrograde labelling could clarify the nature and distribution of receptors on CVPNs. For example, an antibody for the 5-HT_{1a} receptor exists (Sotelo *et al*, 1990) and could be used to demonstrate the presence of these receptors on CVPNs, which would support the results of pharmacological studies and the observations in chapter 4. Another useful technique which could be used with a similar objective may be *in situ* hybridisation, which enables the detection of cells capable of synthesising a neurotransmitter or its receptor, although it is unable to distinguish the distribution of receptors in neuronal membranes. This has been carried out for the 5-HT_{1A} receptor in the central nervous system, but the identity of the neurones which had the capability to express these receptors was not investigated (Pompeiano, Palacios and Mengod, 1992).

8:2.2. Origin of Inputs to CVPNs.

Combination of retrograde labelling of VPNs with anterograde labelling revealed that neurones in the NTS were a source of input to VPNs in the NA of the cat. However, these VPNs were not identified as CVPNs and further experiments are required to determine this point. Other areas which may be sources of input to CVPNs may be revealed by studies involving retrograde labelling from the VLM. Such studies have resulted in labelled neurones in the medullary reticular formation, other levels of the NA and VLM, lateral and medial parabrachial nuclei, Kolliker-Fuse nucleus, paraventricular nucleus of the hypothalamus, lateral hypothalamic nuclei, dorsal medial hypothalamus, ventral and lateral parts of the peri-aqueductal gray, central nucleus of the amygdala, bed nucleus of the stria terminalis, substantia inominata, medial prefrontal cortex, inferior colliculus and locus coeruleus (see table 1.2; Denavit-Saubie and Riche, 1977; Bystrzycka, 1980; Stuesse and Fish, 1984; terHorst *et al*, 1984; Lovick, 1985; vanBockstaele *et al*, 1989; Loewy and Burton, 1978; Ross *et al*, 1985; Dampney *et al*, 1987; Cunningham and Sawchenko, 1990a Nunez-Abades *et al*, 1990; Smith *et al*, 1989; Takayama *et al*, 1990). Although anterograde tracer injected into some of these areas and others have labelled fibres in the NA (see section 1:3.7), further studies are required to establish VPNs as post-synaptic targets of these labelled fibres.

8.3. Summary.

These experiments have used anatomical techniques to study the organisation of CVPNs in the medulla of the rat, and have subsequently shown the chemistry and origin of some of the synaptic inputs of VPNS in the NA of the cat and rat. However, there is clearly a great deal of knowledge to be acquired before we have a complete understanding of the nature and function of inputs which influence VPNS. Further experiments on these topics in addition to investigating inputs affecting other neurones in the VLM will better our understanding of the regulation and maintenance of the cardiovascular system.

References.

Agarwal, J.C. and Calaresu, F.R. (1991). Enkephalins, substance P and acetylcholine microinjected into the nucleus ambiguus elicit vagal bradycardia in rats. *Brain. Res.* 563 pp. 203-208.

Aldskogius, H. (1978). Fine structural changes in nerve cell bodies of the adult rabbit dorsal vagal nucleus during axon reaction. *Neuropath. App. . Neurobiol.* 4(3)pp. 322-41.

Alexander, R.S. (1946). Tonic and reflex functions of medullary sympathetic cardiovascular centres. *J. Neurophysiol.* 9 pp. 205-217.

Allen, J.M. and Bloom, S.R. (1986). Neuropeptide Y: a putative neurotransmitter. *Neurochem. Int.* 8(1)pp. 1-8

Altschuler, S.M., Xinman, B., Bieger, D., Hopkins, D.A. and Miselis, R.R. (1989). Viscerotopic representation of the upper alimentary tract in the rat: sensory ganglia and nuclei of the solitary and trigeminal tracts. *J. Comp. Neurol.* 283(2)pp. 248-268.

Altschuler, S.M., Ferenci, D.A., Lynn, R.B. and Miselis, R.R. (1991). Representation of the cecum in the lateral dorsal motor nucleus of the vagus nerve and commissural subnucleus of the nucleus tractus solitarii in rat. *J. Comp. Neurol.* 304(2)pp. 261-274.

Armstrong, D.M., Saper, C.B., Levey, A.I., Wainer, B.H. and Terry, R.D. (1983). Distribution of cholinergic neurones in rat brain: demonstrated by the immunocytochemical localization of choline acetyltransferase. *J. Comp. Neurol.* 216(1)pp. 53-68.

Bacon, S.J. and Smith, D.A. (1988). Preganglionic sympathetic neurones innervating the rat adrenal medulla: immunocytochemical evidence of synaptic input from nerve terminals containing substance P, GABA or 5-hydroxytryptamine. *J. Auton. Nerv. Syst.* 24 pp. 97-122

Beckstead, R.M., Morse, J.R. and Norgren, R. (1980). The nucleus of the solitary tract in the monkey: projections to the thalamus and brainstem nuclei. *J. Comp. Neurol.* 190(2)pp. 259-282.

Bennett, J.A., Kidd, C., Latif, A.B. and McWilliam, P.N. (1981). A horseradish peroxidase study of vagal motoneurons with axons in cardiac and pulmonary branches of the cat and dog. *Q. J. Exp. Physiol.* 66(2)pp. 145-154.

Berger, A.J. (1979). Distribution of carotid sinus nerve afferent fibers to solitary tract nuclei of the cat using transganglionic transport of HRP. *Neuroscience Letts.* 14(23)pp. 153-158.

Berk, M.L. and Finkelstein, J.A. (1982). Efferent connections of the lateral hypothalamic area of the rat: an autoradiographic investigation. *Brain Res. Bull.* 8(5)pp. 511-526.

Berthoud, H.R., Carlson, N.R. and Powley, T.L. (1991). Topography of efferent vagal innervation of the rat gastrointestinal tract. *Am. J. Physiol.* 260 (1 pt2) R200-7.

Bianchi, A.L., Grelot, L., Iscoe, S. and Romnes, J.E. (1988). Electrophysiological properties of rostral medullary respiratory neurons in the cat: an intracellular study. *J. Physiol.* 407 pp. 293-310

Bieger, D. (1984). Muscarinic activation of rhombencephalic neurones controlling oesophageal peristalsis in the rat. *Neuropharmacology.* 23 pp. 1451-1464

Bieger, D. and Hopkins, D.A. (1987) Viscerotopic representation of the upper alimentary tract in the medulla oblongata in the rat: the nucleus ambiguus. *J. Comp. Neurol.* 262(4) 546-562

Blessing, W.W. (1990). Distribution of glutamate decarboxylase-containing neurons in rabbit oblongata with attention to intramedullary and spinal projections. *Neuroscience.* 37 (1) pp. 171-185.

Blessing, W.W. (1991) Inhibitory vasomotor neurons in the caudal ventrolateral medulla. *N.I.P.S.* 6 pp. 139-141

Blessing, W.W. and Li, Y.W. (1989). Inhibitory vasomotor neurons in the caudal region of the medulla oblongata. *Prog. in Brain Res.* 81 pp. 83-97

Blessing, W.W. and Reis, D.J. (1982). Inhibitory cardiovascular of neurons in the caudal ventrolateral medulla of the rabbit: relationship to the area containing the A1 noradrenergic cells. *Brain Res.* 253(1-2)pp. 161-171.

Blessing, W.W., Howe, P.R.C., Joh, T.H., Oliver, J.R. and Willoughby, J.O. (1986). Distribution of tyrosine hydroxylase and neuropeptide Y-like immunoreactive neurones in rabbit medulla oblongata, with attention to colocalisation studies, presumptive adrenaline synthesising perikarya, and vagal preganglionic neurones. *J. Comp. Neurol.* 248(2)pp. 285-300.

Blessing, W.W., Oliver, J.R., Hodgson, A.H., Joh, T.H. and Willoughby, J.O. (1987). Neuropeptide Y-like immunoreactive C1 neurones in the rostral ventrolateral medulla of the rabbit project to sympathetic preganglionic neurones in the spina cord. *J.A.N.S.* 18 pp. 121-129.

Blessing, W.W., Willoughby, J.O. and Joh, T.H. (1985). Evidence that catcholanmine synthesising perikarya in rat medulla oblongata do not contribute axons to the vagus nerve. *Brain Research.* 348(2)pp. . 397-400.

Bobillier, P., Seguin, S., Petitjean, F., Salvart, D., Touret, M. and Jouvot, M. (1976). The raphe nuclei of the cat brain stem: a topographical atlas of their efferent projections as revealed by autoradiography. *Brain Res.* 113(3)pp. 449-486.

Bockstaele, E.J.V., Peribone, V.A. and Aston-Jones, G. (1989). Diverse afferents converge on the nucleus paragigantocellularis in the rat ventrolateral medulla: retrograde and anterograde tracing studies. *J. Comp. Neurol.* 290(4)pp. 561-584.

Bonham, A.C. and Jeske, I. (1989). Cardiorespiratory effects of DL-homocysteic acid in caudal ventrolateral medulla. *Am. J. Physiol.* 256(3)H688-696.

Bons, N., Mestre, N., Petter, A., Danger, J.M., Pelletier, G. and Vaudry, H. (1990). Localisation and characterisation of neuropeptide Y in the brain of *Microcebus murinus* (Primate, Lemurian). *J. Comp. Neurol.* 298(3) pp. . 343-361.

Borison, H.L. and Domjan, D. (1970) Persistence of the cardioinhibitory response to brainstem ischemia after destruction of the area postrema and the dorsal vagal nuclei. *J. Physiol.* 211 pp. 264-277.

Bunzl-Federn, E (1889). Der centrale Ursprung des Nervus Vagus. *Monatschr. Psychiat. Neurol.* 5 pp. 1-22.

Butcher, L.L. and Woolf, N.J. (1984). Histochemical distribution of acetylcholinesterase in the central nervous system: Clues to the localisation of cholinergic neurones. In - Handbook of Chemical Neuroanatomy, Vol. 3: Classical transmitters and transmitter receptors in the CNS. Edited by Bjorklund, Hokfelt and Kuhar. Elsevier, Amsterdam. pp. . 1-50.

Bystrzycka, E.K. (1980). Afferent projections to the dorsal and ventral respiratory nuclei in the medulla oblongata of the cat studied by the HRP techniques. *Brain. Res.* 185(1)pp. 59-66.

Bystrzycka, E.K. and Nail, B.S. (1984). The source of the respiratory drive to nasolabialis motoneurons in the rabbit: an HRP study. *Brain. Res.* 266(2)pp. 183-191.

Calaresu, F.R. and Cottle, M.K. (1965). Origin of cardiomotor fibres in the dorsal vagal nucleus in the cat: a histological study. *J. Physiol.* 176 pp. 252-260.

Calaresu, F.R. and Pearce, J.W. (1965). Effects on heart rate of electrical stimulation of medullary vagal structures in the cat. *J. Physiol.* 176 pp. 241-251.

Calaresu, F.R. and Yardley, C.P. (1988). Medullary basal sympathetic tone. *Ann. Rev. Physiol.* 50 pp. 511-524.

Carrive, P., Bandler, R. and Dampney, R.A.L. (1988). Anatomical evidence that hypertension associated with the defence reaction in the cat is mediated by a direct projection from a restricted portion of the midbrain periaqueductal grey to the subretrofacial nucleus of the medulla. *Brain Res.* 460(2)pp. . 339-345.

Ceccatelli, S., Millhorn, D.E., Hokfelt, T. and Goldstein, M. (1989). Evidence of the occurrence of an enkephalin-like peptide in adrenaline and noradrenaline neurons of the rat medulla oblongata. *Exp. Brain Res.* 74(3)pp. 631-640

Chen, H.I. and Chai, C.Y. (1976). Integration of the cardio-vagal mechanism in the medulla oblongata of the cat. *Am. J. Physiol.* 231(2)pp. 451-461.

Chernicky, C.L., Barnes, K.L., Ferrario, C.M. and Conomy, J.P. (1983). Brainstem distribution of neurons with efferent projections in the cervical vagus of the dog. *Brain Res. Bull.* 10(3)pp. 345-351.

Chernicky, C.L., Barnes, K.L., Ferrario, C.M. and Conomy, J.P. (1987). Projections of the carotid sinus nerve to the medulla in the dog. *Brain. Res. Bull.* 18(3)pp. 437-445

Chitravanshi, V.C., Agarwal, S.K. and Calaresu, F.R. (1991). Microinjection of glycine into the nucleus ambiguus elicits tachycardia in spinal rats. *Brain. Res.* 566 pp. 290-294.

Chiurugi, E. and Mollica, A. (1954). Contributo alla localizzazione del centro vagale cardioinibitore. *Arch. Fisiol.* 54 pp. 249-267.

Chronwall, B.M., DiMaggio, D.A., Massari, V.J., Pickel, V.M., Ruggiero, D.A. and T.L. Donohue. (1985). The anatomy of neuropeptide Y containing neurones in rat brain. *Neuroscience.* 15(4) pp. 1159-1181

Ciriello, J. (1983). Brainstem projections of aortic baroreceptor afferent fibres in the rat. *Neurosci. Letts.* 36(1)pp. 37-42

Ciriello, J and Calaresu, F.R. (1978). Vagal bradycardia elicited by stimulation of the external cuneate nucleus in the cat. *Am. J. Physiol.* 235(5). pp. R286-293.

Ciriello, J and Calaresu, F.R. (1980) Distribution of vagal cardioinhibitory neurons in the medulla of the cat. *Am. J. Phys.* 238(1)pp. R57-R64

Clement, M.E. and McCall, R.B. (1990). Studies on the site and mechanism of the sympathoexcitatory action of 5-HT₂ agonists. *Brain Research*. 515 (1-2). pp.299-302.

Ciriello, J. and Calaresu, F.R. (1982). Medullary origin of vagal preganglionic axons to the heart of the cat. *J. Auton. Nerv. Syst.* 5(1) pp. 9-22.

Ciriello, J., Hrycyshyn, A.W. and Calaresu, F.R. (1981a). Horseradish peroxidase study of brain stem projections of carotid sinus and aortic depressor nerves in the cat. *J. Auton. Nerv. Syst.* 4(1) pp. 43-61.

Claps, A. and Torrealba, F. (1988). The carotid body connections: a WGA-HRP study in the cat. *Brain Res.* 455(1) pp. 123-133.

Cohen, D.H., Schnall, A.M., MacDonald, R.L. and Pitts, L.H. (1970). Medullary cells of origin of vagal cardioinhibitory fibres in the pigeon. 1. Anatomical studies of peripheral vagus nerve and the dorsal motor nucleus. *J. Comp. Neurol.* 140(2) pp. 299-320.

Connelly, C.A., Ellenberger, H.H. and Feldman, J.L. (1989) Are there serotonergic projections from raphe and retrotrapezoid nuclei to the ventral respiratory group in the rat? *Neurosci. Letts.* 105(1-2) 34-40.

Connelly, J.C., McCallister, L.W. and Kaufman, M.P. (1987). Stimulation of the caudal ventrolateral medulla decreases total lung resistance in dogs. *J. App. I. Physiol.* 63(3) pp. 912-917.

Consolazione, A., Milstein, C., Wright, B. and Cuello, A.C. (1981). Immunocytochemical detection of 5-HT with monoclonal antibodies. *J. Histochem. Cytochem.* 29(12) pp. 1425-1431.

Contreras, R.J., Gomez, M.M. and Norgren, R. (1980). Central origins of cranial nerve parasympathetic neurones in the rat. *J. Comp. Neurol.* 190(3) pp. 373-394.

Cottle, M.K. and Calaresu, F.R. (1975). Projections from the nucleus and tractus solitarius in the cat. *J. Comp. Neurol.* 161(2) pp. 143-158.

Cuello, A.C. and Kanawaza, I. (1978) The distribution of substance P immunoreactive fibres in the rat CNS. *J. Comp. Neurol.* 178(1) pp. 129-156

Cunningham, E.T. and Sawchenko, P.E. (1989). A circumscribed projection from the nucleus of the solitary tract to the nucleus ambiguus in the rat: anatomical evidence for somatostatin-28-immunoreactive interneurons subserving reflex control of esophageal motility. *J. Neurosci.* 9 (5) pp. 1668-1682.

Cunningham, E.T., Simmons, J.R., Swanson, L.W. and Sawchenko, P.E. (1989). Enkephalin immunoreactivity and messenger RNA are expressed in a discrete projection from the nucleus of the solitary tract to the nucleus ambiguus in the rat. *Abs. Soc. Neurosci.* 15 : 451

Cunningham, E.T. and Sawchenko, P.E. (1990a). Brainstem projections from the nucleus of the solitary tract in rat. *Abstracts Soc. Neurosci.* 16(2) 507.9

Cunningham, E.T. and Sawchenko, P.E. (1990b). Central neural control of esophageal motility: a review. *Dysphagia* 5(1)pp. 35-51.

Dahlstrom, A. and Fuxe, K.(1964). Evidence for the existence of mono-amine containing neurons in the central nervous system. 1. Demonstration of monoamines in the cell bodies of brain stem neurons. *Acta Phys, Scand.* 62 (suppl. 1.232) pp. 1-55.

Dalton, D.W. (1986). The cardiovascular effects of centrally administered 5-hydroxytryptamine in the conscious normotensive and hypertensive rat. *J. Auton. Pharmacol.* 6(1)pp. . 67-75.

Daly, M. de B. (1991). Some reflex cardioinhibitory responses in the cat and their modulation by central inspiratory neuronal activity. *J. Physiol.* 439 pp. . 559-577.

Dampney, R.A.L., Czarchurski, J., Dembowski, K., Goodchild, A.K. and Seller, H (1987). Afferent connections and spinal projections of the pressor region in the rostral ventrolateral medulla of the cat. *J. Auton. Nerv. Syst.* 20(1)pp. 73-86.

Dampney, R.A.L. and Moon, E.A. (1980). Role of ventrolateral medulla in vasomotor response to cerebral ischemia. *Am. J. Physiol.* 239 (3)H349-358

Denavit-Saubie, M and Riche, D. (1977). Descending input from the pneumotaxic system to the lateral respiratory nucleus of the medulla. An anatomical study with the horseradish peroxidase technique. *Neuroscience Letters*. 6 pp. 121-126.

Davidson, N.S., Goldner, S. and McCloskey, D.I. (1976). Respiratory modulation of baroreceptor and chemoreceptor reflexes affecting heart rate and cardiac vagal efferent nerve activity. *J. Physiol.* 259(2)pp. . 523-530.

Davies, R.O. and Kalia, M. (1981). Carotid sinus nerve projection to the brainstem in the cat. *Brain Res. Bull.* 6(6)pp. 531-541

Delgado-Garcia, J.M., Lopez-Barneo, J., Serra, R. and Gonzalez-Baron, S. (1983). Electrophysiological and functional identification of different neuronal types within the nucleus ambiguus in the cat. *Brain. Res.* 277(2)pp. 233-240.

DeVito, J.L., Clausing, K.W. and Smith, O.A. (1974). Uptake and transport of horseradish peroxidase by cut end of the vagus nerve. *Brain. Res.* 82(2)pp. 269-271.

DiMicco, J.A., Gale, K., Hamilton, B. and Gillis, R.A. (1979). GABA receptor control of parasympathetic outflow to the heart: characterisation and brainstem localisation. *Science.* 204 pp. 1106-1109.

Dockray, G.J. (1986) Neuropeptide Y: in search of a function. *Neurochem. Int.* 8(1)pp. 9-11.

Donoghue, S., Garcia, M., Jordan, D. and Spyer, K.M. (1984). The central projections of carotid baroreceptors and chemoreceptors in the cat: a neurophysiological study. *J. Phys. (Lond).* 347 pp. 397-410.

Dugin, S.F., Zakharow, S.T., Samonina, G.E. and Udelnov, M.G. (1976). The effect of electrical stimulation of the vagal nuclei in anaesthetised and unaesthetised cats. *Sechenov Physiol. J. USSR* 62(3)pp. 382-386

Ellenberger, H.H. and Feldman, J.L. (1988). Monosynaptic transmission of respiratory drive to phrenic motoneurons from brainstem bulbospinal neurons in rats. *J. comp. Neurol.* 269(1)pp. 47-57.

Ellenberger, H.H. and Feldman, J.L. (1990a) Brainstem Connections of the rostral ventral respiratory group of the rat. *Brain Res* (1) 513: 35-42

Ellenberger, H.H. and Feldman, J.L. (1990b) Subnuclear organisation of the lateral tegmental field of the rat 1: Nucleus ambiguus and ventral respiratory group. *J. Comp. Neurol.* 294(2) p. 202-211

Ellenberger, H.H., Feldman, J.L. and Zhan, W.Z. (1990). Subnuclear organisation of the lateral tegmental field of the rat. 11. Catecholamine neurones and ventral respiratory group. *J. Comp. Neurol.* 294(2) p. 212-222

Ellenberger, H., Haselton, J.R., Liskowsky, D.R. and Schneiderman, N. (1983). The location of chronotropic cardioinhibitory vagal motoneurons in the medulla of the rabbit. *J. Auton. Nerv. Syst.* 9(2-3) pp. 513-529

Ellenberger, H.H., Vera, P.L., Haselton, J.R., Haselton, C.L. and Schneiderman, N. (1990). Brainstem projections to the phrenic nucleus: an anterograde and retrograde HRP study in the rabbit. *Brain. Res. Bull.* 24 pp. 163-174.

Ermirio, R., Ruggeri, P., Cogo, C.E., Molinari, C. and Calaresu, F.R. (1991). Neuronal and cardiovascular responses to ANF microinjected into nucleus ambiguus. *Am. J. Physiol.* 260(6)R1089-R1094.

Everitt, B.J., Hokfelt, T., Terenius, L., Tatemoto, K., Mutt, V. and Goldstein, M. (1984). Differential coexistence of NPY like immunoreactivity with catecholamines in the central nervous system of the rat. *Neuroscience.* 11(2) pp. 443-462

Fedorko, L. and Merrill, E.G. (1984). Axonal projections from the rostral expiratory neurones of the Botzinger Complex to medulla and spinal cord in the cat. *J. Physiol.* 350 pp. 487-496

Feldman, J.L. (1986). Neurophysiology of breathing in mammals. *In* F.R. Bloom (ed): *Handbook of Physiology, Section 1. The Nervous System. iv. Intrinsic Regulatory Systems of the Brain.* Am. Physiol. Soc. pp. 463-524.

Ford, T.W. and McWilliam, P.N. (1986). The effects of electrical stimulation of myelinated and non-myelinated vagal fibres on heart rate in the rabbit. *J. Physiol.* 380 pp. 341-347.

Ford, T.W., Bennett, J.A., Kidd, C. and McWilliam, P.N. (1990). Neurones in the dorsal motor vagal nucleus of the cat with non-myelinated axons projecting to the heart and lungs. *Exp. Physiol.* 95(4)pp. 459-473.

Fox, E.A. and Powley, T.L. (1985). Longitudinal columnar organisation within the dorsal vagal motor nucleus represents separate branches of the abdominal vagus. *Brain Res.* 341(2)pp. 269-282.

Gebber, S.L., Barman, S.M. and Kocsis, B (1990). Coherence of medullary unit activity and sympathetic nerve discharge. *Am. J. Physiol.* 259(3)R561-R571.

Gebber, S.L. (1990). Central determinants of sympathetic nerve discharge. *in "Central Regulation of Autonomic Functions"*. pp.126-144. Edited by Loewy and Spyer.

Geis, G.S. and Wurster, R.D. (1980a). HRP localisation of cardiac vagal preganglionic somata. *Brain. Res.* 182(1)pp. 19-30.

Geis, G.S. and Wurster, R.D. (1980b). Cardiac responses during stimulation of the dorsal vagal motor nucleus and nucleus ambiguus in the cat. *Circ. Res.* 46(5)pp. 606-611.

Geis, G.S., Kozelka, J.W. and Wurster, R.D. (1981). Organisation and reflex control of vagal cardiomotor neurons. *J. A. N. S.* 3(2-4)pp. . 437-450.

Gerfen, C.R. and Sawchenko, P.E. (1984). An anterograde neuroanatomical tracing method that shows the detailed morphology of neurons, their axons and terminals: immunohistochemical localisation of an anterogradely transported plant lectin, *Phaseolus vulgaris*-leucoagglutinin (PHAL). *Brain res.* 290(2)pp. . 219-238.

Getz, B. and Sirnes, T. (1949). The localisation within the dorsal motor vagal motor nucleus. *J. Comp. Neurol.* 90(1) pp. 95-110.

Gilbey, M.P., Jordan, D., Richter, D.W. and Spyer, K.M. (1984). Synaptic mechanisms involved in the inspiratory modulation of vagal cardio-inhibitory neurones in the cat. *J. Physiol.* 356 pp. 65-78.

Gilbey, M.P., Jordan, D., Spyer, K.M. and Wood, L.M. (1985). The inhibitory action of GABA on cardiac vagal motoneurons in the cat. *J. Physiol.* 361 49P.

Graham, R.C. and Karnovsky, M.J. (1966) The early stages of absorption of injected horseradish peroxidase in the proximal tubules of the mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* 14 pp. . 291-392

Granata, A.R. and Kitai, S.T. (1992). Intracellular analysis *in vivo* of different barosensitive bulbospinal neurones in the rat rostral ventrolateral medulla. *J. Neuroscience.* 12 (1) pp. 1-20.

Gray, E.G. (1973). The Synapse. *in Oxford Biology Reader* No. 35. Edited by J.J. Head. pp. . 2-35.

Gray, T.S. and Morley, J.E. (1986) Neuropeptide Y: anatomical distribution and possible function in mammalian nervous system. *Life Sci.* 38(5) pp. 389-401

Grelot, L., Barillot, J.C. and Bianchi, A.L. (1988). Pharyngeal motoneurons: respiratory-related activity and responses to laryngeal afferents in the decerebrate cat. *Exp. Brain Res.* 78(2)pp. 336-334.

Gunn, C.G. , Sevelius, G., Puiggari, M.S. and Myers, F.K. (1968). Vagal cardiomotor mechanisms in the hindbrain of the dog and cat. *Am. J. Physiol.* 214(1)pp. 258-262

Guertzenstein, P.G. and Silver, A. (1974). Fall in blood pressure produced in discrete regions of the ventral surface of the medulla by glycine and lesions. *J. Physiol.* 242(1)pp. 489-503

Guyenet, P.G. (1990) Role of the ventral medulla in blood pressure regulation. *in* Central Regulation of Autonomic Functions *Editors* Loewy, A.D. and Spyer, K.M.

Gwyn, D.G., Leslie, R.A and Hopkins, D.A. (1985). Observations on the afferent and efferent organisation of the vagus nerve and the innervation of the stomach in the squirrel monkey. *J. Comp. Neurol.* 239(2)pp. 163-175.

Halliday, G.M., Li, Y.W., Oliver, J.R, Joh, T.H., Cotton, G.H., Howes, P.R.C., Geffen, L.B. and Blessing, W.W. (1988). The distribution of neuropeptide Y-like immunoreactive neurons in the human medulla oblongata. *Neuroscience* 26 (1) pp. 179-191

Hamilton, R.B., Pritchard, T.C. and Norgren, R. (1987). Central distribution of the cervical vagus nerve in Old and New world primates. *J. Auton. Nerv. Syst.* 19 (2) pp. 153-169

Hancock, M.B. (1988). Evidence for direct projections from the nucleus of the solitary tract onto medullary adrenaline cells. *J. Comp. Neurol.* 276(3)pp. . 460-467.

Hanker, J.S., Yates, P.E., Metz, C.B. and Rustioni, A. (1977) A new specific, sensitive and non-carcinogenic reagent for the demonstration of horseradish peroxidase. *Histochem. J.*, 9(4)pp. 789-792.

Harfstrand, A., Fuxe, K., Aganati, L.F., Benfenati, F. and Goldstein, M. (1986). Receptor autoradiographical evidence for high densities of ¹²⁵I-neuropeptide Y binding sites in the NTS of the normal male rat. *Acta. Phys. Scand.* 128 (2) pp. 195-200

Harfstrand, A (1987) Brain neuropeptide Y mechanisms. Basic aspects and involvement in cardiovascular and neuroendocrine regulation. *Acta. Phys. Scand. Supp. I.* 565 pp. 1-83.

Harfstrand, A., Fuxe, K., Terenius, L. and Kalia, M. (1987). Neuropeptide Y immunoreactive perikarya and nerve terminals in the rat medulla oblongata: relationship to cytoarchitecture and catecholaminergic cell groups. *J. Comp. Neurol.* 260(1)pp. 20-35.

Haselton, J.R., Padrid, P.A. and Kaufman, M.P. (1991). Activation of neurones in the rostral ventrolateral medulla increases bronchomotor tone in dogs. *J. App. I. Physiol.* *71(1)* pp. 210-216.

Haxhiu, M.A., Deal, E.C., Norcia, M.P., vanLunteren, E. and Cherniack, N.S. (1987). Effect of N-methyl-D-aspartate applied to the ventral surface of the medulla on the trachea. *J. App. I. Physiol.* *63(3)* pp. 1268-1274.

Haxhiu, M.A., Deal, E.C., vanLunteren, E. and Cherniack, N.S. (1989). Central modulatory effects of tachykinin peptides on airway tone. *J. Auton. Nerv. Syst.* *28(2)* pp. 105-115.

Helke, C.J., Charlton, C.G. and Wiley, R.G. (1985). Suicide transport of ricin demonstrates the presence of substance P receptors on medullary somatic and autonomic motor neurones. *Brain Res.* *328(1)* pp. 190-195

Helke, C.J., Shults, C.W., Chase, T.N. and O'Donohue, T.L. (1984) Autoradiographic localisation of substance P receptors in the medulla: effect of vagotomy and nodose ganglionectomy. *Neuroscience* *12(1)* pp. 215-223

Herbert, H., Moga, M.M. and Saper, C.B. (1990). Connections of the parabrachial nucleus with the nucleus of the solitary tract and the medullary reticular formation in the rat. *J. Comp. Neurol.* *293(4)* pp. 540-580.

Herkenham, M. (1987). Mismatches between neurotransmitter and receptor localisations in brain: observations and implications. *Neuroscience.* *23(1)* pp. 1-38.

Hokfelt, T., Fuxe, K., Goldstein, M. and Johansson, O. (1974). Immunohistochemical evidence for the existence of adrenaline neurons in the rat brain. *Brain Res.* *66(1)* pp. 235-251

Hokfelt, T., Lundberg, J.M., Tatemoto, K., Mutt, V., Ternius, L., Polak, J., Bloom, S., Sasek, Elde, R and Goldstein, M. (1983) Neuropeptide and FMRF amide neuropeptide like immunoreactivities catecholaminergic neurones in the rat medulla oblongata. *Acta Phys. Scand.* *117(2)* pp. 315-318.

Hokfelt, T., Ljungdahl, A., Steinbusch, H., Verhofstad, A., Nilsson, G., Brodin, E., Pernow, B. and Goldstein, I.J. (1978) Immunohistochemical evidence of substance P like immunoreactivity in some 5-HT containing neurones in the rat CNS. *Neurosci.* 3(pp. 517-538

Holstege, G., Graveland, G., Bijker-Biemand, C. and Schuddeboom, I. (1983). Location of motoneurons innervating soft palate, pharynx and upper oesophagus. Anatomical evidence for a possible swallowing centre in the pontine reticular formation. *Brain Behav. Evol.* 23(pp. 47-62.

Holstege, G. (1989). Anatomical study of the final common pathway for vocalisation in the cat. *J. Comp. Neurol.* 284(2)pp. 242-252.

Holtman, J.R. (1988). Immunohistochemical localisation of serotonin- and substance P- containing fibers around respiratory muscle motoneurons in the nucleus ambiguus of the cat. *Neuroscience.* 26(1) pp. . 169-178.

Holtman, J.R., Marion, L.J. and Speck D.F. (1990) Origin of Serotonin-Containing Projections to the Ventral Respiratory group in the Rat. *Neurosci.* 37 (2): 541-542

Hopkins, D.A. and Holstege, G. (1978). Amygdaloid projections to the mesencephalon, pons, and medulla oblongata in the cat. *Exp. Brain. Res.* 32(pp. 529-547.

Hopkins, D.A. and Armour, J.A. (1982). Medullary cells of origin of physiologically identified cardiac nerves in the dog. *Brain. Res. Bull.* 8(4)p359-365.

Hopkins, D.A. and Armour, J.A. (1984). Localisation of sympathetic postganglionic and parasympathetic preganglionic neurones which innervate different regions of the dog heart. *J. Comp. Neurol.* 229(2) pp. 186-198.

Hopkins, D.A., Gootman, P.M., Gootman, N., DiRusso, S.M. and Zeballas, M.F. (1984). Brainstem cells of origin of cervical vagus and cardiopulmonary nerves in the neonatal pig (*sus scrofa*). Brain. Res. 306(2)pp. 63-72.

Hopkins, D.A. and Holstege, G. (1978) Amygdaloid projections to the mesencephalon, pons and medulla oblongata in the cat. Exp. Brain. Res. 32(4)pp. 529-547.

Houser, C.R., Crawford, G.D., Barber, R.P., Salvaterra, P.M. and Vaughn, J.E. (1983). Organisation and morphological characteristics of cholinergic neurones: an immunocytochemical study with a monoclonal antibody to choline acetyltransferase. Brain Research. 226(2)pp. 97-119.

Housley, G.D., Martin-Body, R.L., Dawson, N.J. and Sinclair, J.D. (1987). Brain stem projections of the glossopharyngeal nerve and its carotid sinus branch in the rat. Neuroscience. 22(1) pp. 237-250

Howe, P.R.C., Costa, M., Furness, J.B., and Chalmers, J.P. (1980). Simultaneous demonstration of phenylethanolamine-N-methyltransferase immuno-fluorescent and catecholamine fluorescent nerve cell bodies in the rat medulla oblongata. Neuroscience. 5(4)pp. . 2229-2238.

Iriuchijima, J. and Kumada, M. (1964). Activity in single vagal efferent fibres to the heart. Jap. J. Physiol. 14 pp. 479-487.

Izzo, P.N. (1991). a note on the use of biocytin in anterograde tracing studies in the central nervous system: application at both light and electron microscopic levels. J. Neurosci. Meths. 36(2-3) pp. . 155-166

Izzo, P.N., Jordan, D. and Ramage, A.G. (1988). Anatomical and pharmacological evidence supporting the involvement of serotonin in the central control of cardiac vagal motoneurons in the anaesthetised cat. J. Phys. 406: 19P

Jewett, D.L. (1964). Activity of single efferent fibres in the cervical vagus nerve of the dog, with special reference to possible cardio-inhibitory fibres. *J. Physiol.* 175 pp. . 321-357.

Johansson, O., Hokfelt, T., Pernow, B, Jeffcoate, S.L., White, N., Steinbusch, H.W.M., Verhofstad, Emson, P.C. and Spindel, E. (1981). Immunohistochemical support for three putative transmitters in one neuron: coexistence of 5-HT, substance P and thyrotropin releasing hormone-like immunoreactivity in medullary neurons projecting to the spinal cord. *Neuroscience.* 6 (10) pp. . 1857-1881.

Johnson, S.M. and Getting, P.A. (1991). Electrophysiological properties of neurons within the nucleus ambiguus of adult guinea pigs. *J. Neurophysiology.* 66(3) pp. 744-761.

Jordan, D., Khalid, M.E.M., Schneiderman, N. and Spyer, K.M. (1982). The location and properties of preganglionic vagal cardiomotor neurones in the rabbit. *Pflugers Arch.* 395 pp. 244-250

Jordan, D. and Spyer, K.M. (1987). Central neural mechanisms mediating respiratory-cardiovascular interactions. *In:* "Neurobiology of the Cardio-Respiratory System." Edited by E.W. Taylor, Studies in Neuroscience, University of Manchester Press, Manchester, U.K. pp. . 342-368.

Kalia, M. (1981). Brain stem localisation of vagal preganglionic neurons. *J. Auton. Nerv. Syst.* 3⁽²⁻⁴⁾ pp. 451-481

Kalia, M., Feldman, J.L. and Cohen, M.I. (1979) Afferent projections to the inspiratory neuronal regions of the ventrolateral nucleus of the tractus solitarius tract in the cat. *Brain Res.* 171(1)pp. 135-141

Kalia, M. and Welles, R.V. (1980). Brain stem projections of the aortic nerve in the cat: a study using tetramethylbenzidine as the substrate for horseradish peroxidase. *Brain Res.* 188(1)pp. 23-32

King, K.A. and Holtman, J.R. (1990). Characterization of the effects of activation of ventral medullary serotonin receptor subtypes on cardiovascular activity and respiratory motor outflow to the diaphragm and the larynx. *J. Pharmacol. Exp. Therapeutics.* 252(2) pp. 655-674.

Kalia, M. and Mesulam, M.M. (1980a). Brainstem projections of sensory and motor components of the vagus complex in the cat: the cervical vagus and nodose ganglion. *J. Comp. Neurol.* 193 pp. 435-465

Kalia, M. and Mesulam, M.M. (1980b). Brainstem projections of sensory and motor components of the vagus complex. 2. Laryngeal, tracheal, pulmonary, cardiac and gastrointestinal branches. *J. Comp. Neurol.* 193 pp. 467-508.

Kalia, M. and Sullivan, J.M. (1982) Brainstem projections of sensory and motor components of the vagus nerve in the rat. *J. Comp. Neurol.* 211 pp. 248-264.

Karim, M.A. and Leong, S.K. (1980) Neurons of origin of cervical vagus nerves in the rat and monkey. *Brain. Res.* pp. 208-210.

Katona, P.G., Poitras, J., Barnett, U. and Terry, B. (1970). Cardiac vagal efferent activity and heart period in the carotid sinus reflex. *Am. J. Physiol.* 218 pp. 1030-1037.

Katz, D.M. and Karten, H.J. (1983). Visceral representation within the nucleus of the tractus solitarius in the pigeon (*Columbia livia*) *J. Comp. Neurol.* 218 pp. 42-73.

Kerr, F.W.L. (1969). Preserved vagal visceromotor function following destruction of the dorsal motor nucleus. *J. Physiology.* 202 pp. 755-769.

Kerr, F.W.L. and Pershaw, R.M. (1969). Secretomotor function of the dorsal motor nucleus of the vagus. *J. Physiol.* 205 pp. 405-415.

King, G.W. (1980). Topology of ascending brainstem projections to nucleus parabrachialis in the cat. *J. Comp. Neurol.* 191 pp. 615-638.

King, M.A., Louis, P.M., Hunter, B.E. and Walker, O.W. (1989). Biocytin: a versatile anterograde neuroanatomical tract-tracing alternative. *Brain res.* 497 pp. 361-367.

Kiriakopoulos, E.T. and Caverson, M.M. (1991). Function of vasopressinergic and oxytocinergic projections to the nucleus ambiguus in the control of vagal cardiomotor neuron activity. I.B.R.O. World Congress of Neuroscience. P19.19.

Kitchell, R.L., Stromberg, M.W. and Davis, L.H. (1956). Comparative studies of the dorsal vagal motor nucleus in ruminants and non-ruminants. *Anat. Rec.* 124 p319.

Kosaka, K. and Yagita, K. (1908). Experimentelle Untersuchungen über den Ursprung des N. Vagus und die zentralen Endigungen der dem Plexus nodosus entstammenden sensiblen Vagusfasern sowie über den Verlauf ihrer sekundären Bahn. *Neurol. Med. Chir.* (Tokyo). 4 pp. . 29-49.

Kreuter, F., Richter, D.W., Camerer, H. and Senekowitsch, R. (1977). Morphological and electrical description of medullary respiratory neurons of the cat. *Pflugers Arch.* 372 pp. 7-16.

Kunze, D.L. (1972). Reflex discharge patterns of cardiac vagal efferent fibres. *J. Physiol.* 222 pp. 1-15.

Laubie, M., Schmitt, H. and Vincent, M. (1979). Vagal bradycardia produced by microinjections of morphine-like drugs into the nucleus ambiguus in anaesthetized dogs. *Eur. J. Pharmacol.* 59 pp. 287-291.

Lawn, A.M. (1966a). The localisation in the nucleus ambiguus of the rabbit, of the cells of origin of motor nerve fibers in the glossopharyngeal nerve and various branches of the vagus by means of retrograde degeneration. *J. Comp. Neurol.* 127 pp. . 293-306.

Lawn, A.M. (1966b). The nucleus ambiguus of the rabbit. *J. Comp. Neurol.* 127 pp. . 307-320.

Laughton, W.B. and Powley, T.L. (1987). Localisation of efferent function in the dorsal motor nucleus of the vagus. *Am. J. Physiol.* 252 R13-25.

Leslie, R.A. (1985). Neuroactive substances in the dorsal vagal complex of the medulla oblongata: nucleus of the tractus solitarius, area postrema, and dorsal motor nucleus of the vagus. *Neurochem. Int.* 7(2) pp. 191-211.

Leslie, R.A., Gwyn, D.G. and Hopkins, D.A. (1982). The central distribution of the cervical vagus nerve and gastric afferent and efferent projections in the rat. *Brain. Res. Bull.* 8(1)pp. 37-43.

Levy, M.N. and Martin, P. (1984) Parasympathetic control of the heart. *in* *Nervous Control of Cardiovascular Function* (edited by W.C. Randall). pp. . 68-94.

Li, Y.Q., Rao, Z.R. and Shi, J.W. (1990) Substance P like immunoreactive neurons in the NTS of the rat send their axons to the nucleus accumbens. *Neurosci. letts.* 120(2) pp. 194-196.

Li, Y.W. and Blessing, W.W. (1989). Axonal projections from caudal ventrolateral medulla to rostral ventrolateral medulla, nucleus tractus solitarius and spinal cord in the rabbit. *Soc. Neurosci. Abs.* 15 p.593

Li, Y.W., Wesselingh, S.L., and Blessing, W.W. (1992). Projections from the rabbit caudal medulla to C1 and A5 sympathetic premotor neurones, demonstrated with Phaseolus Leucoagglutinin and Herpes Simplex Virus. *J. Comp. Neurol.* 317(4)pp. 379-395.

Lipski, J. (1981). Antidromic activation of neurones as an analytical tool in the study of the central nervous system. *J. Neurosci. Meths.* 4(1)pp. 1-32.

Ljungdahl, A., Hokfelt, T. and Nilsson G. (1978a) Distribution of substance P-like immunoreactivity in the CNS of the rat. I. Cell bodies and terminals. *Neuroscience* 3(4)pp. 861-943

Llewellyn-Smith, I.J., Minson, J.B., Wright, A.P. and Hodgson, A.J. (1990). Cholera toxin B-gold, a retrograde tracer that can be used in light and electron microscopic immunocytochemical studies. *J. Comp. Neurol.* 249(2)pp. 179-191.

Loewi, O. (1921). *Über humorale Übertragbarkeit der Herznervenwirkung*. *Pflügers Archiv*. 189 pp. . 239-242. "On the humoral propagation of cardiac nerve action", translated into English by I. Cook and M. Lipkin Jr., 1972, in "Cellular Physiology: A Source Book." Holt, New York pp. . 464-466.

Loewi, O. and Navratil (1926). *Über humorale Übertragbarkeit der Herznervenwirkung. X. Über das Schicksal des Vagusstoffs*. *Pflügers Archiv*. 214 pp. . 678-688. "On the humoral propagation of cardiac nerve action. X. The fate of the vagus substance", translated into English by I. Cook and M. Lipkin Jr., 1972, in "Cellular Physiology: A Source Book." Holt, New York pp. . 478-485.

Loewy, A.D. and Burton, H. (1978). *Nuclei of the solitary tract: efferent projections to the lower brainstem and spinal cord of the cat*. *J. Comp. Neurol.* 181(2)pp. 421-450.

Loewy, A.D., McKellar, S. and Saper, C.B. (1979). *Direct projections from A5 catecholamine cell group to the intermediolateral cell column*. *Brain Res.* 174(2)pp. 309-314.

Loewy, A.D. (1990). *Central Autonomic Pathways*. In "Central Regulation of Autonomic Function " Oxford University Press. ISBN 0-19-505106-8. pp. . 88-103.

Loewy, A.D., and Spyer, K.M. (1990). *Vagal Preganglionic Neurones*. In " "Central Regulation of Autonomic Function " Oxford University Press. ISBN 0-19-505106-8. pp. . 68-87.

Lorenz, R.G., Saper, C.B., Wong, D.L., Gorenello, R.P. and Loewy, A.D. (1985) *Colocalisation of substance P and PNMT-like immunoreactivity in neurones of the ventrolateral medulla that project to the spinal cord: potential role in control of vasomotor tone*. *Neurosci. Lett.* 55(3)pp. 255-260

Lovick, T.A. (1985). *Projections from the diencephalon and mesencephalon to nucleus paraventricularis lateralis in the cat*. *Neuroscience.* 14 (3) pp. . 853-861.

Lynch, G., Gall, C., Mensah, P. and Cotman, C.W. (1974). Horseradish peroxidase histochemistry: a new method for tracing efferent projections in the central nervous system. *Brain Res.* 65(3) pp. . 373-380.

Lynch, D.R., Walker, M.W., Miller, R.J. and Snyder, S.H. (1989). Neuropeptide Y receptor binding sites in the rat brain: differential localisations with ¹²⁵I-neuropeptide Y and ¹²⁵I-peptide YY imply receptor heterogeneity. *J. Neurosci.* 9(8) pp. 2607-2619

Maccarone, C., and Jarrat, (1986) Neuropeptide Y: a putative neurotransmitter. *Neurochem. Int.* 8 pp. 13-22.

Macrae, I.M., and Reid, J.L. (1988) Cardiovascular significance of neuropeptide Y in the caudal ventrolateral medulla of the rat. *Brain Res.* 456(1)pp1-8

Maley, B.E., Engle, M.G., Humphreys, S., Vascik, D.A., Howes, K.A., Newton, B.W. and Elde, R.P. (1990). Monoamine synaptic structure and localisation in the central nervous system. *J. Electron. Microsc. Technique.* 15(1)pp. . 20-33.

Manier, M., Mouchet, P. and Feuerstein, C. (1987). Immunohistochemical evidence for the co-existence of cholinergic and catecholamine phenotypes in neurons of the vagal motor nucleus of the adult rat. *Neuroscience Letters.* 80(1)pp. . 141-146.

Maqbool, A., Batten, T.F.C. and McWilliam, P.N. (1991) Ultrastructural relationships between GABAergic terminals and cardiac vagal preganglionic motoneurons and vagal afferents in the cat: a combined HRP tracing and immunogold labelling study. *Eur. J. Neurosci.* 3(6)pp. 501-513.

Marfurt, C.F., Turner, D.F. and Adams, C.E. (1988) Stabilisation of tetramethylbenzadine (TMB) reaction product at the electron microscopic level by ammonium molybdate. *J. Neurosci. Meths.* 25(3) 215-223.

- Marson, L. (1989) Evidence for colocalisation of substance P and 5-HT in spinally projecting neurons from the cat medulla oblongata. *Neurosci. Lett.* 96(1) pp. 54-59
- Martel, J.C., St-Pierre, S. and Quirion, R. (1986). Neuropeptide Y receptors in the rat brain: autoradiographic localisation. *Peptides* 7(1) pp. 55-60
- Martin, K., Kong, T.H., Renehan, W., Schurr, A., Dong, W., Zhang, X. and Fogel, R. (1989). Identification and function of brain stem neurons regulating rat ileal water absorption. *Am. J. Physiol.* G266-273. 257(2)
- Massari, V.J., Hornby, P.J., Friedman E.K., Milner, T.A., Gillis, R.A. and Gatti, P.J. (1990). Distribution of neuropeptide Y-like immunoreactive perikarya and processes in the medulla of the cat. *Neuroscience Letters.* 115(pp. 37-42.
- McAllen, R.M. and Spyer, K.M. (1976). The location of cardiac vagal preganglionic motoneurons in the medulla of the cat. *J. Physiol.* 258(1)pp. 187-204.
- McAllen, R.M. and Spyer, K.M. (1978a). Two types of vagal preganglionic motoneurons projecting to the heart and lungs. *J. Physiol.* 282 pp. 353-364.
- McAllen, R.M. and Spyer, K.M. (1978b). The baroreceptor input to cardiac vagal motoneurons. *J. Physiol.* 282 pp. 365-374.
- McAllen, R.M., Neil, J.J. and Loewy, A.D. (1982). Effects of kainic acid applied to the ventral surface of the medulla oblongata on vasomotor tone, the baroreceptor reflex and hypothalamic autonomic responses. *Brain. Res.* 238(1)pp. 65-76.
- McAuley, M.A., MacRae, I.M. and Reid, J.L. (1989) The cardiovascular actions of clonidine and neuropeptide Y in the ventral lateral medulla of the rat. *BR. J. Pharmacol.* 97(4) pp. 1067-1074.

McIlhinney, R.A.J., Bacon, S.J. and Smith, A.D. (1988) A simple and rapid method for the production of cholera B-chain coupled to HRP for neuronal tracing. *J. Neurosci. Meths.* 22(3)189-194.

McKellar, S. and Loewy, A.D. (1982). Efferent projections of the A1 catecholaminergic cell group in the rat: an autoradiographic study. *Brain Res.* 241(1)pp. 11-29.

McLean, J.H. and Hopkins, D.A. (1981). A light and electron microscopic study of the dorsal nucleus of the vagus nerve in the cat. *J. Comp. Neurol.* 195(1)pp. 157-175

McLean, J.H. and Hopkins, D.A. (1982). Ultrastructural identification of labelled neurons in the dorsal motor nucleus of the vagus nerve following injections of horseradish peroxidase into the vagus nerve and the brainstem. *J. Comp. Neurol.* 206(3)pp. 243-252

McLean, J.H. and Hopkins, D.A. (1985a). Ultrastructural studies of the nucleus ambiguus in cat and monkey following injection of HRP into the vagus nerve. *J. Neurocytology.* 14(1)pp. 961-979.

McLean, J.H. and Hopkins, D.A. (1985b). Ultrastructure of the dorsal motor nucleus of the vagus nerve in monkey with a comparison of synaptology in monkey and cat. *J. Comp. Neurol.* 231(2)pp. 162-174.

McWilliam, P.N. and Woolley, D.C. (1988). The actions of myelinated and non-myelinated vagal fibres on atrial contraction in the rabbit. *J. Auton. Nerv. Syst.* 22(1)pp. 67-73.

McWilliam, P.N., Maqbool, A. and Batten, T.F.C. (1989). Distribution of calcitonin gene-related peptide-like immunoreactivity in the nucleus ambiguus of the cat. *J. Comp. Neurol.* 282(1)pp. 206-214.

Menetrey, D. and Basbaum, A.I. (1987) The distribution of substance P, enkephalin and dynorphin immunoreactive neurones in the medulla and their contribution to bulbospinal pathways. *Neuroscience* 23 (1) pp. 173-187

Merrill, E.G. (1970). The lateral respiratory neurones of the medulla: their associations with nucleus ambiguus, nucleus retroambiguus, the spinal accessory nucleus and the spinal cord. *Brain Res.* 24 pp. 11-28.

Merrill, E.G. (1984). Where are the *real* respiratory neurones? *Fed. Proc.* 40 pp. 2389-2394.

Miceli, M.O. and Malsbury, C.W. (1985). Brainstem origins and projections of the cervical and abdominal vagus in the golden hamster: a HRP study. *J. Comp. Neurol.* 237(1)pp. 65-76.

Middleton, S., Middleton, H.H. and Grundfest, H. (1950). Spike potentials and cardiac effects of mammalian vagus nerve. *Am. J. Phys.* 162(2)pp. 553-559.

Mifflin, S.W., Spyer, K.M. and Withington-Wray, D.J. (1988). Baroreceptor inputs to the nucleus tractus solitarius in cat: postsynaptic actions and the influence of respiration. *J. Phys. (Lond).* 399 pp. 399-367.

Millhorn, D.E. and Eldridge, F.L. (1986). Role of ventrolateral medulla in regulation of respiratory and cardiovascular systems. *J. App. I. Physiol.* 61(4) pp. . 1249-1263.

Millhorn, D.E., Hokfelt, T., Seroogy, K., Oertel, W., Verhofstad, A.A.J. and Wu, J.Y. (1987). Immunohistochemical evidence for colocalisation of GABA and serotonin in neurons of the ventral medulla oblongata projecting to the spinal cord. *Brain Res.* 410(1) pp. 179-185

Miller, F.R. and Bowman, J.T. (1916). The cardioinhibitory centre. *Am. J. Physiol.* 39(1)pp. 149-153.

Milner, T.A., Pickel, V.M., Park, D.H., Joh, T.H. and Reis, D.J. (1987). Phenylethanolamine N-methyltransferase-containing neurons in the rostral ventrolateral medulla of the rat 1. Normal ultrastructure. *Brain Research.* 411(1)pp. 28-45.

Milner, T.A., Pickel, V.M, Abate, C. Joh, T.H. and Reis, D.J. (1988). Ultrastructural characterisation of substance P-containing neurons in the RVLM in relation to neurons containing catecholamine synthesising enzymes. *J. Comp. Neurol.* 270(3)pp. . 427-446.

Minson, J., Llewellyn-Smith, I., Neville, A., Somogyi, P. and Chalmers, J.(1990). Quantitative analysis of spinally projecting adrenaline-synthesising neurons of C1, C2 and C3 groups in rat medulla oblongata. *J.Auton. Nerv. Syst.* 30(3)pp. 209-220

Miselis, R.R., Rogers, W.T., Schwaber, J.S. and Spyer, K.M. (1989). Localisation of cardiomotor neurones in the anaesthetised rat; cholera toxin HRP conjugate and pseudorabies labelling. *J. Phys.* 77P

Mitchell, G.A.G. and Warwick, R. (1955). The dorsal vagal nucleus. *Acta Anat.* 25 pp. 371-395.

Mitchell, R.A., Herbert, D.A. and Baker, D.G. (1985). Inspiratory rhythm in airway smooth muscle tone. *J. Aqpp. I. Physiol.* ~~158~~ 58(3) pp. 911-920.

Miura, M. and Okada, J. (1981). Cardiac and non-cardiac preganglionic neurones of the thoracic vagus nerve: an HRP study in the cat. *Jap. J. Phys.* 31 pp. 53-66

Mohlant, M. (1910). Le nerf vagus (premier partie): les connexion anatomique et la valeur fonctionelle du noyau dorsal du vague. *Nervaxe* 11 pp. 137-244.

Morest, K. (1967). Experimental study of the projections of the nucleus of the tractus solitarius and the area postrema in the cat. *J. Comp. neurol.* 130(3)pp. 277-330.

Morilak, D.A., Somogyi, P., McIlhinney, R.A.J. and Chalmers, J. (1989). An enkephalin-containing pathway from the nucleus tractus solitarius to the pressor area of the rostral ventrolateral medulla of the rabbit. *Neuroscience.* 31(1) pp. 187-194.

Murakami, S., Okamura, H., Pelletier, G. and Ibata, Y. (1989). Differential co-localisation of neuropeptide Y and methionine-enkephalin-Arg6-Gly7-Leu8 like immunoreactivity in catecholaminergic neurons in the rat brain stem. *J. Comp. Neurol.* 281(4)pp. 532-544

Nakajima, T., Yashima, Y. and Nakomera, K. (1986). Quantitative autoradiographic localisation of neuropeptide Y receptors in the rat lower brain stem. *Brain Res.* 380(1)pp. 140-150

Neil, E. and Palmer, J.F. (1975). Effects of spontaneous respiration on the latency of the reflex cardiac chronotropic responses to baroreceptor stimulation. *J. Physiol.* 247 16P.

Neuhuber, W.L. and Sandoz, P.A. (1986). Vagal primary afferent terminals in the dorsal motor nucleus of the rat: are they making monosynaptic contacts on preganglionic efferent neurons? *Neuroscience Letters.* 69(2)pp. 126-130.

Nicholas, A.P. and Hancock, M.B. (1988) Evidence for substance P and 5-HT inputs to medullary bulbospinal adrenergic neurones. *Synapse* 2(6) pp. 569-76

Nicholas, A.P. and Hancock, M.B. (1989) Evidence for substance P, serotonin and oxytocin input to medullary catecholamine neurons with diencephalic projections. *Brain Res. Bull.* 22(2) pp. 213-23.

Nicholas A.P. and Hancock M.B. (1990) Evidence for projections from the rostral medullary raphe onto medullary catecholamine neurons in the rat. *Neurosci. Letts.* 108(1-2)22-28

Nicoll, R.A., Schienker, C. and Leeman, S.E. (1980). Substance P as a transmitter candidate. *Ann. Rev. Neurosci.* 3 pp. 227-268

Nomura, S. and Mizuno, N. (1982). Central distribution of afferent and efferent components of the glossopharyngeal nerve: an HRP study in the cat. *Brain. Res.* 236(1)pp. 1-13

Nomura, S. and Mizuno, N. (1983). Central distribution of efferent and afferent components of the cervical branches of the vagus nerve. *Anat. Embryol.* 166(1)pp. 1-18.

Norgren, R. (1978). Projections from the nucleus of the solitary tract in the rat. *Neuroscience*. 3⁽²⁾pp. 207-218.

Norgren, R. and Smith, G.P. (1988). Central distribution of subdiaphragmatic vagal branches in the rat. *J.Comp. Neurol.* 273⁽²⁾ pp. 207-223.

Nosaka, S., Kamaike, T. and Yasanuga, K. (1978). Central vagal organisation in rats: an electrophysiological study. *Exp. Neurol.* 60⁽³⁾pp. 405-419.

Nosaka, S., Yamamoto, T. and Yasunaga, K. (1979a) Localisation of vagal cardioinhibitory preganglionic neurons within rat brain stem. *J. Comp. Neurol.* 186⁽¹⁾ 79-92.

Nosaka, S., Yasunaga, K. and Kawano, M. (1979b). Vagus cardioinhibitory fibres in the rat. *Pflugers Arch.* 379⁽³⁾pp. 281-285.

Nosaka, S., Yasunaga, K. and Tamai, S. (1982). Vagal cardiac preganglionic neurones: distribution, cell types and reflex discharges. *Am. J. Physiol.* 1⁽²⁾ R92-98.

Nunez-Abades, P.A., Portillo, F. and Pasaro, R. (1990). Characterisation of afferent projections to the nucleus ambiguus of the rat by means of fluorescent double labelling. *J. Anat.* 172 pp. 1-15.

Onai, T., Saji, M. and Miura, M. (1987). Projections of supraspinal structures to the phrenic motor nucleus in rats studied by a horseradish peroxidase microinjection method. *J. Auton. Nerv. Syst.* 21⁽²⁻³⁾ pp. 233-239.

Pagani, F.D., Norman, W.P., Kaskebar, D.K. and Gillis, R.A. (1985). Effects of stimulation of nucleus ambiguus complex on gastroduodenal function. *Am. J. Physiol.* 246 G253-G256.

Pagani, F.D., Norman, W.P. and Gillis, R.A. (1988). Medullary parasympathetic projections innervate specific sites in the feline stomach. *Gastroenterology.* 95⁽³⁾pp. 277-288.

Palkovits, M. (1985). Distribution of neuroactive substances in the dorsal vagal complex of the medulla oblongata. *Neurochemistry International*. 7 pp. 213-219

Palkovits, M. and Jacobowitz, D.M. (1974). Topographic atlas of catecholamine and acetylcholinesterase-containing neurones in rat brain 2. Hindbrain (mesencephalon, rhombencephalon). *J. Comp. Neurol.* 157 pp. 29-42.

Panneton, W.M. and Loewy, A.D. (1980). Projections of the carotid sinus nerve to the nucleus of the solitary tract in the cat. *Brain Res.* 191 pp. 239-244.

Pazos, A. and Palacios, J.M. (1985) Quantitative Autoradiographic Mapping of Serotonin Receptors in the Rat Brain 1. Serotonin-1 Receptors. *Brain Res.* 346 pp. 205-230.

Pernow, B. (1983) Substance P. *Pharmacol. Revs.* 35(2) pp. 85-141

Plecha, D.M., Randall, W.C., Geis, G.S. and Wurster, R.D. (1988). Localisation of vagal preganglionic somata controlling sinoatrial and atrioventricular nodes. *Am. J. Phys.* 24 R703-R708.

Pompeiano, M., Palacios, J.M. and Mengod, G. (1992). Distribution and cellular localisation of mRNA coding for 5-HT_{1A} receptor in the rat brain: correlation with receptor binding. *J. Neurosci.* 12(2) pp. 440-453.

Portillo, F. and Pasaro, R. (1988). Location of bulbospinal neurons and of laryngeal motoneurons within the nucleus ambiguus of the rat and cat by means of retrograde fluorescent labelling. *J. Anat.* 159 pp. 11-18

Price, J.L. and Amaral, D.G. (1981). An autoradiographic study of the projections of the monkey amygdala. *J. Neurosci.* 1 pp. 1242-1259.

Ramage, A.G. (1990). Influence of 5-HT_{1A} receptor agonists on sympathetic and parasympathetic nerve activity. *J. Cardiovascular Pharmacology* 15 suppl 7 pp. S75-S85.

Ramage, A.G. and Fozard, J.R. (1987). Evidence that the putative 5-HT_{1A} receptor agonists, 8-OHDPAT and ipsapirone, have a central hypotensive action that differs from that of clonidine in anaesthetised cats. *Eur. J. Pharmacol.* 38 pp. . 179-191.

Reynolds, E.S. (1963). The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell. Biol.* 17 pp. . 208-212.

Richter, D.W., and Spyer, K.M. (1990). Cardiorespiratory Control. *In* " "Central Regulation of Autonomic Function " Oxford University Press. ISBN 0-19-505106-8. pp. . 68-87.

Rijlant, P. (1936). L'arythmie cardiaque respiratoire. *C.R. Soc. Biol. Paris.* 123 pp. . 997-1001.

Rikard-Bell, G.C., Bystrzycka, E.K. and Nail, B.S. (1984). Brainstem projections to the phrenic nucleus: a HRP study in the cat. *Brain Res. Bull.* 12 pp. 469-477.

Rinaman, L., Card, J.P., Schwaber, J.S. and Miselis, R.R. (1989). Ultrastructural demonstration of a gastric monosynaptic vagal circuit in the nucleus of the solitary tract in rat. *J. Neurosci.* 9 (6) pp. 1985-1996

Rinaman, L. and Miselis, R.R. (1990). Thyrotrophin-releasing hormone immunoreactive nerve terminals synapse on the dendrites of gastric vagal motoneurons in the rat. *J. Comp. Neurol.* 294 pp. . 235-251.

Ritchie, T.C., Westlund, K.N., Bowker, R.M., Coulter, J.D. and Leonard, R.B. (1982). The relationship of the medullary catecholamine-containing neurones to the vagal motor nuclei. *Neurosci.* 7 pp. 1471-1482

Rogers, R.C., Kita, H., Butcher, L.L. and Novin, D. (1980). Afferent projections to the dorsal motor nucleus of the vagus. *Brain Res. Bull.* 5 pp. 365-373.

Ross, C.A., Armstrong, D.M., Ruggiero, D.A., Pickel, V.M., Joh, T.H. and Reis, D.J. (1981a). Adrenaline neurons in the rostral ventrolateral medulla innervate the thoracic spinal cord: a combined immunocytochemical and retrograde transport demonstration. *Neurosci. Letts.* 25(3)pp. 257-262.

Ross, C.A., Ruggiero, D.A. and Reis, D.J. (1981b). Afferent projections to cardiovascular portions of the nucleus of the tractus solitarius in the rat. *Brain Res.* 223(2)pp. 402-408.

Ross, C.A., Ruggiero, D.A., Joh, T.H., Park., D.H. and Reis, D.J. (1984). Rostral ventrolateral medulla: selective projections to the thoracic autonomic cell column from the region containing C1 adrenaline neurons. *J. Comp. Neurol.* 228(2)pp. 168-185

Ross, C.A., Ruggiero, D.A. and Reis, D.J. (1985). Projections from the nucleus tractus solitarii to the rostral ventrolateral medulla. *J. Comp. neurol.* 242(4)pp. 511-534.

Ruggiero, D.A., Giuliano, R., Anwar, M., Stornetta, R. and Reis, D.J. (1990). Anatomical substrates of cholinergic-autonomic regulation in the rat. *J. Comp. Neurol.* 292(1)pp. 1-53

Saji, M. and Miura, M. (1991). Co-existence of glutamate and choline acetyltransferase in a major sub-population of laryngeal motoneurons of the rat. *Neuroscience Letters.* 123(2)pp. . 175-178.

Saper, C.B. (1983) A Simple Inexpensive and Reliable Nanolitre Syringe. *Brain. Res. Bull.* 10(3)403-405.

Sawchenko, P.E., Swanson, L.W., Grzanna, R., Howe, P.L.R., Bloom, S.R. and Polak, J.M. (1985). Colocalisation of neuropeptide Y immunoreactivity in the brainstem catecholaminergic neurones that project to the paraventricular nucleus of the hypothalamus. *J. Comp. Neurol.* 241(2)pp. 138-153

Sawchenko, P.E., Cunningham, E.T.Jr and Levin, M.C. (1987). Anatomic and biochemical specificity in central autonomic pathways. In: "Organisation of the Autonomic Nervous System: Central and Peripheral Mechanisms." J. Ciriello, F.R. Calaresu, L.P. Renaud and C. Polosa (Editors), Liss, New York. pp. . 267-261.

Schwaber, J.S. and Schneiderman, N. (1975). Aortic nerve-activated cardioinhibitory neurons and interneurons. *Am. J. Physiol.* 229(3)pp. 783-785.

Schwaber, J.S., Kapp, B.S., Higgins, G.A. and Rapp, P.R. (1982). Amygdaloid and basal forebrain direct connections with the nucleus of the solitary tract and the dorsal motor nucleus. *J. Neurosci.* 2(10)pp. 1424-1438.

Schwaber, J.C. and Cohen, D.H. (1978). Electrophysiological and electron microscope analysis of the vagus nerve of the pigeon, with particular reference to the cardiac innervation. *Brain. Res.* 147(1)pp. 65-78

Severinghaus, J.W. and Stupfel, M. (1955). *J. App. I. Physiol.* 8 pp. 81-87.

Shapiro, R.E. and Miselis, R.R. (1985a). The central neural connections of the area postrema of the rat. *J. Comp. Neurol.* 234(3)pp. 344-364.

Shapiro, R.E. and Miselis, R.R. (1985b). The central organisation of the vagus nerve innervating the stomach. *J. Comp. Neurol.* 238(4)pp. 473-488

Shipley, M.T. (1982). Insular cortex projections to the nucleus of the solitary tract and brain stem visceromotor regions in the mouse. *Brain Res. Bull.* 8(3)pp. 138-148.

Smith, J.K. and Barron, K.W. (1990). Cardiovascular effects of L-glutamate and tetrodotoxin microinjected into the rostral and caudal ventrolateral medulla in normotensive and spontaneously hypertensive rats. *Brain Res.* 506(1)pp. 1-8

Smith, J.C., Morrison, D.E., Ellenberger, H.H., Otto, M.R., and Feldman, J.L. (1989) Brainstem Projections to the Major Respiratory Neuron Populations in the Medulla of the Cat. *J. Comp. Neurol.* 281(1) 69-96

Smolen, A.J. and Truex, R.C. (1977). The dorsal vagal motor nucleus of the vagus nerve of the cat; localisation of preganglionic neurones by quantitative histological methods. *Anat. Rec.* 189(4)pp. 555-556.

Somogyi, P., Hodgson, A.J. and Smith, A.D. (1979) An approach to tracing networks in the cerebral cortex and basal ganglia. Combination of Golgi staining, retrograde transport of horseradish peroxidase and anterograde degeneration of synaptic boutons in the same material. *Neurosci.* 4:1805-1852.

Somogyi, P., Minter, J.B., Morilak, D., Llewellyn-Smith, I., McIlhinney, J.A. and Chalmers, J. (1989). Evidence for an excitatory amino acid pathway in the brainstem and for its involvement in cardiovascular control. *Brain Res.* 496(1-2)pp. 401-407.

Sotelo, C., Cholley, B., Mestikawy, S., Gozlan, H and Hamon, M. (1991) Direct immunohistochemical evidence of the existence of 5HT1A autoreceptors on serotonergic neurons in the midbrain raphe. *Eur. J. Neurosci.* 2(12) pp. 1144-1154

Strack, A.M., Sawyer, W.B., Platt, K.B. and Loewy, A.D. (1989). CNS cell groups regulating the sympathetic outflow to adrenal gland as revealed by transneuronal cell body labelling with pseudorabies virus. *Brain Res.* 491(2)pp. 274-296

Stuesse, S.L (1982) Origins of cardiac vagal preganglionic fibres: a retrograde transport study. *Brain Res.* 236(1)pp. 15-25.

Stuesse, S.L. and Fish, S.E. (1984). Projections to the cardioinhibitory region of the nucleus ambiguus of the rat. *J. Comp. Neurol.* 229(2)pp. 271-278

Steinbusch, H.W.M. (1981) Distribution of serotonin-immunoreactivity in the central nervous system of the rat - Cell bodies and terminals. *Neurosci.* 6(4): 557-618.

Sugimoto, T., Itoh, K., Mizuno, N., Nomura, S. and Konishi, A. (1979). The site of origin of cardiac vagal preganglionic fibres of the vagus nerve: an HRP study in the cat. *Neurosci. Letts.* 12(1)pp. 53-58.

Szentogathai, J. (1952). The general visceral efferent column of the brainstem. *Acta Morphol. Acad. Sci. Hung.* 2 pp. 313-328.

Taber, E. (1961). The cytoarchitecture of the brainstem of the cat. 1. Brain stem nuclei of the cat. *J. Comp. Neurol.* 116(1)pp. 27-69

Tache, Y. (1988). CNS peptides and regulation of gastric acid secretion. *Ann. Rev. Physiol.* 50 pp. . 19-39.

Takayama, K., Okada, J. and Miura, M. (1990). Evidence that neurons of the central amygdaloid nucleus directly project to the site concerned with circulatory and respiratory regulation in the ventrolateral nucleus of the cat: a WGA-HRP study. *Neurosci. Letts.* 109(3)pp. 241-246.

Tatemoto, K., Carlquist, M. and Mutt, V. (1982) Neuropeptide Y - a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. *Nature* 296 pp. 659-660

Tatemoto, K. (1982) Neuropeptide Y: complete amino acid sequence of the brain peptide. *Proc. Natl. Acad. Sci. USA* 79 pp. 5485-5489

Tayo, E.K. and Williams, R.G. (1988). Catecholaminergic parasympathetic efferents within the dorsal motor nucleus of the vagus in the rat: a quantitative analysis. *Neuroscience Letters.* 90(2)pp. . 1-5

terHorst, G.J., Luiten, P.G.M. and Kuipers, F. (1984). Descending pathways from hypothalamus to dorsal motor vagus and ambiguous nuclei in the rat. *J. A. N. S.* 11(1)pp. . 59-75.

Thomas, M.R. and Calaresu, F.R. (1974a). Localisation and function of medullary sites mediating vagus bradycardia. *Am. J. Physiol.* 226(6)pp. 1344-1349.

Thomas, M.R. and Calaresu, F.R. (1974b). Medullary sites involved in hypothalamic inhibition of reflex vagal bradycardia in the cat. *Brain Res.* 80(1)pp. 1-16.

Thor, K.B. and Helke, C.J. (1987) 5-HT and Substance P containing projections to the NTS of the rat. *J. Comp. Neurol.* 265 (2) pp. 275-293.

Thor, K.B. and Helke, C.J. (1988). Catecholamine synthesising neuronal projections to the NTS of the rat. *J. Comp. Neurol.* 268(2) pp. . 264-280.

Todo, K. (1977). Vagal preganglionic innervation of the cat heart. *Jap. Cir. J.* 41(12)pp. 1341-1352.

Todo, K., Yamamoto, T., Satomi, H., Ise, H., Takatama, H. and Takahashi, K. (1977). Origins of vagal preganglionic fibres to the sino-atrial and atrio-ventricular node regions in the cat heart as studied by the HRP method. *Brain Res.* 130(3)pp. 545-550.

Tohyama, M., Sakai, K., Salvert, D., Touret, M. and Jouvett, M. (1979). Spinal projections from the lower brainstem in the cat as demonstrated by the horseradish peroxidase technique. 1. Origins of the reticulospinal tracts and their funicular trajectories. *Brain. Res.* 173(3)pp. 383-403.

Trojanowski, J.Q., Gonatas, J.O. and Gonatas, N.K. (1982). Horseradish peroxidase conjugates of cholera toxin and lectins are more sensitive retrogradely transported markers than free HRP. *Brain Res.* 231(1)pp. 33-50.

Tseng, C., Mosqueda-Garcia, R., App. alsamy, M. and Robertson, D. (1988). Cardiovascular effects of neuropeptide Y in rat brain stem nuclei. *Circ. Res.* 64(1)pp. 55-61.

Tucker, D.C., Saper, C.B., Ruggiero, D.A. and Reis, D.J. (1987). Organisation of central adrenergic pathways: 1. Relationships of ventrolateral medullary projections to the hypothalamus and the spinal cord. *J. Comp. Neurol.* 259(4)pp. . 591-603.

Van Bockstaele, E.J.V., Peribone, V.A. and Aston-Jones, G. (1989). Diverse afferents converge on the nucleus paragigantocellularis in the rat ventrolateral medulla: retrograde and anterograde tracing studies. *J. Comp. Neurol.* 290 (4) pp. 561-584.

Wenthold, R.J., Altschuler, R.A. and Hampson, D.R. (1990). Immunocytochemistry of neurotransmitter receptors. *J. Electron Microscopy Techniques.* 15 pp. 81-96.

- Urbanski, R.W., Murugaian, J., Krieger, A.J. and Sapru, H.N. (1989). Cardiovascular effects of substance P receptor stimulation in the ventrolateral medullary pressor and depressor areas. *Brain Res.* 491(2)pp. 383-389.
- Wallach, J.H. and Loewy, A.D. (1980). Projections of the aortic nerve to the nucleus tractus solitarius in the rabbit. *Brain Res.* 188(1)pp. 247-251.
- Wan, X.C., Trojanowski, J.Q. and Gonatas, J.O. (1982). Cholera toxin and wheat germ agglutinin conjugates as neuroanatomical probes: their uptake and clearance, transganglionic and retrograde transport and sensitivity. *Brain. Res.* 243(2)pp. 215-224
- Weiss, G.K. and Priola, D.V. (1972). Brainstem sites for activation of vagal cardioaccelerator fibers in the dog. *Am. J. Physiol.* 223 pp. 300-304.
- Westlund, K.W., Bowker, R.M., Ziegler, M.G. and Coulter, J.D. (1983). Noradrenergic projections to the spinal cord of the rat. *Brain Res.* 263(1)pp. 15-31.
- Westlund, K.W., Bowker, R.M., Ziegler, M.G. and Coulter, J.D. (1984). Origins and terminations of descending noradrenergic projections to the spinal cord of the monkey. *Brain Res.* 292(1)pp. 1-16
- Widdicombe, J.G. (1966). Action potentials in parasympathetic and sympathetic efferent fibres to the trachea and lungs of dogs and cats. *J. Physiol.* 186 pp. 56-58.
- Willette, R.N., Barcas, P.P., Krieger, A.J. and Sapru, H.N. (1983a). Vasopressor and depressor areas in the rat medulla: identification by micro-injection of L-glutamate. *Neuropharmacology.* 22(9) pp. 1071-1079
- Willette, R.N., Abbott, J.K., Barcas, P.P. and Sapru, H.N. (1983b). Medullary gamma-amino butyric acid (GABA) receptors and the regulation of blood pressure in the rat. *J. Pharm. Exp. Therapeutics.* 226 (3) pp. 893-899

Withington-Wray, D.W. and Spyer, K.M. (1988). The brain stem localisation of vagal preganglionic neurones in the ferret, *Mustela Putorius furo*. Q. J. Exp. Physiol. 73(3)pp. 439-441

Woolley, D.C., McWilliam, P.N., Ford, T.W. and Clarke, R.W. (1987). The effect of selective stimulation of non-myelinated vagal fibres on heart rate in the rabbit. J. Auton. Nerv. Syst. 21(2-3)pp. 215-221.

Yamazoe, M., Shiosaka, S., Emson, P.C. and Toyhama, M. (1985) Distribution of neuropeptide Y in the lower brainstem: an immunohistochemical analysis. Brain. Res. 335(1)pp. 109-120

Zagon, A. and Smith, A.D. (1990). Projections from the rostral ventrolateral medulla to identified spinal sympathetic preganglionic neurones and serotonergic medullary raphe neurones in the rat. Abs. Soc. Neurosci. 16(2) 97.6.

Zheng, Y., Barillot, J.C. and Bianchi, A.L. (1991). Are the post-inspiratory neurons in the decerebrate rat cranial motoneurons or interneurons? Brain Res. 551(1-2)pp. 256-266.

Appendix One - Immunocytochemical Procedures.

In immunocytochemical procedures the following controls should be carried out. These are taken from a series of books entitled "Molecular Neuroanatomy" edited by VanLeeuwen, Buijs, Pool and Pach and published in 1988 by Elsevier, Volume 3, " Techniques in the Behavioural and Neural Sciences", chapter 14 "Antigen Identity in Immunocytochemistry", written by Pool and Buijs.

These tests cannot guarantee antiserum specificity. Many factors can influence both antigen and antibody e.g. fixation alters different antigens in different manners, which may result in an antigen being altered beyond recognition by the antibody. In addition, antibodies are often raised against a small portion of the immunogen and thus related (but different) molecules in the tissue may share antigenic sites with this immunogen. Tests should thus be carried out under the same conditions as in tissue.

An antiserum raised against A.
Sections of an area known to contain A.

Positive reaction.

Do the reagents alone cause this staining? - must test method specificity and antibody specificity.

Method Specificity.

Incubate some sections as before.

Incubate others without primary antiserum, in pre-immune serum from the same animal as the antibody. (If no pre-immune serum available then use non-immune serum from same species). If staining is present in sections with primary antiserum but not in those without, staining is due to primary antiserum.

At this stage pre-adsorption of primary antiserum with the component to be localised shows that the reaction between the tissue and antiserum was caused only by antibodies that are able to react with the component used for the adsorption (this does not mean that these antibodies are specific to that component).

Serum Specificity.

Determine if the antiserum detects only the antigen you want or cross-reacts with other antigens. This requires identification of all antigens in the tissue (e.g. blotting techniques, gel iso-electric focussing). Alternatively the reaction of the antiserum with other antigens known to be in the tissue can be carried out. This of course leaves in the unknowns.

Thus if satisfy these demands should have a purified antiserum which reacts with only one tissue component.

Final test.

No reaction after adsorption of purified antiserum with its homologous antigen.

No reaction on a section of the same tissue lacking the antigen.

Tests for 5-HT Antibody.

The antibody to 5-HT used was a monoclonal antibody (Serotec, YC5/HLK). Therefore, there should be no contaminating antibodies present. Specificity tests marked with * have been carried out by Consolazione *et al* (1981).

Positive staining in medullary areas containing 5-HT, previously shown by aldehyde fluorescent techniques.

No staining if primary antibody omitted.

*No staining if antibody pre-adsorbed with antigen (serotonin-albumin).

*Antibody known to cross react with dopamine, serotonin and tryptamine in a liquid media. However, in the tissue, when 5-HT synthesis was blocked by prior administration of a tryptophan hydroxylase inhibitor, no staining occurred. Staining still occurred on blockage of catecholamine synthesis.

Thus - no reaction after adsorption with antigen and no reaction on a section of the same tissue with no 5-HT present.

Tests for Neuropeptide Y antibody.

This antiserum was kindly supplied by Dr. R. Corder, who carried out the specificity tests of the antibody (*). This was a rabbit antiserum raised against porcine NPY, but which cross-reacts 100% with rat NPY.

Positive staining of soma and axonal fibres in rat medulla.

No staining if primary antibody omitted. However, there was no pre-immune serum available to test for detectable contaminating antibodies. No staining if incubated in non-immune serum from another rabbit.

*No staining if pre-adsorbed with human NPY (same as rat NPY).

*Cross-reactivities for the antibody tested by radioimmunoassay:

1. Peptide YY <0.01% at 1 μ M, 0.1% at 10nM.
2. Tryptic fragments of NPY, namely NPY₁₋₁₉, NPY₂₀₋₂₅, NPY₂₆₋₃₃ slight non-parallel crossreactivity, <1% at 10nm.
3. No cross reaction at any concentration tested - ACTH, angiotensin II, atrial natriuretic peptide, rat CRF, CGRP, dynorphin, human beta-endorphin, met-enkephalin-Arg-Phe, galanin, rat GHRF, human GHRF, neurotensin, somatostatin, substance P, VIP, [Arg⁸]-vasopressin.

No tests to show if antibody still reacts on tissue with NPY removed.

Thus antibody appears to be specific to NPY. However, cross-reactivities not detected in RIA may appear in tissue (possibly due to fixation procedures) and this cannot be ruled out. In addition, the antibody may detect other antigens in the tissue which have not been tested for cross-reactivity.

Tests for substance P Antiserum.

This antiserum was raised in rabbit. It was purchased from Eugene Tech. International who carried out the tests indicated by *.

Positive staining occurred in medullary sections.

No staining if the primary antibody omitted. Thus staining not due to other steps in the procedure. However, no pre-immune serum available to test for detectable contaminating antibodies. No staining when incubated in non-immune serum from another rabbit.

*No staining upon simultaneous incubation of rat brain tissue with substance P (0.1 μ m - 10 μ m) and antibody (information supplied by Eugene Tech Int.).

*A solid phase assay determined that the antisera binds a substance P glutaraldehyde conjugate with greater affinity than pure substance P.

*Staining still occurred when the antiserum was pre-incubated with thyrotropin releasing hormone, bombesin and a portion of substance P containing amino acids 9-11.

No tests carried out for staining when substance P neurones absent.