Central pathways activated by cardiac vagal afferent fibres

in the rat

by

David Colin Eric Bulmer B.Sc.

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Department of Physiology, University College London, Royal Free Campus.

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Declaration

All experiments reported within this thesis were performed by myself between July 1999 and April 2001. No part of this thesis has been previously submitted for any other degree.

David Bulmer
Abstract

The heart receives a rich innervation of afferent vagal nerve fibres. The majority of these fibres are chemosensitive and unmyelinated and terminate in the left ventricle. Research into the central pathways activated by cardiac vagal afferent fibres has focused mainly on the mechanosensitive myelinated vagal afferent fibres originating from endings in the atrium of the heart, which are thought to play a role in the control of blood volume.

The aim of this study was to investigate the pathways involved in relaying chemosensitive cardiac vagal afferent fibre input within the CNS of the rat. Within this thesis the experimental evidence derived from in vivo experiments using electrophysiological and immunohistochemical methods designed to demonstrate these projections is presented and critically evaluated.

Initial experiments examined the pharmacology of the coronary chemoreflex or Bezold-Jarisch reflex in the rat. Activation of this reflex has been shown to produce a bradycardia and hypotension. The afferent limb of this reflex is carried by chemosensitive unmyelinated vagal afferent fibres from the heart. On the basis of the cardiovascular and respiratory effects observed following left ventricular drug administration both before and after vagotomy, the 5-HT$_3$ agonist phenylbiguanide (PBG) was selected as the most suitable chemical stimulant of cardiac C-fibres in the rat.

The expression of the activity dependent protein product of the c-fos oncogene throughout the CNS was determined in response to electrical stimulation of the cardiac branch of the vagus. A large increase in c-fos expression was observed in the parabrachial nucleus (PBN). The PBN has previously been shown to act as a major relay of afferent vagal input from the nucleus tractus solitarii (NTS) to other higher centres of the CNS.

To investigate whether the PBN receives cardiac vagal afferent input via a direct projection from the NTS, single unit extracellular recordings were made from NTS neurones. A population of neurones were found that responded to both electrical and chemical stimulation of cardiac vagal afferent fibres and were activated antidromically following electrical stimulation of the PBN, indicating that they both received input from cardiac vagal afferent fibres and sent direct projections to the PBN.

The results of this thesis demonstrate that the PBN is activated by cardiac vagal afferent fibre stimulation and that it receives input from cardiac vagal afferent fibres via a monosynaptic projection from the NTS.
Acknowledgements

I am especially grateful to my supervisor, Prof. K.M. Spyer, for the opportunity to undertake this PhD, and his endless support throughout its duration.

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Finally, I would like to thank my parents, Fiona, my family and friends for all their support.

I dedicate this thesis to my grandparents.
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## Introduction

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<tr>
<td>5-HT</td>
<td>5-Hydroxytryptamine</td>
</tr>
<tr>
<td>A5</td>
<td>A5 noradrenergic cell group</td>
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<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
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<tr>
<td>AP</td>
<td>Area postrema</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>BNST</td>
<td>Bed nucleus of the stria terminalis</td>
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<tr>
<td>BPM</td>
<td>Beats per minute</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cLPBN</td>
<td>Central lateral parabrachial subnucleus</td>
</tr>
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<td>CNA</td>
<td>Central nucleus of the amygdala</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<td>CP-NTS</td>
<td>NTS neurone that received cardiac and pulmonary afferent vagal input</td>
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<td>CREB</td>
<td>cAMP response element binding protein</td>
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<td>CVLM</td>
<td>Caudal ventrolateral medulla</td>
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<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
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<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DBP</td>
<td>Diastolic blood pressure</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DVN</td>
<td>Dorsal vagal motor nucleus</td>
</tr>
<tr>
<td>DRN</td>
<td>Dorsal raphe nucleus</td>
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<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>eCP-NTS</td>
<td>NTS neurone that received excitatory cardiac and pulmonary afferent vagal input</td>
</tr>
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<td>eLPBN</td>
<td>External lateral parabrachial subnucleus</td>
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<td>Electromyogram</td>
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<td>γ-Aminobutyric acid</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>iCP-NTS</td>
<td>NTS neurone that received inhibitory cardiac and pulmonary afferent vagal input</td>
</tr>
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<td>IEG</td>
<td>Immediate early genes</td>
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<td>i.p.</td>
<td>intra peritoneal</td>
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<td>i.v.</td>
<td>intra venous</td>
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<td>MABP</td>
<td>Mean arterial blood pressure</td>
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<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<td>NRM</td>
<td>Nucleus raphe magnus</td>
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<tr>
<td>PAG</td>
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</tr>
<tr>
<td>PB</td>
<td>Phosphate buffer</td>
</tr>
<tr>
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<td>Phenylbiguanide</td>
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</tr>
<tr>
<td>PBGS</td>
<td>Phosphate buffered goat serum</td>
</tr>
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<td>PBN</td>
<td>Parabrachial nucleus</td>
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<td>pCO₂</td>
<td>Partial pressure of carbon dioxide</td>
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<td>PDG</td>
<td>Phenyldiguanide</td>
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<td>PGE₁</td>
<td>Prostaglandin E₁</td>
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<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PMR</td>
<td>Paramedian raphe nucleus</td>
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<td>PNA</td>
<td>Phrenic nerve activity</td>
</tr>
<tr>
<td>PNZ</td>
<td>Perinuclear zone of the hypothalamus</td>
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<tr>
<td>pO₂</td>
<td>Partial pressure of oxygen</td>
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<tr>
<td>PSTH</td>
<td>Post stimulus triggered histogram</td>
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<tr>
<td>PVN</td>
<td>Paraventricular nucleus of the hypothalamus</td>
</tr>
<tr>
<td>RAR</td>
<td>Pulmonary rapidly adapting receptors</td>
</tr>
<tr>
<td>RVLM</td>
<td>Rostral ventrolateral medulla</td>
</tr>
<tr>
<td>SAR</td>
<td>Pulmonary slowly adapting receptors</td>
</tr>
<tr>
<td>SBP</td>
<td>Systolic blood pressure</td>
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<tr>
<td>SNP</td>
<td>Sodium nitroprusside</td>
</tr>
<tr>
<td>SON</td>
<td>Supraoptic nucleus of the hypothalamus</td>
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<tr>
<td>SRF</td>
<td>Serum response factor</td>
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VLM ventrolateral medulla

WGA-HRP Wheat germ agglutinin-horseradish peroxidase
Chapter 1  Introduction

Cardiac vagal afferent fibres
Initial evidence for the existence of an afferent vagal innervation of the heart came from experiments, which demonstrated that changes in heart rate and blood pressure could be evoked either by distending the chambers of the heart or following the application of chemicals to the heart. These changes could be abolished by section of the vagus nerve indicating that they involved the activation of the vagus nerve and were not due to a direct effect upon the heart.

One of the earliest demonstrations of the reflex cardiovascular responses which could be elicited from the heart was provided by the now seminal experiments of von-Bezold and Hirt in 1867 (for review see (Dawes & Comroe, 1954; Krayer, 1961)). Bezold described a reflex mediated bradycardia, hypotension and apnoea following intravenous administration of veratrum alkaloids, in the cat. These cardiovascular and respiratory responses were abolished following section of the vagi, which led von-Bezold and Hirt to hypothesis that veratridine was acting upon vagal afferent endings in the heart and lungs. Later in 1915, a second reflex suggested to originate from vagal afferent endings in the heart was described by Bainbridge, in the dog. Bainbridge showed a reflex mediated tachycardia could be elicited in response to an increase in venous filling following injections of saline into the jugular vein sufficient to raise diastolic pressure. This response could be abolished by vagotomy indicating that the afferent pathway lay in the vagus. A decade later de Burgh Daly & Verney (1927) demonstrated the existence of a vagally mediated reflex bradycardia in response to an increase in ventricular pressure following occlusion of the ascending aorta or a decrease in extracardiac pressure. It was suggested that this reflex was due to the activation of mechanosensitive vagal afferent fibres located within the left ventricle of the heart.

With the advent of improved techniques for recording single or few fibre activity in nerve slips teased from the cervical and cardiac branch of the vagus, scientists have been able to confirm the presence of vagal afferent fibres with endings located in the heart, which respond to mechanical or chemical stimulation. However it is from these early experiments, demonstrating the existence of different vagally mediated reflex responses to mechanical and chemical stimulation of the heart that the template for the
understanding of cardiac vagal afferent fibres was laid. In recognition of the pioneering work of von-Bezold which was latter expanded most notably by Jarisch in a series of electrophysiological studies from 1937 onwards (Dawes & Comroe, 1954; Krayer, 1961) the depressor reflex elicited by chemical activation of cardiac vagal afferent fibres subsequently became known as the Bezold-Jarisch reflex.

1.2 Afferent vagal innervation of the heart

Histological and functional studies in cats with intact vagi or following supranodose vagotomy have been used to establish the diameter and composition of afferent and efferent fibres in the cardiac branch of the vagus (Daly & Evans, 1953; Agostoni et al., 1957). Overall the majority of fibres in the cardiac branch of the vagus are unmyelinated (2000 – 2500 fibres) compared to about 500 myelinated fibres. Furthermore the vast majority of fibres are afferent, only about 500 fibres are efferent nearly all of which were unmyelinated. Therefore at least in the cat the greatest proportion of the vagal innervation of the heart is by afferent fibres of which up to 75% is unmyelinated

Within the heart no specific receptor specialisation has been described attached to unmyelinated cardiac vagal afferent fibres, and stimulus transduction is therefore believed to be mediated by the properties of receptors and channels present in the bare nerve endings (Hainsworth, 1991). By comparison complex unencapsulated endings attached to myelinated nerve fibres have been described in the heart and are located predominantly on the endocardial surface of the atria although their presence has also been reported over the epicardial surface of the heart (Nonidez, 1937; Miller & Kasahara, 1964). Combined histological and electrophysiological studies have demonstrated that these atrial endings are mechanosensitive and are attached to myelinated vagal afferent fibres (Holmes, 1957; Coleridge et al., 1957).

A rich network of fine fibres, the end net, has also been described over the endocardial surface of the atria and ventricles of the heart and represents the only presumed nervous structure reported on the endocardial surface of the ventricles of the heart (Woollard, 1926; Holmes, 1957; Miller & Kasahara, 1964). These fibres are thought to be of
nervous origin although components still remain after degeneration studies indicating
that some fibres may have a motor function. Contributions from branches of complex
unencapsulated endings have been reported to these fine fibre networks and they may
also receive fibres from unmyelinated vagal afferent fibres although whether this end
net contributes to the termination of cardiac vagal afferent fibres in the heart is
unknown.

Ventricular afferent fibres

1.3 Myelinated and unmyelinated afferent fibres

Electrophysiological recordings have demonstrated the existence of myelinated and
unmyelinated cardiac vagal afferent fibres with receptive fields in either the right or left
ventricle in cats (Paintal, 1955; Thoren, 1977; Thames et al., 1977; Thoren, 1980; Gupta
& Thames, 1983) and dogs (Coleridge et al., 1964; Sleight & Widdicombe, 1965;
Muers & Sleight, 1972). The consensus of opinion from the results of these studies was
that the innervation of the ventricles by unmyelinated afferent fibres is greater on the
left than the right (Coleridge et al., 1964; Sleight & Widdicombe, 1965; Muers &
Sleight, 1972; Thoren, 1980). However the innervation by myelinated afferent fibres
may be similar to both ventricles (Coleridge et al., 1964).

In the rat the presence of cardiac vagal afferent fibres with endings in the left and right
ventricles has only recently been reported (Ustinova & Schultz, 1994a, b; Schultz &
Ustinova, 1996, 1998; Sun et al., 2001). Earlier studies performed by Thoren et al.,
(1979) failed to fully determine the presence of cardiac vagal afferent fibres with
endings in the cardiac ventricles and only reported the presence of unmyelinated
afferent fibres which terminated in the atria of the heart. However Thoren did report
finding 2 fibres that were activated by probing the ventricular surface although their
precise location could not be fully determined. The comparative failure of Thoren to
detect cardiac vagal afferent fibres with ventricular endings in the rat may be due to the
stimuli used to excite cardiac vagal afferent fibres. Thoren occluded the aorta in order to
increase ventricular pressure to determine the presence of mechanosensitive afferent
fibres. In contrast Schultz and co-workers topically applied capsaicin to determine the
presence of chemosensitive afferent fibres. Schultz observed that the majority (17 / 23) of ventricular fibres were chemosensitive and only responded to aortic occlusion sufficient to produce a gross distension of the heart. Consequently Thoren may not have activated these fibres and hence excluded the majority of ventricular vagal afferent fibres from his study. In agreement with Thoren, Schultz and co-workers reported that all cardiac vagal afferent fibres recorded had conduction velocities which were consistent with the activation of unmyelinated fibres (Ustinova & Schultz, 1994a, b; Schultz & Ustinova, 1996).

A greater innervation of the left ventricle compared to the right ventricle by unmyelinated afferent fibres has also been reported in the rat (Ustinova & Schultz, 1994a; Sun et al., 2001).

1.4 Mechanosensitivity

Mechanosensitive afferent fibres have been studied that respond with an increase in discharge in response to experimentally induced increases in ventricular pressure in the rat, cat and dog (Paintal, 1955; Coleridge et al., 1964; Muers & Sleight, 1972; Thoren, 1977; Thames et al., 1977; Thoren, 1980; Gupta & Thames, 1983; Ustinova & Schultz, 1994a). The increase in firing rate has been found to correlate with changes in end diastolic pressure rather than changes in systolic pressure within the ventricles indicating that distension during diastole is the more important stimulus (Thoren, 1977; Thames et al., 1977; Thoren, 1980; Gupta & Thames, 1983; Ustinova & Schultz, 1994a). However Thoren (1977) also observed that during ventricular fibrillation when the heart became grossly distended no increase in activity occurred and that activity only increased when contraction of the ventricle resumed suggesting that the inotropic state of the heart was also an important influence on the firing of mechanosensitive afferent fibres. In agreement with this observation increases in cardiac inotropic state induced by administration of sympathomimetic agents or electrical stimulation of sympathetic efferents have been shown to increase activity in mechanosensitive afferent fibres and reductions in inotropic state induced by β adrenoceptor antagonists have been shown to reduce activity in mechanosensitive afferent fibres (Sleight & Widdicombe, 1965; Muers & Sleight, 1972; Thoren, 1977; Thoren, 1980; Gupta & Thames, 1983).
would therefore appear that although mechanosensitive afferent fibres can be activated by ventricular contraction, the discharge frequency is influenced predominantly by increased diastolic filling. However, this is not always the rule, by contrast some mechanosensitive ventricular afferent fibres are activated during reduced ventricular filling, a low ventricular volume with an increased inotropic state shown to be a particularly strong stimulus for mechanosensitive ventricular afferent fibres (Oberg & Thoren, 1972a, b).

In the cat and dog, mechanosensitivity has been observed in both myelinated and unmyelinated afferent fibres, although myelinated fibres appear to be more sensitive to mechanical stimulation and respond with a greater overall discharge (Gupta & Thames, 1983). Typically mechanosensitive fibres discharge with an ongoing activity exhibiting a cardiac rhythm (1 – 2 impulses per cardiac cycle), although this is more common in myelinated fibres compared with unmyelinated afferent fibres (Coleridge et al., 1964; Gupta & Thames, 1983). However unmyelinated mechanosensitive afferent fibres with no ongoing activity or activity with a irregular firing pattern (1 – 2 Hz) begin to discharge with a cardiac rhythm once firing rate is increased by mechanical stimulation (Muers & Sleight, 1972; Thoren, 1977; Gupta & Thames, 1983). In the rat only fibres possessing ongoing cardiac related activity were found to be mechanosensitive (Ustinova & Schultz, 1994a) and in that regard despite being unmyelinated resemble more the myelinated afferent fibres found in cats and dogs than the unmyelinated afferent fibres.

Mechanosensitive vagal afferent fibres have been localised in the cat and dog to the endocardial, myocardial and epicardial surface of the heart although only myelinated afferent fibres appear to be located on the endocardial surface (Coleridge et al., 1964; Sleight & Widdicombe, 1965; Muers & Sleight, 1972; Gupta & Thames, 1983). In addition, a population of epicardial myelinated cardiac vagal endings in the dog has been localised in or near to coronary arteries (Brown, 1965) and has been demonstrated to respond to independent increases in ventricular or coronary artery pressure (Drinkhill et al., 1993). The response to changes in coronary arterial pressure is greater than for changes in ventricular pressure within normal physiological limits and may contribute to the increased sensitivity of myelinated afferent fibres compared to unmyelinated afferent fibres observed by (Gupta & Thames, 1983).
1.5 Chemosensitivity

An increase in the firing rate of ventricular vagal afferent fibres has been observed following administration of exogenous chemical mediators such as veratridine (Coleridge et al., 1964; Drinkhill et al., 1993), nicotine (Sleight & Widdicombe, 1965; Muers & Sleight, 1972), capsaicin (Coleridge et al., 1964; Ustinova & Schultz, 1994b) and phenylbiguanide (a 5-HT₃ receptor agonist) (Kaufman et al., 1980; Wacker et al., 2002) to the myocardial surface of the heart or following injection into the coronary arteries, the chambers of the heart or the pericardium. Similar applications of endogenous chemical mediators such as bradykinin (Kaufman et al., 1980), arachidonic metabolites; PGE₂, PGI₂ & PGF₂α, leukotrienes and thromboxane A₂ (Baker et al., 1979; Sun et al., 2001; Wacker et al., 2002), substance P, adenosine and ATP (Armour et al., 1994) have also been shown to increase discharge in ventricular afferent fibres. These observations have been further extended recently in a study by Thompson et al., (2000) who have reported an increase in activity within guinea-pig nodose ganglion neurones following epicardial application of an extensive range of chemical mediators.

In the rat, chemosensitive afferent fibres are unmyelinated and where ongoing activity is present it is of low frequency (1 - 2 Hz) with an irregular discharge pattern (Ustinova & Schultz, 1994b). In the cat and dog responses to chemical mediators have been observed in myelinated and unmyelinated afferent fibres, although predominantly in unmyelinated fibres. Ongoing activity is usually irregular in these fibres although chemosensitive vagal afferent fibres with a cardiac rhythm have been reported usually in myelinated afferent fibres (Coleridge et al., 1964; Sleight & Widdicombe, 1965; Muers & Sleight, 1972). The majority of chemosensitive afferent fibres are located in the left ventricle, and are found in the myocardium or epicardial surface (Coleridge et al., 1964; Sleight & Widdicombe, 1965; Muers & Sleight, 1972).
1.6 Mechanosensitivity vs chemosensitivity

Ventricular vagal afferent fibres as described above can be classified with respect to their responses to mechanical and chemical stimuli. Whether or not the same afferent fibre is responsive to both types of stimuli, depends on the strength and type of stimuli used (Sleight & Widdicombe, 1965; Muers & Sleight, 1972; Kaufman et al., 1980; Drinkhill et al., 1993; Armour et al., 1994; Ustinova & Schultz, 1994a). For example mechanosensitive afferent fibres can be classified by their response to events, which increase ventricular pressure, such as aortic occlusion whilst other authors may use distortion of the epicardial surface with probes. Similarly chemosensitivity may be defined by the application of a variety of chemical mediators with a diverse range of actions (Thompson et al., 2000), some of which can be classified as endogenous mediators e.g. bradykinin, whilst other as clearly exogenous e.g. veratridine. Consequently in studies using bradykinin Kaufman et al. (1980), reported that mechanosensitive ventricular vagal afferent fibres defined by their response to aortic occlusion did not respond to epicardial application of bradykinin indicating a clear separation of mechanosensitive and chemosensitive vagal afferent fibres based on these stimulation parameters. However Kaufman also reported that probing of the epicardial surface could excite some chemosensitive ventricular vagal afferent fibres. Studies conducted by Armour et al. (1994) concluded that only 12% of nodose ganglion neurones responded to both probing of the epicardial surface and topical application of bradykinin, demonstrating that even with this wider definition of mechanosensitivity two clear populations of ventricular vagal afferent fibres exist. By contrast 80% of sympathetic ventricular afferent fibres studied by Baker et al. (1980) were demonstrated to respond to aortic occlusion and bradykinin.

Observations reported by Ustinova & Schultz (1994a) in the rat similarly suggest the existence of different populations of ventricular afferent fibres, demonstrating the existence of mechanosensitive vagal afferent fibres, which did not respond to epicardial application of capsaicin. However although the chemosensitive afferent fibres recorded did not respond to aortic occlusion at increases in left ventricular end diastolic pressure up to 28 mmHg, which were sufficient to activate mechanosensitive afferent fibres, further increases in pressure did result in the activation of chemosensitive afferent fibres.
fibres. It would therefore appear that the strength of mechanical stimuli must be considered when determining whether a fibre is mechanosensitive or not.

In contrast to studies conducted using capsaicin or bradykinin, a high proportion of ventricular vagal afferent fibres classified as chemosensitive by their response to nicotine or veratridine were also demonstrated to be mechanosensitive responding to increased ventricular pressure (Sleight & Widdicombe, 1965; Muers & Sleight, 1972; Drinkhill et al., 1993). This clearly demonstrates the importance of the choice of chemical mediator when classifying the response of afferent fibres to mechanical and chemical stimuli.

Based on these responses it has been suggested that ventricular vagal afferent fibres are a heterogeneous population of fibres ranging from fibres, which are solely mechanosensitive to those that are solely chemosensitive with a mixture of fibres that respond to both stimuli depending on the strength of the stimulus or the type of chemical mediator employed.

1.7 Reflex effects

1.7.1 Mechanical stimulation

Initial investigation of the reflex effects elicited by the activation of mechanosensitive ventricular cardiac vagal afferent fibres reported a fall in heart rate and a dilation of systemic resistance and capacitance vessels (Daly & Verney, 1927; Salisbury et al., 1960; Oberg & Thoren, 1973a; Fox et al., 1977). However the stimulus used in these studies e.g. aortic occlusion, application of negative pressure by means of a cardiometer or infusion of saline, would have also produced changes in other reflexogenic areas such as the aortic arch, left atrium and pulmonary circulation and so it is unclear whether these changes were solely due to the activation of ventricular afferent fibres. Subsequent studies have used cardiac bypass systems to prevent changes in pressure within other reflexogenic areas whilst distending the left ventricle using inflatable balloons (Zelis et al., 1977; Kostreva et al., 1979; Hoka et al., 1988a) or by increasing aortic root pressure (Challenger et al., 1987; Tutt et al., 1988a). In agreement with earlier findings distension of the left ventricle in these studies was reported to elicit a
relaxation of both resistance and capacitance vessels, however, no change or small falls in heart rate were reported in contrast to earlier studies. Investigation of the effects of left ventricular distension on regional vascular beds have demonstrated a vasodilatation in skeletal muscle and hindlimb resistance vessels and a vasodilatation of splanchnic capacitance vessels (Zelis et al., 1977; Challenger et al., 1987; Hoka et al., 1988b). No change was however reported within cutaneous resistance and capacitance vessels following ventricular distension (Zelis et al., 1977; Challenger et al., 1987). Earlier studies by Oberg (1973) have also reported a vasodilatation with renal resistance vessels and skeletal muscle capacitance vessels following aortic occlusion, however, it is unclear whether these responses were solely due to the activation of ventricular mechanosensitive afferent fibres.

Increasing coronary artery pressure has also been shown to elicit a vagally mediated reflex fall in blood pressure and heart rate which is consistent with the presence of epicardial ventricular vagal afferent fibres on or near to the coronary arteries (Brown, 1965; Brown, 1966). Through the continual refinement of their partial heart bypass methodology over the last 15 years, Hainsworth and co-workers have developed a system which allows quantitative changes in left ventricular and coronary artery pressure to be made independent of each other, whilst also maintaining constant perfusion pressures within other reflexogenic areas such as the left atrium and aortic arch and carotid sinus. The results of these experiments have demonstrated only modest decreases in systemic vascular resistance and heart rate following physiological changes in left ventricular pressure (al Timman et al., 1993; Wright et al., 2000; Drinkhill et al., 2001). Whilst increasing coronary artery pressure independent of changes in ventricular pressure, elicited a significant decrease in systemic resistance greater than that produced by increasing ventricular pressure, although no significant decrease in heart rate, was observed (al Timman et al., 1993; Wright et al., 2000). The results of these studies indicate that the predominant reflex effect is elicited by distension of the coronary arteries, which would suggest the results of previous studies demonstrating reflex changes in response to ventricular distension were possibly due to a concomitant distension of the coronary arteries. Furthermore the significant changes in systemic resistance occurred at lower coronary artery pressures (60 to 90 mmHg) than compared to the ventricular pressure require to elicit a significant change in systemic resistance (150 mmHg and greater). The change in systemic resistance elicited by coronary artery
mechanoreceptors was also found to be of similar magnitude to that elicited by stimulation of carotid and aortic baroreceptors although changes in systemic resistance occurred at lower pressure ranges for coronary artery mechanoreceptors (Wright et al., 2000). Based on these observations it has been proposed that coronary artery mechanosensitive vagal afferent fibres represent a third set of baroreceptors the "coronary baroreceptors" which act to regulate smaller changes in blood pressure than those sensed by the conventional aortic and carotid baroreceptors. No interaction has been reported between the reflex effects elicited by ventricular distension with those elicited by the carotid or coronary baroreceptors or those elicited by the activation of chemosensitive ventricular afferent fibres (Vukasovic et al., 1989; Wright et al., 2001).

Importantly, the depressor reflexes observed by increasing left ventricular pressure only occurred when ventricular pressure was elevated sufficiently to alter end diastolic pressure, consistent with the reported electrophysiological data (Thoren, 1977; Thames et al., 1977; Thoren, 1980; Gupta & Thames, 1983; Drinkhill et al., 1993). By contrast increases in the ionotrophic state of the heart and reduced ventricular filling, which have been reported to increase activity in mechanosensitive ventricular afferent fibres (Sleight & Widdicombe, 1965; Muers & Sleight, 1972; Thoren, 1977, 1980; Gupta & Thames, 1983) did not produce a significant changes in the fall in systemic resistance observed at a given ventricular or coronary artery pressure (Tutt et al., 1988b; al Timman & Hainsworth, 1992; Drinkhill et al., 2001). This would suggest that the activation of ventricular vagal afferent fibres and fall in systemic resistance reported previously in response coronary artery catecholamine injection or stimulation of sympathetic efferent fibres were elicited by the secondary change in systolic pressure opposed to the direct change in inotrophic state (Fox et al., 1977).

In addition to changes in cardiovascular function distension of the left ventricle has been reported to produce a vagally mediated depression in respiratory activity eliciting a reduction in phrenic nerve activity and a increase in breath-breath interval or apnoea (Kostreva et al., 1979; Crisp et al., 1989). Furthermore aortic occlusion has also been reported to produce a reflex gastric relaxation as part of vomiting efforts (Abrahamsson & Thoren, 1972), and a decrease in plasma renin activity (Gorman & Chen, 1989) although the precise role of mechanosensitive ventricular afferent fibres in these actions is still unclear.
In contrast to the left ventricle, distension of the right ventricle does not appear to produce any prominent reflex responses. Earlier studies have reported the presence of a reflex fall in heart rate and systemic vascular resistance, and an increase in respiratory activity (Aviado et al., 1951; Zelis et al., 1977; Kostreva et al., 1979). However the reliability of these observation has been called into question due to the possibility that other reflexogenic areas may also have been activated, most notably that the high distending pressures used may have also activated afferent fibres in the left ventricle. A more recent study designed to address these short comings failed to report any changes in heart rate, vascular resistance or respiration in response to changes in right ventricular pressure within physiological pressures (Crisp et al., 1988). Considering distension of the left ventricle independent of a change in coronary artery pressure produces only small changes in systemic resistance an absence of reflex responses would not be unexpected from the more sparsely innervated right ventricle.

1.7.2 Chemical stimulation

The term “Bezold-Jarisch reflex” was originally developed to define the vagally mediated reflex bradycardia and hypotension arising from stimulation of chemosensitive vagal afferent fibres in the heart. The apnoea produced in the original experiments of von Bezold and Hirt is due to the stimulation of vagal afferent fibres in the lungs, which also produce a smaller bradycardia and hypotension than that observed from the stimulation of cardiac receptors by veratridine in the dog and cat (Dawes, 1947). In 1954, Dawes and Comroe proposed a reclassification of the terminology surrounding the Bezold-Jarisch reflex due to the increasing use of the Bezold-Jarisch reflex to refer to reflex depressor effects from the heart and lungs rather than the heart alone (Dawes & Comroe, 1954). They proposed that the depressor reflex arising from the stimulation of vagal afferent fibres in the heart be term the “coronary chemoreflex” and that the depressor reflex and apnoea arising from the stimulation of vagal afferent fibres in the lungs be term the “pulmonary chemoreflex”. This classification will be adopted here, with the term Bezold Jarisch reflex reserved for experiments where both cardiac and pulmonary vagal afferent fibres were activated.
In an innovative series of experiments Dawes (1947) injected veratridine and nicotine into coronary arteries supplying the left ventricle, right ventricle, right atrium and left atrium respectively demonstrating that the coronary chemoreflex arose from the activation of chemosensitive afferent fibres in the left ventricle, the majority of which are unmyelinated (Coleridge et al., 1964; Gupta & Thames, 1983). Further investigation has demonstrated that these fibres are predominantly although not exclusively located within the inferior posterior wall of the left ventricle (Walker et al., 1978; Inoue & Zipes, 1987). Although not reported in the experiments of Dawes, a reflex bradycardia and hypotension has also been observed following the stimulation of chemosensitive afferent fibres in the right ventricle (Waldrop & Mullins, 1987). The coronary chemoreflex has also been demonstrated in the rat although the relative contribution of umyelinated afferent fibres in the atria and ventricle to the reflex in the rat is unknown (Veelken et al., 1990).

The bradycardia produced by the coronary chemoreflex is mediated by an increase in vagal cardioinhibitory drive to the heart and is abolished by atropine pre-treatment (Sleight, 1964; Zucker & Cornish, 1981; Barron & Bishop, 1982; Daly et al., 1988). The hypotension occurs as a result of both the fall in heart rate and a reduction in total peripheral resistance (Sleight, 1964; Zucker & Cornish, 1981). Vasodilatation of resistance vessels has been reported following stimulation of the coronary chemoreflex within the coronary (Trimarco et al., 1984; Hintze & Kaley, 1984; Clozel et al., 1990), iliac (Barron & Bishop, 1982; Gorman et al., 1984), skeletal muscle (Bergel & Makin, 1967; Oberg & Thoren, 1973a; Hintze & Kaley, 1984; Daly et al., 1988), cutaneous (McGregor et al., 1986) and abdominal circulation (Hainsworth et al., 1986). The latter involves a vasodilatation within the splanchnic and hepatic but not the splenic vascular beds (Bergel & Makin, 1967).

Within the coronary and iliac vascular beds the reduction in vascular resistance is mediated by both cholinergic vasodilator activity and a reduction in sympathetic vascular tone (Gorman et al., 1984; Trimarco et al., 1984). However, within the skeletal muscle and cutaneous vascular beds responses appear to be mediated solely by a reduction in sympathetic activity despite the original suggestion by Bergel (1964) that a cholinergic vasodilator pathways was involved (Oberg & Thoren, 1973a; McGregor et al., 1986; Daly et al., 1988).
The response within the renal vascular bed to stimulation of chemosensitive cardiac vagal afferent fibres is unclear as some authors have reported a vasodilatation within resistance vessels that is sympathetically mediated (Oberg & Thoren, 1973a; Hintze & Kaley, 1984), whilst other authors have reported no change in renal vascular resistance (Barron & Bishop, 1982; Gorman et al., 1984). However a reduction in renal sympathetic nerve activity has been reported by many authors following stimulation of chemosensitive cardiac vagal afferent fibres (Oberg & Thoren, 1973a; Thames, 1979; Reimann & Weaver, 1980; Weaver et al., 1984; Higuchi et al., 1988; Veelken et al., 1990; Minisi, 1998). It would appear that the failure to observe a change in renal vascular resistance is due to compensatory mechanisms maintaining vascular resistance in the renal bed rather than an absence of an inhibitory effect on sympathetic vascular tone. Similarly an inhibition of splenic sympathetic nerve activity has also been reported despite the absence of a change in splenic vascular resistance in response to the activation of chemosensitive cardiac vagal afferent fibres (Weaver et al., 1984). A increase in adrenal sympathetic nerve activity has also been reported in response to pericardial injection of PBG (Higuchi et al., 1988). By contrast in the same study aortic occlusion was reported to result in a decrease in adrenal sympathetic nerve activity, which was abolished by pericardial procaine, demonstrating that it was elicited by cardiac receptors.

In addition to producing vasodilatation within resistance vessels stimulation of chemosensitive cardiac vagal afferent fibres also been shown to elicit a vasodilatation within skeletal muscle, cutaneous and abdominal capacitance vessels (Oberg & Thoren, 1973a; Hainsworth et al., 1986; McGregor et al., 1986). Nganele & Hintze (1990) observed that activation of chemosensitive cardiac vagal afferent fibres also elicited a reduction in left ventricular end diastolic pressure and diameter, which they attributed to a vasodilatation within capacitance vessels mediated by a withdrawal of sympathetic constrictor tone.

Stimulation of chemosensitive cardiac vagal afferent fibres has also been shown to produce a reduction in plasma renin particularly following haemorrhage and a reduction in vasopressin secretion elicited by haemorrhage (Thames, 1977; Thames et al., 1980). A small but significant decrease in tidal volume and minute respiratory volume and
gastric relaxation / initiation of vomiting efforts have also been reported (Sleight, 1964; Abrahamsson & Thoren, 1972; Abrahamsson & Thoren, 1973; Daly et al., 1988).

The activation of chemosensitive cardiac vagal afferent fibres has been reported to have an interactive effect on the baroreflex (Chen, 1979; Holmberg et al., 1983; Hainsworth et al., 1986; McGregor et al., 1986). The decrease in heart rate and blood pressure elicited by the coronary chemoreflex has been reported to be diminished by increasing baroreceptor distending pressures. Conversely activation of chemosensitive cardiac afferent fibres has been reported to decrease the sensitivity of the baroreflex.

Atrial afferent fibres

The atria of the heart in the cat and dog contain both myelinated and unmyelinated vagal afferent fibres (Paintal, 1953a; Coleridge et al., 1973; Thoren, 1976; Thames et al., 1977). In the rat atria only umyelinated vagal afferent fibres have been reported (Thoren et al., 1979; Ustinova & Schultz, 1994a; Hines & Hodgson, 2000).

1.8 Atrial afferent fibres in the cat and dog

In the cat and dog myelinated vagal atrial afferent fibres have been demonstrated to fire with distinct patterns of ongoing activity related to the atrial pressure wave. Three types of atrial receptor with myelinated fibres have been reported, the type A, the type B and the intermediate type receptor (Paintal, 1953a; Coleridge et al., 1957; Kappagoda et al., 1976). The discharge of fibres associated with the type A receptor is in phase with the "a" wave of the atrial pressure trace and the discharge of fibres associated with the type B receptor occurring during the "v" wave of the atrial pressure trace. Fibres associated with the intermediate type receptor fire with characteristics of both type A and B receptors. Although Paintal originally suggested that the different atrial receptors had different functional effects, current experimental evidence strongly suggests that the different atrial receptors are in fact the same mechanosensitive receptor that responds to distension of the atria (Hainsworth, 1991). The differences in firing patterns are thought to arise from when in the cardiac cycle the receptor is activated by distension and hence
it is possible to convert fibres with a firing pattern characteristic of the type A receptor to that of a type B receptor and vice versa by increasing or decreasing atrial pressure / distension respectively (Kappagoda et al., 1976, 1977). Between such transformations the firing pattern becomes that of the intermediate type receptor and on removal of stimuli the firing pattern reverts back to its original state.

By contrast to the distinct firing patterns of myelinated atrial vagal afferent fibres, unmyelinated atrial vagal afferent fibres in the cat and dog have a sparse spontaneous discharge that is often irregular in pattern, few fibres demonstrating a cardiac rhythm (Coleridge et al., 1973; Thoren, 1976).

Activity in both myelinated and unmyelinated atrial vagal afferent fibres is increased in response to increases in atrial pressure and in closed chested animals is modulated by respiration (Whitteridge, 1948; Paintal, 1953a; Coleridge et al., 1957; Thoren, 1976; Baertschi & Gann, 1977; Thames et al., 1977; Kidd et al., 1978). The respiratory modulation of atrial vagal afferent fibres is thought to be related to transmural pressure being greatest during end inspiration and early expiration (Thames et al., 1977). In unmyelinated atrial vagal afferent fibres silent fibres or fibres with an irregular discharge often begin to fire with a firing pattern related to the atrial “v” wave when firing rate is increased by raising atrial pressure (Thoren, 1976; Thames et al., 1977). Application of chemical mediators such as veratridine, capsaicin, PDG and bradykinin has also been demonstrated to increase activity in unmyelinated atrial vagal afferent fibres, however myelinated afferent fibres do not appear to be chemosensitive (Paintal, 1953b, 1955; Coleridge et al., 1973; Kaufman et al., 1980).

1.9 Atrial afferent fibres in the rat

Although no myelinated atrial afferent fibres have been observed in the rat, Thoren (1979) described two subtypes of unmyelinated atrial vagal afferent fibres, one subtype, which, responded with a high frequency discharge to increase in atrial pressure and had ongoing activity with a cardiac rhythm that was related mainly to the “v” wave of the atrial pressure trace but also the “a” wave, similar to the myelinated afferent fibres found in the cat and dog. The second subtype responded with a low frequency
discharge to increase in atrial pressure and had ongoing activity with irregular discharge
and hence was similar to the unmyelinated atrial afferent fibres observed in the cat and
dog. Ustinova and Schultz (1994) also observed two subtypes of unmyelinated atrial
afferent fibres in the rat, one with ongoing discharge with a cardiac rhythm and one with
an irregular ongoing discharge of which only the latter fibre subtype responded to
epicardial application capsaicin. In the cat and dog only unmyelinated afferent fibres
are reported to be chemosensitive and so the observations of Ustinova and Schultz are
consistent with the concept of 2 sub-populations of atrial afferent fibres in the rat, one
that behave more like the myelinated afferent fibres found in the cat and dog and one
that behaves more like the unmyelinated afferent fibres found in the cat and dog.

In addition to responding to changes in atrial pressure and applications of chemical
mediators a respiratory modulation of atrial vagal afferent activity has also been
reported in the rat, although this was only found in fibres with cardiac related discharge
(Ustinova & Schultz, 1994a).

1.10 Reflex effects

It was originally shown by Bainbridge in the dog, that through an increase of venous
return a reflex acceleration of heart rate could be elicited which was abolished by
section of the vagi or cardiac accelerator nerves (Bainbridge, 1915). More recent studies
also in the dog, employing the use of balloon tipped cannula to selectively distend the
atria alone, have elicited a similar reflex tachycardia, indicating that the original
observations of Bainbridge were due to the activation of vagal atrial afferent fibres
following distension of the atria by increasing venous return (Ledsome & Linden, 1968;
Linden, 1987). Although distension of the atria through the use of indwelling balloon
cannula has been shown to activate all three types of myelinated vagal afferent fibres
and unmyelinated vagal afferent fibres, selective cooling of the vagus at temperatures
which block myelinated afferent fibres but not unmyelinated afferent fibres has
demonstrated that the reflex tachycardia is mediated through the activation of
myelinated vagal afferent fibres alone (Kidd et al., 1978; Kappagoda et al., 1979a;
Linden et al., 1982a). In further agreement with the earlier observations of Bainbridge
the efferent pathway of this reflex tachycardia has been shown to be an increase in
cardiac sympathetic nerve activity and is abolished by section of cardiac sympathetic efferent fibres, bretylium tosyalte blockade of post ganglionic noradrenaline release and β-adrenoceptor blockade (Ledsome & Linden, 1964; Karim et al., 1972; Kappagoda et al., 1972). It was also suggested by Bainbridge that a reduction in vagal inhibitory tone may have also been responsible for the reflex tachycardia, however despite the suggestion of a small efferent vagal response particularly after spinal lesion (Burkhart & Ledsome, 1974; Mason & Ledsome, 1974), other authors have reported no change in efferent vagal nerve activity in response to atrial distension in intact or decerebrate animals and suggested that inadequate localisation of the stimulus was responsible for any vagal component observed (Walters et al., 1982; Walters & Mary, 1986; Walters et al., 1986). Whether or not there is a small efferent vagal component to the reflex tachycardia elicited by atrial distension the principle efferent pathway clearly lies in the sympathetic cardiac efferent fibres. Interestingly distension of the atrium did not result in a positive inotropic response (Furnival et al., 1970). Furthermore the cardiac sympathetic efferent fibres activated by atrial distension were found to be separate to those activated by baroreceptor or chemoreceptor stimulation indicating that a separate population of cardiac sympathetic preganglionic neurones are activated by atrial vagal afferent compared with those stimulated by the baroreceptors or chemoreceptors (Linden et al., 1982b).

Atrial distension has also been demonstrated to elicit a reduction in renal sympathetic nerve activity and a increase in renal blood flow (Karim et al., 1972; Mason & Ledsome, 1974). The reduction in renal sympathetic nerve activity and increase in renal blood flow, like the increase in cardiac sympathetic nerve activity was found by selective cooling of the vagus to be mediated through the activation of myelinated vagal afferent fibres (Linden et al., 1980; Karim et al., 1982). However, in contrast to the response in cardiac sympathetic efferent fibres the same renal sympathetic efferent fibres modulated by atrial distension were also modulated by baroreceptor or chemoreceptor stimulation demonstrating that the same pool of renal sympathetic preganglionic neurones are affected by atrial vagal afferent fibres, baroreceptors and chemoreceptors (Kidd et al., 1981; Linden et al., 1981). No response was observed in lumbar or splenic sympathetic nerve activity following atrial distension (Karim et al., 1972).
In addition to eliciting an increase in cardiac sympathetic nerve activity and a decrease in renal sympathetic nerve activity, atrial distension has also been shown to produce a reflex vagally mediated diuresis and natriuresis (Henry & Pearce, 1956; Ledsome et al., 1961; Ledsome & Linden, 1968; Kappagoda et al., 1974a; Sivananthan et al., 1981; Fater et al., 1982; Schultz et al., 1982); a fall in plasma concentration of vasopressin (Bennett et al., 1983; Ledsome et al., 1983; Bennett et al., 1984), renin (Drinkhill et al., 1988; Drinkhill et al., 1989) and ACTH / cortisol (Kappagoda et al., 1985; Drinkhill & Mary, 1989); and a variable, transient fall in blood pressure (Carswell et al., 1970; Burkhart & Ledsome, 1977; Kappagoda et al., 1979a). With the exception of the fall in blood pressure which is indicated to be mediated by unmyelinated atrial vagal afferent fibres (Kappagoda et al., 1979a), these reflex effects have been demonstrated to be elicited by myelinated vagal afferent fibres (Henry & Pearce, 1956; Sivananthan et al., 1981; Bennett et al., 1984; Kappagoda et al., 1985; Knapp et al., 1986; Drinkhill et al., 1988).

The diuresis is mediated by humoral events being observed in isolated kidneys perfused with blood from the same animal, and surgically or pharmacologically denervated kidneys. By contrast the natriuresis appears to be predominantly mediated by neuronal and hemodynamic changes being reduced or abolished by surgical or pharmacological denervation of the kidneys, unless accompanied by concomitant hemodynamic changes elicited by the reflex increase in heart rate (Linden & Sreeharan, 1981; Sreeharan et al., 1981). The nature of the humoral agent or agents responsible for the diuresis still remains to be elucidated. The reduction in plasma vasopressin concentration may play a role as infusions of vasopressin have been shown to elicit a reduction in the diuretic response to atrial distension (Lydtin & Hamilton, 1964). Whilst changes in the concentration of vasopressin similar to those elicited by atrial distension has been demonstrated to elicit a similar diuretic response to atrial distension in hypophysectomized dogs (Bennett & Linden, 1989). However, other studies have failed to observe a change in the diuretic response to atrial distension following vasopressin infusion (Ledsome et al., 1961) and diuresis was still observed following ablation of the pituitary indicating that vasopressin is not the sole humoral agent involved (Kappagoda et al., 1975).
Several studies have provided evidence indicating that a possible diuretic agent may be involved in the diuresis elicited by atrial distension. This substance was detected by applying plasma taken from dog to the renal tubules of the blood sucking bug *Rhodnius prolixus* were a reduction in tubular secretion was only observed in response to plasma taken from dogs in which atrial distension had occurred (Kappagoda *et al.*, 1979b). This response could be abolished by selective cooling of the vagus during atrial distension indicating that the release of the putative diuretic agent was dependent upon the activation of myelinated vagal afferent fibres (Knapp *et al.*, 1983). The identity of this putative diuretic agent remains unknown, however, it has been shown to be stable at pH 3.2, of low molecular weight, soluble in ethyl acetate, weakly acidic and lipophilic and is not deactivated by boiling water (Knapp *et al.*, 1981; Pither *et al.*, 1985).

Although with the exception of the transient decrease in blood pressure the majority of the reflex effects described have been attributed to the activation of myelinated vagal afferent fibres, such fibres have not been demonstrated in the rat (Thoren *et al.*, 1979; Ustinova & Schultz, 1994a; Hines & Hodgson, 2000). However, distension of the atria or vena cava in the rat has similarly been shown to elicit a increase in heart rate (Kaufman *et al.*, 1981) and a decrease in renal sympathetic nerve activity (Pyner *et al.*, 2002) which are abolished by vagotomy and a diuresis and natriuresis (Kaufman & Stelfox, 1987; Kaufman, 1990), although no change in plasma renin concentration has been reported (Kaufman, 1987). It would therefore appear that many of the reflex effects elicited by the activation of myelinated mechanosensitive atrial vagal afferent fibres in larger animals can also be elicited in the rat albeit by unmyelinated afferent fibres, possibly by the sub-type described by Thoren to have characteristics resembling myelinated afferent fibres in the cat and dog (Thoren *et al.*, 1979).

**Sympathetic afferent fibres**

Although not the focus of this thesis the heart has also been shown to receive both myelinated and unmyelinated sympathetic afferent fibres whose fibre endings have been located within all four chambers of the heart (Malliani *et al.*, 1973; Uchida *et al.*, 1974; Uchida & Murao, 1974a; Uchida, 1975; Nishi *et al.*, 1977; Casati *et al.*, 1979; Baker *et al.*, 1980; Maksymowicz & Szulczyk, 1983). Similar to cardiac vagal afferent fibres,
myelinated sympathetic afferent fibres predominantly discharge with a cardiac rhythm and have been located on both the endocardial and epicardial surfaces of the heart (Malliani et al., 1973; Uchida, 1975; Baker et al., 1980; Maksymowicz & Szulczyk, 1983). Whilst unmyelinated sympathetic afferent fibres preferential innervate the epicardial surface of the heart and have an irregular ongoing activity (approximately 1Hz) (Casati et al., 1979; Uchida, 1975; Baker et al., 1980; Maksymowicz & Szulczyk, 1983).

However, unlike cardiac vagal afferent fibres, the majority of sympathetic afferent fibres are polymodal responding to both chemical and mechanical stimuli (Baker et al., 1980; Gnecchi-Ruscone et al., 1995; Foreman et al., 1999; Pan & Chen, 2002). Although, sympathetic cardiac afferent fibres respond to a similar strength and range of mechanical or chemical stimuli as cardiac vagal afferent fibres (Malliani et al., 1973; Uchida et al., 1974; Uchida, 1975; Nishi et al., 1977; Casati et al., 1979; Baker et al., 1980; Lombardi et al., 1981; Bolser et al., 1989).

Stimulation of chemosensitive sympathetic afferent fibres typically through the epicardial application of bradykinin has been shown to elicit predominantly sympathoexcitatory reflex responses, increasing heart rate, blood pressure and renal sympathetic nerve activity in the cat (Reimann & Weaver, 1980; Gorman & Zucker, 1984; Waldrop, 1986; Waldrop & Mullins, 1987; Huang et al., 1995), dog (Staszewska-Barczak & Dusting, 1977; Pagani et al., 1985; Minisi & Thames, 1991; Dibner-Dunlap et al., 1993; Malik & Minisi, 1997) and rat (McDermott et al., 1995; Veelken et al., 1996). The tachycardia is predominantly mediated by an increase in cardiac sympathetic efferent activity, whilst the pressor response is thought to be mediated by an increase in sympathetic vasoconstrictor tone and involves both spinal and supra-spinal relays (Staszewska-Barczak & Dusting, 1977; Pagani et al., 1985; McDermott et al., 1995; Malik & Minisi, 1997).

The reflex response to chemical stimulation of sympathetic afferent fibres does however exhibit some species variability. A decrease in heart rate, blood pressure and renal sympathetic nerve activity were observed following epicardial application of bradykinin in the vagotomised monkey (Gorman et al., 1983) and rabbit (Niitani et al., 1988). These depressor reflexes elicited by chemical stimulation of cardiac sympathetic afferent fibres have been suggested to be the result of both surgery and anaesthesia.
which may lead to changes in the central excitatory state that facilitated the production of depressor reflexes by cardiac sympathetic afferent fibres (Pagani et al., 1985; Malliani et al., 1986). However, regardless of the cause it is notable that depressor reflexes elicited by chemical stimulation of cardiac afferent fibres are not necessarily due to the sole activation of cardiac vagal afferent fibres.

There is little information concerning the reflex response to stimulation of mechanosensitive sympathetic afferent fibres, however increasing coronary artery pressure in vagotomised animals elicits a spinal sympathetic reflex increasing efferent cardiac sympathetic activity. Given the polymodal nature of the majority of cardiac sympathetic afferent fibres it would be reasonable to surmise that mechanical stimulation of cardiac sympathetic afferent fibres would produce similar reflex responses to those elicited by chemical stimulation. It is therefore intriguing that distension of the cardiac chambers has not be reported to elicit pressor / sympathoexcitatory responses, following vagotomy (Fox et al., 1977; Kostreva et al., 1979; Crisp et al., 1989).

Central termination of cardiac vagal afferent fibres input

1.11 NTS

Anatomical tracing studies have demonstrated that the nucleus tractus solitarii (NTS) is the principle site of termination of cardiac vagal afferent fibres within the CNS (Kalia & Mesulam, 1980; Xie et al., 1999). Consistent with this observation, electrophysiological studies have demonstrated the synaptic activation of NTS neurones in response to chemosensitive and mechanosensitive cardiac vagal afferent input (Kappagoda et al., 1974b; Baertschi et al., 1975; Keith et al., 1975; Ward et al., 1977; Donoghue et al., 1981; Bennett et al., 1985; Hines et al., 1994; Wilson et al., 1996; Paton, 1998a; Silva-Carvalho et al., 1998; Seagard et al., 1999). Furthermore pharmacological blockade or electrolytic destruction of the NTS has been demonstrated to reduce or abolish chemosensitive and mechanosensitive cardiac vagal afferent reflexes (Lee et al., 1972; Burkhart et al., 1977; Verberne & Guyenet, 1992; Vardhan et
In the cat, cardiac vagal afferent terminal labelling following injection of HRP throughout the heart was reported predominantly in the dorsolateral subnuclei of the NTS and the area postrema, with moderate labelling also observed in the dorsal, medial, parvocellular and ventrolateral subnuclei of the NTS (Kalia & Mesulam, 1980). Sparse labelling was reported in the commissural nucleus of the NTS. Electrophysiological studies have also demonstrated that the majority of NTS neurones activated synaptically by cardiac vagal nerve stimulation are located in the area postrema and the medial, lateral and ventrolateral subnuclei of the NTS in the cat (Donoghue et al., 1981; Bennett et al., 1985; Silva-Carvalho et al., 1998). In the dog, recordings from NTS neurones activated by mechanosensitive cardiac vagal afferent fibres were also found predominantly in the medial NTS (Seagard et al., 1999).

In the rat a strong pattern of afferent labelling was observed similarly in the medial, dorsolateral and ventrolateral subdivisions of the NTS following injection of WGA-HRP into the walls of the heart (Xie et al., 1999). However unlike the cat dense afferent terminal labelling was also found in the commissural NTS but no labelling was observed in the area postrema. Electrophysiological studies in both the rat (Hines et al., 1994; Wilson et al., 1996) and mouse (Paton, 1998a) have also reported the presence of NTS neurones activated by cardiac vagal afferent input in the commissural NTS.

The difference in the intensity of the terminal labelling observed in the commissural NTS between the cat and rat may merely reflect an anatomical rather than a functional difference. The medial NTS is considered to be a rostral extension of the commissural NTS based on their cytoarchitectual and connectional similarities (Loewy, 1990).

The absence of cardiac vagal afferent fibre labelling in the area postrema of the rat compared to the cat represents a major species difference. However, the area postrema may not be important in the central processing of cardiac vagal reflexes. The coronary chemoreflex in the dog elicited following intra-coronary injection of veratridine was not reported to be significantly different in area postrema lesioned animals compared to intact animals (Hasser et al., 1988).
1.12 Function of NTS

The NTS is regarded as a major nucleus for the relay and integration of visceral inputs within the CNS. The NTS is the principle site for the termination of all vagal afferent fibres within the CNS (Kalia & Mesulam, 1980), and also receives afferent inputs from the facial, glossopharyngeal and trigeminal nerves (Torvik, 1956; Davies & Kalia, 1981; Contreras et al., 1982; Hamilton & Norgren, 1984; Grelot et al., 1989), as well as visceral and cutaneous inputs via projections from the spinal cord (Menetrey & Basbaum, 1987) (including cardiac sympathetic afferent fibres (Tjen et al., 1997)). Whilst the NTS also sends efferent and receives afferent projections from many central autonomic areas, facilitating both the relay of visceral inputs within the CNS, but also integrating descending control of visceral input from higher CNS areas (Loewy, 1990; Saper, 1995). The visceral inputs into the NTS are viscerotopically arranged (Jordan & Spyer, 1986; Loewy, 1990). The medial and commissural NTS receive afferent input from throughout the abdominal and thoracic viscera and so are believed to be involved in the relay of general visceral information to higher autonomic centres. Whilst the density of inputs to the subnuclei of the intermediate NTS vary depending on where in the body they arise. For example pulmonary afferent fibres terminate predominantly in the ventral and ventrolateral subnuclei of the NTS (Kalia & Mesulam, 1980; Xie et al., 1998) whereas cardiac afferent fibres terminate predominantly in the dorsolateral NTS (Kalia & Mesulam, 1980; Xie et al., 1999) and gastrointestinal afferent fibres in the parvocellular NTS (Leslie et al., 1982; Shapiro & Miselis, 1985). This separation of visceral input with the subnuclei of the intermediate NTS is thought to allow the NTS to mediate the diverse reflex effects elicited by the activation of the different visceral afferent fibres received by the NTS.

1.13 Processing of cardiac vagal inputs in the NTS

1.13.1 Pharmacology

Recordings from NTS neurones have demonstrated that the excitation by cardiac vagal afferent input is mediated in part by the activation of non-NMDA and to a lesser extent NMDA glutamate receptors and NK-1 receptors (Wilson et al., 1996; Seagard et al., 1996; Tjen et al., 1997).
1999; Paton, 1998b). These observations are further supported by the reduction in the coronary chemoreflex and Bezold-Jarisch reflex in response to microinjection of glutamate and NK-1 receptor antagonists into the NTS (Verberne & Guyenet, 1992; Vardhan et al., 1993; Vayssettes-Courchay et al., 1997; Paton, 1998b). In addition, 5-HT may also have a role in the processing of cardiac vagal afferent input within the NTS. Microinjection of 5-HT$_3$ antagonists (Pires et al., 1998) and agonists (Sevoz et al., 1996), and 5-HT$_4$ agonists (Edwards & Paton, 1999) have all been shown to inhibit the Bezold-Jarisch reflex in the rat. In addition ionophoretic application of 5-HT$_2$ agonists mimics the effect of combined cardiac and pulmonary afferent fibre stimulation on recorded NTS neurones (Sevoz-Couche et al., 2000). However, in all these studies responses were observed to stimulation of combined cardiac and pulmonary afferent fibres and so the role of 5-HT in the processing of only cardiac vagal afferent input is unclear.

1.13.2 Integration of cardiac vagal input

A separation in the processing of mechanosensitive and chemosensitive cardiac vagal afferent input is observed within the NTS similar to that observed in the periphery. Paton (1998a) reported that in the mouse the majority (30/43) of NTS neurones excited by cardiac vagal afferent fibres fibre stimulation using veratridine did not also respond to distension of the left side of the heart following perfusate injection into the left ventricle. Furthermore none of the NTS neurones excited by veratridine stimulation of cardiac vagal afferent fibres responded to an increase in right atrial pressure. Similarly findings were also reported by Hines (1994) in the rat who observed that only 5/27 vagally evoked NTS neurones that were excited following distension of the right atrium were also excited by stimulation of combined cardiac and pulmonary afferent fibres following right atrial injection of PBG. Considering the differences in reflex changes in heart rate produced by the stimulation of the coronary chemoreflex (Daly, 1991), compared with increasing ventricular pressure (Wright et al., 2000) or atrial distension (Kappagoda et al., 1979a) this separation in the processing of these different cardiac vagal inputs would be expected in the NTS.
By contrast the NTS neurones reported by Paton (1998a), which were excited by veratridine but were insensitive to ventricular distension possessed a high degree of convergent chemoreceptor input and virtually no convergent input from baroreceptor stimulation. Conversely the NTS neurones excited by ventricular distension received a high degree of convergent baroreceptor input and little chemoreceptor input. Similar findings were also reported in a previous study by Paton (1998c) were a high degree of convergent input onto NTS neurones excited by chemosensitive pulmonary C-fibres stimulation from chemosensitive cardiac vagal afferent fibres and chemoreceptor stimulation was observed. By comparison a sparse convergent input was reported from mechanosensitive cardiac vagal afferent fibres and following baroreceptor stimulation. In the cat NTS neurones excited by chemical stimulation of cardiac vagal afferent fibres also received a strong convergent input from chemoreceptor afferent fibres and chemosensitive pulmonary C-fibre stimulation but not from baroreceptor stimulation (Silva-Carvalho et al., 1998).

The high degree of convergence of mechanosensitive input from cardiac vagal afferent fibres and the baroreceptors onto NTS neurones could be expected as both types of afferent fibres respond to distension and produce a reduction in vascular resistance as a consequence. This is particularly true for the coronary baroreceptors (which will be activated by ventricular distension) and the aortic and carotid sinus baroreceptors (Wright et al., 2000). The same can also be said for the high degree of convergent inputs from chemosensitive pulmonary and cardiac vagal afferent fibres which respond to the same endogenous and exogenous chemical mediators and both produce a reflex bradycardia and hypotension when activated (Daly, 1991; Wilson & Bonham, 1997). Stimulation of chemoreceptor afferent fibres also produces a reflex bradycardia (Daly, 1991) and can be activated by chemical mediators that stimulate cardiac and pulmonary afferent fibres (Jarisch et al., 1951; Brophy et al., 1999). The findings of these and other studies have led the authors to postulate that the NTS channels information to produce specific output patterns of cardiorespiratory activity (Spyer, 1994; Mifflin, 1996; Paton, 1998a, c; Silva-Carvalho et al., 1998).

It is notable that the majority of NTS neurones recorded in these studies were located in either the commissural NTS of the rat and mouse or the medial subnucleus in the cat, areas of the NTS that receive inputs from widely dispersed viscera (Loewy, 1990).
the lateral nuclei of the intermediate NTS, which receive specific projections from
different cardiorespiratory afferent inputs, a limited degree of convergent inputs may
occur. This would explain how cardiorespiratory afferent fibres with a high degree of
convergent input in the medial or commissural NTS can still produce differences in
their reflex responses.

1.14 Relay of cardiac vagal input from the NTS

1.14.1 Vagal preganglionic neurones

The bradycardia produced by coronary chemoreflex activation is vagally mediated
indicating that cardiac vagal afferent input received by the NTS is further relayed to
vagal cardioinhibitory preganglionic neurones in the brainstem (Sleight, 1964; Zucker
& Cornish, 1981; Barron & Bishop, 1982; Daly et al., 1988). This has been confirmed
by electrophysiological recordings which have demonstrated that vagal preganglionic
neurones in both the nucleus ambiguus (NA) (Lipski et al., 1976) and dorsal vagal
motor nuclei (DVN) (Jones et al., 1998) are synaptically activated by combined cardiac
and pulmonary vagal stimulation. Furthermore electrolytic lesion of either the DVN or
NA reduces the bradycardic component of the Bezold Jarisch reflex indicating an
involvement of both nuclei (Lee et al., 1972). The depressor component was still
observed following lesions indicating that changes were specific for the destruction of
the DVN and NA and not afferent and efferent vagal projections to or from the NTS. In
an intriguing series of experiments (Daly et al., 1988; Daly, 1991) demonstrated that the
bradycardia elicited by carotid chemoreceptor, arterial baroreceptor and cardiac receptor
stimulation was reduced by lung inflation and central inspiratory activity, whilst the
bradycardia elicited by the pulmonary chemoreflex was not. One possible explanation
for this observation is that the pulmonary chemoreflex involved the activation of a
different population of cardiac vagal motoneurones to those stimulated by cardiac
receptor, baroreceptors and chemoreceptors, a population that was not respiratory
modulated. However recent studies demonstrating the activation of cardiac vagal motor
neurones with respiratory modulated activity in the NA by pulmonary C-fibre vagal
afferent stimulation have cast doubt over this suggestion (Wang et al., 2000).
The pathway by which cardiac vagal afferent input activates cardiac vagal motor neurones is unknown, although lower pontine transection did not alter the magnitude of the bradycardia elicited by the coronary chemoreflex indicating that it is contained within the medulla (Lee et al., 1972). Furthermore electrolytic lesion or pharmacological blockade of the caudal ventrolateral medulla (CVLM) using glutamate antagonists markedly attenuates the Bezold-Jarisch indicating that the relay of cardiac and pulmonary afferent input to the NA involves a relayed through the CVLM (Verberne et al., 1989).

1.14.2 Medullary areas.

A well established intra-medullary depressor pathway has been proposed to mediate the central processing of the arterial baroreflex (for review see (Guyenet, 1990). This pathway is thought to involve an excitatory projection from NTS neurones, which receive baroreceptor inputs to the CVLM utilising glutamate as a neurotransmitter, and a further inhibitory GABAergic projection from the CVLM to sympathoexcitatory neurones within the rostral ventrolateral medulla (RVLM). Microinjection studies within the CVLM and RVLM have demonstrated that the reflex depressor and sympathoinhibitory effects of the Bezold-Jarisch reflex similarly involves the glutamergic modulation of neurones in CVLM and the GABAergic modulation of neurones in the RVLM (Verberne et al., 1989; Verberne & Guyenet, 1992). Furthermore electrophysiological recordings have demonstrated that combined cardiac and pulmonary afferent stimulation inhibits barosensitive neurones in the RVLM and excites barosensitive and non-barosensitive neurones in the CVLM (Verberne & Guyenet, 1992; Vayssettes-Courchay et al., 1997). The results of these studies demonstrate that cardiac and pulmonary vagal afferent input is further relayed from the NTS to the CVLM and RVLM, and suggests that the baroreflex and Bezold-Jarisch reflex may share a common depressor pathway through the medulla. Whether the RVLM or the CVLM specifically receives cardiac vagal afferent input is unclear from these studies as both cardiac and pulmonary afferent fibres were stimulated. However the strong convergence of chemosensitive pulmonary and cardiac vagal afferent input within the NTS (Paton, 1998c) would indicate that the coronary chemoreflex would involve a similar depressor pathway as the Bezold-Jarisch reflex.
In addition to the CVLM and RVLM, chemosensitive cardiac and combined cardiac and pulmonary vagal afferent input has also been demonstrated to elicit predominantly inhibitory, but also excitatory responses in medullary raphe neurones (Evans & Blair, 1993; Vayssettes-Courchay et al., 1997). Inhibitory responses were reported predominantly in barosensitive sympathoexcitatory raphe neurones in one study (Vayssettes-Courchay et al., 1997) and spinally projecting raphe neurones in another study (Evans & Blair, 1993). Whilst, excitatory responses were predominantly observed in baroinsensitive or non-spinally projecting raphe neurones respectively in these studies. The majority of these neurones were reported to be in the nucleus raphe magnus, which is consistent with this observation an reduction in serotonin accumulation in the nucleus raphe magnus and its rostral continuation the nuclei raphe medianus-centralis, mediated by cardiac vagal afferent fibres following coronary artery occlusion (Sole et al., 1983).

Mechanosensitive cardiac vagal afferent input is thought to be relayed from the NTS to the ventrolateral medulla (VLM). Transection of the brainstem at the level of the inferior peduncle did not alter the magnitude of the reflex tachycardia elicited by distension of the left atrial / pulmonary vein junction indicating that the sympathetic premotor neurones which mediate the increase in heart rate following atrial distension were located within the medulla (Burkhart & Ledsome, 1977). Furthermore, electrophysiological investigations have demonstrated a change in discharge patterns of neurones located in the ventral reticular formation deep to the hypoglossal nucleus following atrial distension (Keith et al., 1975).

The majority of the data indicating a role for medullary regions in the central processing of mechanosensitive cardiac vagal afferent input is derived from studies in which changes in neuronal activity have been detected using c-fos expression in response to hypervolemia (elicited by infusion of plasma expanders) or hypovolemia (elicited by haemorrhage or graded vena caval occlusion) (Chan & Sawchenko, 1994; Krukoff et al., 1995; Badoer et al., 1997; Thrivikraman et al., 1997; Randolph et al., 1998; Potts et al., 2000; Cunningham et al., 2002a, 2002b). The results of these studies have demonstrated an enhanced expression of c-fos in both the CVLM and RVLM in addition to the NTS, area postrema (AP), A5 noradrenergic cell group (A5), locus coeruleus (LC), parabrachial nucleus (PBN), paraventricular nucleus (PVN), supraoptic
nucleus (SON) and perinuclear zone (PNZ) of the hypothalamus, the bed nucleus of the stria terminalis (BNST) and the central nucleus of the amygdala (CNA), following either hypovolemia or hypervolemia. Whilst hypovolemia and hypervolemia have been reported to stimulate atrial and ventricular vagal afferent fibres (Oberg & Thoren, 1972a, b; Kidd et al., 1978; Gupta & Thames, 1983) such a generalised stimulus undoubtedly could elicit c-fos expression through a number of different pathways. However, expression of c-fos in response to hypovolemia and hypervolemia has been reported not to differ between barointact and barodenervated animals leading the authors of this study to suggest that c-fos expression was primarily a consequence of inputs from cardiac receptors and circulating hormones under their experimental conditions (Potts et al., 2000). Furthermore a significant reduction in c-fos expression was observed in the NTS, VLM, LC, PVN and SON but not the AP following intrapericardial injection of procaine indicating that cardiac afferent fibres contributed to the enhanced c-fos expression within these areas but not in the AP (Cunningham et al., 2002a). Although intra-pericardial procaine injection will also eliminate sympathetic cardiac afferent fibres, it is reasonable to assume that at least part of the c-fos expression in these studies was mediated through the activation of cardiac vagal afferent fibres.

1.14.3 Supramedullary areas

As indicated in the previous paragraph hypovolemia or hypervolemia has been demonstrated to elicit an enhanced expression of c-fos within several supramedullary area most notably the PVN and SON, but also the LC and PBN. Whilst the precise contribution of cardiac vagal afferent input to these observed changes in c-fos expression is unclear due to the generalised nature of the stimulus, other studies have examined changes in these supramedullary areas using balloon distension of the atria, as a more specific stimulus. Electrophysiological recordings have demonstrated an inhibitory effect in identified neurosecretory neurones in the PVN and SON in response to atrial distension which was abolished by vagotomy (Koizumi & Yamashita, 1978; Menninger, 1979a, b). In the rat this response has been demonstrated to occur only in the vasopressin secreting neurones of the SON but not the oxytocin secreting neurones, the PVN was not examined (Grindstaff et al., 2000a; Grindstaff & Cunningham, 2001a).
However, an enhanced expression of c-fos has been demonstrated within the PVN following atrial distension in the rat (Deng & Kaufman, 1995; Pyner et al., 2002).

The pathway by which cardiac vagal afferent input from the NTS inhibits the magnocellular neurones of the hypothalamus is unclear. However a GABAergic projection from the PNZ of the lateral hypothalamus to the magnocellular neurones of the supraoptic nucleus has been shown to be excited following atrial distension (Grindstaff & Cunningham, 2001b). In addition electrophysiological recordings have demonstrated that neurones within the dorsal vagal complex were excited by atrial distension project to the locus subcoeruleus (Ward et al., 1977) and that atrial distension excites neurones in the LC (Jhamandas et al., 1988). The LC has been demonstrated to be involved in the baroreceptor mediated inhibition of vasopressin release via a projection to the diagonal band of brocca which in turn projects to the PNZ of the hypothalamus (Cunningham et al., 1994; Grindstaff et al., 2000b). A similar pathway may exist for atrial cardiac vagal afferent input especially as a high percentage of PNZ neurones inhibited by atrial stretch afferent fibres are also inhibited by baroreceptor input suggesting a common afferent pathway (Grindstaff & Cunningham, 2001b).

Recordings have also been made from neurones excited by distension of the right atrium in both the insular cortex (Cechetto & Saper, 1987) and parabrachial nucleus (Jhamandas et al., 1991a). However, the majority of neurones recorded in these studies did not show any response to right atrial distension suggesting that these areas were not prominently involved in the processing of atrial mechanosensitive cardiac vagal input.

In contrast to the literature on the supramedullary areas activated following stimulation of mechanosensitive cardiac vagal afferent fibres there is only a sparse literature on the activation of supramedullary areas following the stimulation of chemosensitive cardiac vagal afferent fibres. What literature that exists has demonstrated an inhibitory effect on spinally projecting PVN neurones (Lovick & Coote, 1988) and an enhanced expression of c-fos in the PAG (Clement et al., 1996), following stimulation of combined cardiac and pulmonary vagal afferent fibres by intravenous injection of 5-HT. The precise role of cardiac vagal afferent fibres in these studies is however unclear. The scanty literature on the supramedullary pathways involved in the processing of cardiac vagal input, in part, may stem from the observation that pontine transection does not effect the Bezold-Jarisch reflex (Lee et al., 1972). Thus, indicating that supramedullary areas are not
important in the central processing of the reflex effects elicited by the combined stimulation of cardiac and pulmonary vagal afferent fibres. However, considering the recent literature demonstrating the antinociceptive effects of vagal afferent stimulation (Ren et al., 1990; Randich et al., 1990) (including chemosensitive cardiac and pulmonary vagal afferent fibres (Randich & Gebhart, 1992)), particularly on spinal neurones which receive nociceptive input from the heart (Ammons et al., 1983, 1985; Fu et al., 1992), may involve the activation of supramedullary areas (Brennan et al., 1987; Chandler et al., 1989). The further understanding of the central processing of cardiac vagal afferent input is an important area as yet not investigated.

Aims
The aim of this thesis was therefore to investigate the central pathways involved in the processing of cardiac vagal input with particular emphasis placed on supramedullary areas that receive ascending inputs from the NTS neurones activated by chemosensitive cardiac vagal afferent fibres.
Chapter 2  Pharmacology of the coronary chemoreflex

Introduction
Stimulation of chemosensitive cardiac vagal afferent fibres elicits a chemoreflex that includes hypotension and bradycardia (Dawes & Comroe, 1954). Experimentally exogenous chemical mediators such as veratridine (Dawes, 1947; Paintal, 1955; Coleridge et al., 1964; Walker et al., 1978), nicotine (Sleight, 1964; Sleight & Widdicombe, 1965; Minisi, 1998), capsaicin (Coleridge et al., 1964, 1973; Waldrop & Mullins, 1987; Clozel et al., 1990) and phenyldiguanide (PDG) (Dawes & Comroe, 1954; Paintal, 1955; Coleridge et al., 1973; Baker et al., 1979; Ravi & Dev, 1988) have been shown to stimulate cardiac vagal afferent fibres and elicit the coronary chemoreflex in the cat and dog.

In the rat PEG has been shown to elicit the coronary chemoreflex by stimulating cardiac vagal afferent fibres (Higuchi et al., 1988; Veelken et al., 1990). In addition, intravenous or left ventricular administration of veratridine has also been reported to produce a bradycardia and hypertension in the rat (Chianca et al., 1985; Yamano et al., 1995; Rocha et al., 2000) and capsaicin has been shown to excite afferent cardiac vagal fibres (Schultz & Ustinova, 1998). However, pericardial administration of nicotine in the rat produces a pressor response, indicating that nicotine preferentially stimulates sympathetic cardiac afferent fibres by this route of administration in this species (Veelken et al., 1990).

Endogenous chemical mediators such as prostaglandins and bradykinin, which can be released from the myocardium during distension of the ventricles (Block et al., 1974) or myocardial infarction and hypoxia (Kimura et al., 1973; Berger et al., 1976; Needleman & Kaley, 1978), have also been shown to excite chemosensitive cardiac vagal afferent fibres in the rat (Ustinova & Schultz, 1994b; Schultz & Ustinova, 1998) cat (Hintze et al., 1984; Reimann & Weaver, 1980) and dog (Baker et al., 1979; Kaufman et al., 1980).
The reflex cardiovascular response produced by these endogenous mediators, however, varies greatly. Bradykinin produces contrasting reflex effects depending on the route of administration and anaesthesia. Epicardial or pericardial administration of bradykinin in the rat (Vetterlein et al., 1984; Veelken et al., 1996), cat (Nishi et al., 1977; Baker et al., 1980) and dog (Staszewska-Barczak & Dusting, 1977) produces pressor responses associated with the activation of sympathetic afferent fibres. However, injection of bradykinin into the circulation either via a coronary artery or into the left atrium evokes a depressor response associated with the activation vagal cardiac afferent fibres in the cat (Kaufman et al., 1980) and dog (Neto et al., 1974). The use of anaesthesia also alters the response to bradykinin, for example, injection of bradykinin into coronary artery, elicits a depressor response in anaesthetised dogs (Neto et al., 1974), however in the conscious animal a pressor response is observed (Pagani et al., 1985). The prevalence of pressor responses following bradykinin administration particularly in conscious animals would suggest that bradykinin preferentially stimulates sympathetic cardiac afferent fibres rather than vagal cardiac afferent fibres.

Intravenous or coronary artery injection of PGI₂, PGF₂α, PGE₂, and 6 keto PGE₁ like bradykinin elicits a vagally mediated bradycardia and hypotension in anaesthetised cats and dogs (Chappie et al., 1980; Chiba & Malik, 1980; Hintze & Kaley, 1984; Hintze et al., 1984; Panzenbeck et al., 1988). However, in contrast to bradykinin, coronary artery injection of PGI₂ in the conscious dog does not produce a pressor response but still produces a vagally mediated bradycardia and hypotension (Nganele & Hintze, 1987) similar to that observed in the anaesthetised animal. The prevalence of depressor responses following administration of postaglandins has led authors to speculate that prostaglandins represent the “natural” stimulus for chemosensitive cardiac vagal afferent fibres (Hintze, 1987).

In the rat intravenous injection of PGE₂ has been shown to produce a vagally mediated hypotension in normotensive and spontaneously hypertensive animals due to the activation of cardiac and / or pulmonary afferent fibres (Leach et al., 1973; Chen et al., 1979).

The aim of the experiments in this chapter was to establish the cardiovascular and respiratory responses following injection of exogenous and endogenous chemical mediators into the left ventricle in the rat. The observation of cardiovascular responses
consistent with the activation of the coronary chemoreflex and their abolition following vagotomy could then be used as an indication of cardiac vagal afferent stimulation. This would allow the determination of an appropriate chemical stimulant of cardiac vagal afferent fibres to use in the studies reported subsequently. In addition the cardiovascular and respiratory responses to systemic injection of chemical mediators were also examined to determine a potential drug dose, which only elicited cardiovascular responses consistent with the activation of the coronary chemoreflex following left ventricular injection. Such a dose could then be used in c-fos studies to selectively activate the coronary chemoreflex but not the pulmonary chemoreflex following re-circulation via the right side of the heart.

**Materials and Methods**

All experiments were performed in accordance with the Animals Scientific Procedures Act 1986.

2.2 Anaesthesia and femoral cannulation

Male Sprague Dawley rats (300-360g) were anaesthetised with sodium pentobarbitone (Sagatal; Rhone Merieux Ltd, Harlow, U.K.), induction 60mg/kg i.p. and supplemented with 18mg/kg i.v. as required following cannulation of the femoral vein with a saline filled polythene tubing (Portex Ltd., Hythe, Kent, U.K. external diameter 0.96 mm, internal diameter 0.58 mm). The femoral artery was cannulated (Portex Ltd., external diameter 0.96 mm, internal diameter 0.58 mm) for the measurement of blood pressure. The depth of anaesthesia was determined throughout the experiment by the absence of a withdrawal response to a noxious toe pinch and the stability of cardiovascular and respiratory variables.

Body temperature was measured with a rectal temperature probe and maintained at 37 ± 1 °C by means of a servo-controlled heating pad (Harvard apparatus Ltd, Edenbridge, Kent, U.K.).
2.3 Surgery

Both cervical vagi were dissected carefully free of the surrounding tissue and a loose suture placed around them. A cannula was advanced into the left ventricle via the right carotid artery for drug and control (pH matched) saline injections. Correct placement of the cannula was established by observing the changes in the blood pressure signal recorded from the cannula on entering the left ventricle, and confirmed post mortem.

The trachea was intubated below the larynx and the animal allowed to breathe 40% oxygen / nitrogen gas mixture. ECG and diaphragmatic EMG were recorded using percutaneous lacquer insulated copper wire electrodes, placed across the chest in a lead II configuration. In some experiments respiratory activity was monitored by recording phrenic nerve activity. The phrenic nerve was exposed on the right hand side by a ventral approach, following the removal of the clavicle and subsequent section of the underlying subclavian vein. The phrenic nerve was cut peripherally and the central cut end prepared for recording using bipolar silver wire (Advent Ltd, Halesworth, Suffolk, U.K.) electrodes and insulated using soft paraffin.

At the end of all experiments rats were humanely killed by anaesthetic overdose (Sagatal, 100mg/kg i.v.).

2.4 Measurement of cardiovascular and respiratory variables

All recorded variables were displayed continuously on a computer monitor in a chart recorder style and simultaneously stored on a computer hard disk using the Spike 2 on line data capture programme and 1401 plus computer interface (C.E.D. Cambridge, U.K.). Blood pressure was recorded at a sampling frequency of 300 Hz, all other recordings were sampled at 1000 Hz.

Blood pressure was monitored by means of a disposable pressure transducer (NeuroLog NL 108T2, Digitimer, Welwyn Garden City, Herts. U.K.) and amplified using a pressure amplifier (NeuroLog NL 108, Digitimer). ECG, diaphragm EMG and phrenic nerve activity were recorded using a Digitimer NL100 head-stage and amplified (NeuroLog NL104; gain 5-10K) and filtered (NeuroLog NL125; frequency band 100-5000 Hz). Diaphragmatic EMG and phrenic nerve activity were integrated either off-
line using a Spike 2 data analysis programme or on-line using a NeuroLog NL703 EMG integrator with a time constant of 50ms.

Once amplified and filtered, signals were relayed to the analogue to digital converter inputs of the 1401 plus interface via a digital recorder (VR-100B, Instrutech Corp., Great Neck, N.Y., USA) from which data could be sent to a VCR for storage on tape.

Throughout the experiment arterial blood gas samples were regularly analysed using a pH/blood gas analyser (Model 238 Corning, Halstead, Essex, U.K.).

2.5 Drug administration

2.5.1 Phenylbiguanide (PBG)

The cardiovascular and respiratory effects of left ventricular injection of incrementing doses of PBG (2, 5, 10, 20 and 40 µg, line filled with 200 µg/ml PBG, pH 10) were examined in male Sprague Dawley rats (n = 4; 325 – 350g) (see Figure 1a for schematic protocol). Initially 2 control pH matched 0.9% saline injections were made at 15 min intervals of the two highest volumes of PBG to be injected (i.e. 100 and 200 µl) and the ventricular cannula loaded with PBG. Thirty minutes later incrementing doses of PBG were injected into the left ventricle at 15 min intervals and the femoral artery cannula loaded with PBG. Following a further 30 min the same doses of PBG were this time injected systemically via the femoral artery cannula at 15 min intervals. A bilateral cervical vagotomy was performed and after a 30 min recovery period the effects of injection of PBG into the left ventricle were re-examined.

2.5.2 Prostaglandin E₂

The cardiovascular and respiratory effects of left ventricular injections of PGE₂ (250, 500, 1000, 2500 ng, line filled with 5 µg/ml PGE₂, pH 7) were examined in rats (n = 4; 300 – 345g) using the same protocol described above for PBG.

2.5.3 Veratridine

The cardiovascular and respiratory effects of left ventricular injection of veratridine (200 µg / ml, pH 7) were examined in a total of 11 rats split into 3 groups described below.
Group I (n = 4; 300 – 360g), (see Figure 1b for schematic protocol). The effects of incrementing doses of veratridine (4, 6, 8, 10 and 20 μg) given at 15 min intervals were examined. As previously using PBG this was preceded by 2 control pH-matched 0.9% saline injections of the two highest volumes of veratridine injected (i.e. 50 and 100 μl). A bilateral cervical vagotomy was then performed and 30 min latter the effects of veratridine were re-examined.

Group II (n = 4; 325 – 360g), the equivalent protocol to that used for Group I was repeated except veratridine was this time injected in decrementing doses (e.g. 20, 10, 8, 6 and 4 μg).

Group III (n = 3; 330 – 350g), (see Figure 1c for schematic protocol), 4 injections of veratridine (5 μg) were made into the left ventricle at 15 min intervals. This was followed by further injections of veratridine (5 μg) at 10, 5, 2 and 1 min to test for a possible sensitisation of cardiovascular and respiratory responses, which were observed from the studies in Groups I & II.

2.6 Data Analysis

2.6.1 Blood pressure and heart rate

Mean arterial blood pressure (MABP) was calculated from diastolic (DBP) and systolic blood pressure (SBP) (MABP = DBP ± 1/3(SBP – DBP)) measured over a 1 min period prior to injection and from the peak change following injection. The onset latency of the peak change in blood pressure was measured from the end of drug injection.

Heart rate was derived off line from the blood pressure recording using Spike 2 software. Each pressure pulse was classified as an “event” which was then counted per minute in 1 s bins to give heart rate in beats per minute (b.p.m.). In some animals heart rate was derived from the blood pressure recording on-line using the wave mark function within Spike 2. Heart rate was measured over a 1 min period prior to injection and the peak change measured following completion of injection. The latency of any change in heart rate was also determined.

For intraventricular injection of drugs changes in peak heart rate and MABP were only measured if they occurred with an onset latency no greater than 3s following injection.
2.6.2 Respiratory interval

The respiratory interval was measured as the time between the peaks of the integrated phrenic nerve or diaphragm EMG activity. The interval was calculated before left ventricular injection as the average of the 5 intervals prior to injection. Respiratory interval was then plotted for the next 10 intervals following injection. The peak change in respiratory interval was calculated as well as the onset latency of any observed change. The onset latency was determined as the first interval of the series of respiratory bursts leading to the peak change showing a >20% change compared to before injection. If the peak change was less than 20% of the pre-injection baseline the latency of onset was determined to the peak change.

2.6.3 Statistical analysis of data

All data was expressed as mean ± sem unless otherwise stated. Changes in heart rate, MABP and breath-breath interval before and after drug or saline injection were analysed statistically using Student’s paired t-test comparison of absolute values; significance was set at P<0.05. Pairwise comparison of these changes before and after bilateral cervical vagotomy, and between control saline and drug injection were made using an unpaired two sample Student’s t-test, significance was set at P<0.05.

2.7 Drugs and solutions

Phenylbiguanide (PBG)(ICN Biochemicals Inc.) a 5-HT3 receptor agonist was dissolved in saline 0.9% w/v.

Prostaglandin E2 (Sigma Chemical Co., Poole, U.K.) was dissolved in saline 0.9% w/v.

Veratridine (Sigma). Stock (10mg/ml) was dissolved in Polyethylene glycol (Sigma), subsequent dilutions made in saline 0.9% w/v.

Saline (0.9 % w/v) (Baxter Healthcare Ltd, Thetford, Norfolk, U.K.)
Figure 1  Schematic representation of the drug administration protocols used in the study of the coronary chemoreflex in the rat.

A  Phenylbiguanide (PBG) or PGE$_2$ administration.

B  Veratridine administration in Group I and II experiments.

C  Veratridine administration in Group III.
(a) Drug loaded Drug loaded Bilateral vagotomy

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15 min

(b) Drug loaded Bilateral vagotomy

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15 min

(c)

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\[d = \text{drug}\]
\[s = \text{saline}\]
\[a = \text{arterial}\]

15 min
Results

The baseline values and changes in heart rate, MABP and breath-breath interval for each dose of the drugs injected can be found tabulated in the appendix of this thesis.

2.8 Resting cardiovascular and respiratory variables

In 19 rats, resting heart rate was 362 ± 7 b.p.m., mean arterial blood pressure (MABP) was 100 ± 4 mmHg and breath-breath interval was 1.0 ± 0.1 s before drug injections. Arterial blood gas measurements were pH of 7.38 ± 0.2, pCO\textsubscript{2} of 37 ± 2 mmHg and pO\textsubscript{2} of 120 ± 7 mmHg.

2.9 Response to vehicle

Left ventricular injections of saline pH 7 and pH 10 did not produce any significant changes in heart rate and MABP except following injection of the highest volume (500\textmu l pH 7) which resulted in a small but significant decrease in heart rate (8.6 ± 2.4 b.p.m.) and MABP (10.7 ± 3.0 mmHg). These changes in heart rate and MABP occurred during the period of injection.

Small but significant decreases in breath-breath interval (maximum 0.11 ± 0.1 s) were also observed following left ventricular injection of 500 and 100\textmu l of saline pH 7 and 100 \textmu l of saline pH 10. The onset latency of these changes ranged from 0.9 ± 0.4 s to 2.1 ± 0.2 s following the completion of injection.

2.10 Response to PBG

Left ventricular injection of PBG (2 – 40 \mu g) produced a significant dose related decrease in heart rate and MABP (Figure 2). The maximum fall in heart rate and MABP produced by injection of 40\mu g PBG was 149 ± 42 b.p.m. and 34 ± 8 mmHg respectively. Following bilateral cervical vagotomy this decrease in heart rate observed after left ventricle injection of PBG was abolished (98% reduction in the bradycardia produced by 40 \mu g PBG) and the decrease in MABP significantly attenuated (68%
reduction in the hypotension produced by 40 μg PBG) (Figure 2). The onset latency of these changes was rapid, with a maximum latency of 0.3 ± 0.2 s for heart rate and 1.3 ± 0.4 s for MABP.

Breath-breath interval also increased following injection of PBG at doses from 5 μg to 40 μg in 3 / 4 rats tested with onset latencies up to 3s from injection (Figure 2). However, overall the change in breath-breath interval following PBG injection did not reach significance (p>0.05) when compared with pre-injection values or control pH matched (pH 10) saline injections. The lowest dose tested (2 μg) produced a significant decrease of 0.2 ± 0.1 s in breath-breath interval, which occurred 0.8 ± 0.3 s following injection of PBG. (Figure 2). These changes in breath-breath interval following PBG injection was not affected by bilateral cervical vagotomy (Figure 2).

Intra-arterial injection of PBG only produced significant decreases in heart rate (maximum fall of 122 ± 62 b.p.m.) and MABP (maximum fall of 35 ± 5 mmHg), at the two highest doses of PBG, 20 μg and 40 μg (Figure 3). The onset latencies of these changes ranged from 4.2 ± 0.4 s to 5.5 ± 0.6 s. Significant decreases in breath-breath interval were also observed following arterial injection of PBG at 20 and 40 μg with onset latencies of 4.1 ± 0.7 s and 2.7 ± 0.3 s.

2.11 Response to PGE₂

Left ventricular injection of PGE₂ produced a significant reduction in MABP at all doses of PGE₂ tested (Figure 4), however no significant changes in heart rate were observed. The hypotension produced by left ventricular injection of PGE₂ was unaffected by bilateral cervical vagotomy (eg. 2500ng PGE₂: 39 ± 0.3 mmHg before vagotomy vs 39 ± 6.6 mmHg following vagotomy). The onset latency of the decrease in MABP following PGE₂ injection occurred within 1s of injection (range: 0.2 ± 0.2 s to 0.6 ± 0.4 s).

Significant decreases in breath-breath interval (e.g. 0.14 ± 0.04 s at 500ng PGE₂, Figure 4) were also observed following left ventricle injection of PGE₂, however, both before and after vagotomy, the onset latency of these changes was long (e.g. 6.8 ± 1.1 s at 500ng PGE₂).
Arterial injection of PGE$_2$ only produced a significant decrease in MABP at the two highest doses (1000 and 2500 ng) delivered, which occurred at onset latencies of $1.28 \pm 0.43$ and $1.54 \pm 0.55$ s respectively (Figure 5).

2.12 Response to veratridine

**Group I**

Left ventricular injections of veratridine in ascending order of dose produced a significant dose related increase in breath-breath interval up to a maximum value of $31 \pm 4$ s (Figure 6a). Bilateral cervical vagotomy reversed the respiratory effects of veratridine, breath-breath interval becoming significantly decreased after injection of veratridine following vagotomy eg. $-0.24 \pm 0.06$ at 20 µg veratridine (Figure 6b). The onset latency of these changes in breath-breath interval decreased in a dose related manner from $6.5 \pm 1.8$ s to $2.4 \pm 0.9$ s before vagotomy and from $3.4 \pm 1.6$ s to $0.5 \pm 0.1$ s after vagotomy following the injection of 4 µg and 20 µg of veratridine. No significant changes were observed in heart rate or MABP before or after vagotomy.

**Group II**

Injection of veratridine in a decrementing dose produced a significant decrease in heart rate at 10 µg ($14.5 \pm 2.3$ b.p.m.) and 20 µg ($18.5 \pm 4.7$ b.p.m.) of veratridine, which were not observed following vagotomy. However no significant changes in MABP were observed before or after vagotomy. The onset latency of the significant decreases in heart rate was approximately 1s after veratridine injection.

Increases in the breath-breath interval were also seen following left ventricle injection of veratridine but were smaller than those observed in group I animals, the maximal increase was $4.7 \pm 2.3$ s at 20 µg veratridine (Figure 6a). Following bilateral cervical vagotomy, left ventricle injection of veratridine did produce, however, a decrease in breath-breath interval that was similar in magnitude to those observed in group I animals (e.g. 10 µg veratridine: group I $0.29 \pm 0.04$ s and group II $0.29 \pm 0.06$ s, Figure 6b). The onset latency of the increase in breath-breath interval at 10µg veratridine was
2.4 ± 0.9 s and the onset latencies of the decrease in breath-breath interval following vagotony ranged from 3.2 ± 1.2 s to 0.9 ± 0.1 s.

**Group III**

Repeated administration of 5 μg of veratridine produced an increase in breath-breath interval, which increased in magnitude with repeated administration of veratridine at 15 min intervals to a maximal level of 8.6 ± 1.7 s, p < 0.05 (Figure 6c). Furthermore, the increase in breath-breath interval following left ventricular injection of veratridine did not change by decreasing the interval between veratridine injections (Figure 6c). The onset latency of the changes in breath-breath interval decreased with repeated veratridine administration from 7.7 ± 0.8 s to 2.3 ± 0.4 s.

No significant decreases in MABP and heart rate were observed following repeated injection of veratridine.
Figure 2  Histogram showing the changes (Δ) in cardiovascular and respiratory variables following intraventricular injection of PBG.

Closed columns represent changes before bilateral cervical vagotomy (n = 4). Open columns represent changes after bilateral cervical vagotomy (n = 4). Each column represents the mean changes and the bars show s.e.m.

Changes have been compared statistically before and after PBG injection and before and after vagotomy using paired and unpaired Student's t-test respectively. Significant changes in absolute values before and after PBG injection are denoted by asterix above the s.e.m. bar. Significant differences between changes in variables before and after bilateral cervical vagotomy are denoted by asterix over brackets between columns.

* p<0.05, ** p<0.01, *** p<0.005.
Figure 3  Histogram showing the changes (Δ) in cardiovascular and respiratory variables following systemic injection of PBG.

Each column represents the mean changes and the bars show s.e.m (n = 4).

Changes have been compared statistically before and after PBG injection using paired Student's t-test.

* p<0.05, ** p<0.01, *** p<0.005.
Figure 4  Histogram showing the changes (Δ) in cardiovascular and respiratory variables following intraventricular injection of PGE₂.

Closed columns represent changes before bilateral cervical vagotomy (n = 4). Open columns represent changes after bilateral cervical vagotomy (n = 4). Each column represents the mean changes and the bars show s.e.m.

Changes have been compared statistically before and after PGE₂ injection and before and after vagotomy using paired and unpaired Student's t-test respectively. Significant changes in absolute values before and after PGE₂ injection are denoted by asterix above the s.e.m. bar.

* p<0.05, ** p<0.01, *** p<0.005.
PGE$_2$ (ng)

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Figure 5  Histogram showing the changes (Δ) in cardiovascular and respiratory variables following systemic injection of PGE₂.

Each column represents the mean changes and the bars show s.e.m (n = 4).

Changes have been compared statistically before and after PGE₂ injection using paired Student's t-test.

* p<0.05, ** p<0.01, *** p<0.005.
Figure 6  Histogram showing the changes (Δ) in breath-breath interval following intraventricular injection of veratridine.

A  Before bilateral cervical vagotomy: Closed columns represent group I animals (n = 4). Open columns represent group II animals (n = 4).

B  After bilateral cervical vagotomy: Closed columns represent group I animals (n = 4). Open columns represent group II animals (n = 4).

C  Group III animals (n = 3)

Each column represents the mean changes and the bars show s.e.m.

Changes before and after veratridine injection in Group I & II animals were compared statistically using paired Student's t-test.

Responses were compared statistically between Group I & II animals using unpaired Student's t-test.

Changes in group III animals were statistically compared before and after veratridine injection using paired Student's t-test, and against the first injection using unpaired Student's t-test.

Significant changes in absolute values before and after veratridine injection are denoted by asterix above the s.e.m. bar. Significant differences between changes in variables between Group I & II animals are denoted by asterix over brackets between columns.

* p<0.05, ** p<0.01, *** p<0.005.
Discussion

The data presented demonstrate a different pattern of cardiovascular and respiratory responses following injection of each drug tested. Drugs were injected into the left ventricle of the rat in order to provide rapid access to cardiac vagal afferent fibre endings located either on the endocardial surface of the left ventricle or within the epicardium and myocardium via the coronary arteries. Of the drugs tested only PEG produced a bradycardia and hypotension that was abolished by bilateral cervical vagotomy. Left ventricular injection of veratridine produced an apnoea that was abolished by vagotomy and injection of PGE$_2$ produced a hypotension which was unaffected by vagotomy.

2.13 Veratridine

The increase in breath-breath interval elicited by left ventricular injection of veratridine was subject to sensitisation. Repeat injection of the same dose of veratridine increased the magnitude of the lengthening of breath-breath interval. Furthermore injection of veratridine in incrementing doses resulted in greater increases in breath-breath interval than injection of veratridine in decrementing magnitudes of dose.

It is possible that the sensitisation of respiratory responses to repeated injection of veratridine was due to the accumulation of veratridine within the circulatory system because of the inadequate clearance of the drug between injections. However, this effect would have been greater following the injection of veratridine in a decrementing dose order rather than following the injection of veratridine in an ascending dose order. Similarly, further sensitisation was not observed following a reduction in the time interval between injections, which would have been expected if veratridine was accumulating systemically.

Veratridine has been shown to produce, however, a prolonged stimulation of sensory afferent fibres (up to 310s) following bolus injection (Paintal, 1957; Coleridge et al., 1964, 1973; Matsumoto et al., 2000) and impair sodium channel inactivation (Strichartz et al., 1987). A prolonged stimulation of afferent fibres by repeated injection of
veratridine may represent the explanation for the sensitisation of respiratory responses in this study.

In all experiments bilateral cervical vagotomy abolished the increase in breath-breath interval, indicating that the effects of veratridine were mediated through the activation of vagal afferent fibres. The latency for the appearance of apnoea on left ventricular injection of veratridine ranged from 2 to 8s. This suggests that the activation of cardiac vagal afferent fibres is not involved. As vagotomy abolished these respiratory effects, the increase in breath-breath interval is most probably due to the activation of pulmonary vagal afferent fibres. This is in agreement with observations showing that veratridine stimulates pulmonary stretch receptors (Paintal, 1953b, 1957; Matsumoto et al., 2000) and elicits the pulmonary chemoreflex (Dawes, 1947), both of which produce a vagally mediated apnoea. Furthermore studies in the rat have demonstrated that veratridine injected intravenously produced a vagally mediated apnoea at a dose that was ineffective when injected into the left ventricle, indicating that the site of action was within the pulmonary circulation (Chianca et al., 1985). The shorter latency responses observed at higher doses in this study probably reflects the access of veratridine to pulmonary vagal afferent fibres via the bronchial circulation whilst the longer latency responses represent the time taken for veratridine to reach the pulmonary circulation.

In contrast to studies in the cat (Daly et al., 1988) and dog (McGregor et al., 1986) veratridine produced only small and inconsistent changes in heart rate in this study. This is in agreement with findings previously reported in the rat using a lower dose of veratridine (Chianca et al., 1985). However, by contrast larger doses of veratridine, injected intravenously or into the left ventricle have been reported to produce a significant bradycardia in the rat (Yamano et al., 1995; Rocha et al., 2000), although in these studies the effect of vagotomy were not explored. In consideration of these reports, left ventricular injection of veratridine at doses greater than 20μg were made in initial studies. Although veratridine did produce a bradycardia in some cases, in the majority of rats this preceded total cardiac arrest. Furthermore these responses were still observed following vagotomy (Figure 7), so the use of doses above 20 μg was discontinued. Veratridine has been shown to prolong the cardiac action potential in the
rat by a direct effect on the heart (Nand et al., 1997), which may account for the cardiac
effects seen following injection of large doses of veratridine.
Figure 7  Raw data illustrating the change in heart rate, blood pressure and integrated diaphragm EMG following left ventricular injection of veratridine (a) 30 µg and (b) 40 µg after bilateral vagotomy.

Left ventricle injection of veratridine is indicated by the bar above the recordings.

Note, in figure (A) veratridine elicits a bradycardia and hypotension consistent with the coronary chemoreflex despite a bilateral cervical vagotomy. Whilst, in figure (B) cardiac arrest can be observed following injection of a higher dose of veratridine.
(a) Veratridine (30 µg)

Integrated diaphragm EMG

Heart rate (b.p.m.)

Blood pressure (mmHg)

(b) Veratridine (40 µg)

Integrated diaphragm EMG

Heart rate (b.p.m.)

Blood pressure (mmHg)

2 s
2.14 PGE₂

Left ventricular injection of PGE₂ produced a fall in MABP, but not heart rate, which was unaffected by bilateral cervical vagotomy demonstrating that the hypotension produced by PGE₂ did not involve the activation of vagal afferent fibres. Previous studies in the rat have also demonstrated a fall in blood pressure following the injection of PGE₂, which was attenuated by vagotomy following intravenous but not aortic injection, suggesting that the action of PGE₂ was in part due to the activation of cardiac and/or pulmonary vagal afferent fibres (Chen et al., 1979). The results observed in this study would therefore suggest that the vagally mediated hypotension produced following the intravenous injection of PGE₂ was due to the activation of pulmonary but not cardiac vagal afferent fibres.

A fall in MABP but no change in heart rate has also been reported following left ventricular injection of PGE₂ in the mouse (Paton, 1998a) and most recently following the epicardial application of PGI₂ in the rat (Sun et al., 2001). However despite the apparent failure of PGE₂ and PGI₂ to produce a bradycardia consistent with the activation of the cardiac chemoreflex in these studies, PGE₂ and PGI₂ were shown to stimulate cardiac vagal afferent fibres in these studies.

2.15 PBG

Left ventricular injection of PBG produced a vagally mediated bradycardia and hypotension consistent with the coronary chemoreflex (Dawes & Comroe, 1954) suggesting that PBG could have activated cardiac vagal afferent fibres by this route of administration. Consistent with this hypothesis, the latency of the cardiovascular responses to PBG injection was rapid occurring within 1s of injection. This would indicate that the reflexogenic areas responsible for the bradycardia and hypotension were within a close vicinity of the left ventricle, excluding the possibility that the pulmonary chemoreflex was also activated. The supposition that the stimulation of cardiac vagal afferent fibres contributed to the cardiovascular changes produced by left ventricular injection PBG, is also in agreement with previous studies which have demonstrated a bradycardia and hypotension following intrapericardial injection of
PBG, that was abolished by vagotomy or pericardial procaine injection (Higuchi et al., 1988; Veelken et al., 1990).

However, the bradycardia observed following pericardial administration of PBG was smaller than that observed in this study although the magnitude of the hypotension was similar. The difference in the magnitude of the bradycardia elicited by the different routes of PBG administration may reflect a difference in access to cardiac vagal afferent fibre endings offered by the different routes of administration. The latency of cardiac vagal afferent activation to epicardial application of capsaicin and bradykinin is long (Schultz & Ustinova, 1998) suggesting that cardiac vagal afferent fibre endings are not located on the epicardial surface of the rat heart but deeper in the heart. Therefore PBG injected into the pericardium would have to diffuse through the epicardial layers to gain access to afferent endings. By contrast left ventricular injection of chemicals would offer instant access to the endocardial surface and better access to myocardial tissue via the coronary circulation and hence stimulate a greater number of cardiac vagal afferent fibres producing a larger response.

However, PBG has also been demonstrated to stimulate chemoreceptor afferent fibres although not baroreceptor afferent fibres in the rat (Goldman & Saum, 1984; Brophy et al., 1999). Although the latency of the bradycardia was rapid following left ventricular injection of PBG, which would exclude the activation of receptor sites distal to the heart, PBG would still have reached the peripheral chemoreceptors within this time. An additional vagally mediated bradycardia produced by the stimulation of peripheral chemoreceptors (Daly, 1991) following ventricular, but not pericardial, injection of PBG may therefore underlie the difference in the magnitude of the bradycardia produced by these two routes of administration.

The failure of bilateral cervical vagotomy to completely abolish the hypotension following ventricular injection of PBG and the increase in breath-breath interval which was unaffected by vagotomy, would further demonstrate that PBG also stimulated other afferent fibres in addition to the proposed cardiac vagal afferent fibres. The longer latency of the increase in breath-breath interval would suggest that the afferent fibres responsible for this response where distal to those involved in the bradycardia. The exact nature of the afferent fibres involved cannot be determined from this study.
however, the activation of laryngeal or trigeminal afferent fibres, or a direct effect of PBG on the CNS or nodose ganglion are possibilities.

Systemic injection of PBG at doses 20 μg and 40 μg elicited a decrease in heart rate and blood pressure and a lengthening of breath-breath interval. The long latency of these responses (2.7 to 5.5 s) would indicate that they were mediated by the activation of pulmonary vagal C fibres and possibly cardiac vagal afferent fibres following circulation of PBG through the right side of the heart. Simulation of pulmonary vagal C-fibres by PBG has been demonstrated to elicit a reflex decrease in heart rate, blood pressure and apnoea referred to as the pulmonary chemoreflex (Dawes & Comroe, 1954; Thoren, 1979; Hainsworth, 1991). At a dose of 10 μg systemic injection of PBG did not elicit any significant changes in heart rate, blood pressure or respiration, however, when injected at this dose in to the left ventricular elicited the coronary chemoreflex. From these results it was concluded that left ventricular injection of 10 μg but not 20 μg or 40 μg would be a suitable dose of PBG to use in a further c-fos study in so much as this dose of PBG would stimulate cardiac vagal afferent fibres but did not appear to stimulate pulmonary vagal C-fibres as indicated by the absence of cardiovascular and respiratory changes following systemic injection. However the presence of a hypotensive and respiratory responses to left ventricle injection of PBG following bilateral cervical vagotomy would indicate that PBG also stimulates other afferent fibres in addition to cardiac vagal afferent fibres demonstrating that PBG would be unsuitable for further use in a c-fos study.

Conclusion
In summary only PBG produced cardiovascular effects consistent with the coronary chemoreflex elicited by the stimulation of cardiac vagal afferent fibres. Although the latency of the responses to PBG injection into the left ventricle and the attenuation of responses following bilateral cervical vagotomy suggests that stimulation of cardiac vagal afferent fibres are likely to be involved in the cardiovascular responses observed. The presence of a small hypotension and respiratory affects following vagotomy would also indicate that PBG activates other reflexogenic area by this route of administration. However, using the coronary chemoreflex as an indication of cardiac vagal afferent stimulation it was concluded, that in the rat, PBG was the most suitable chemical
stimulant of cardiac vagal afferent fibres for the use in further studies conducted as part of this thesis.
Chapter 3  Expression of c-fos within the CNS following stimulation of the cardiac branch of the vagus

Introduction

Expression of the c-fos gene visualised through the detection of its protein product Fos has become over the last 15 years an acceptable means of mapping neuronal excitation within the CNS (Hunt et al., 1987; Morgan et al., 1987; Dragunow & Faull, 1989; Bullitt, 1990; Sharp et al., 1993; Li & Dampney, 1994; Guthrie & Gall, 1995; Herbert, 1996; Herdegen & Leah, 1998).

The c-fos gene belongs to a group of over 100 genes classified as immediate early genes (IEGs) that are rapidly and transiently expressed without the need for de novo protein synthesis (Kruijer et al., 1984; Morgan & Curran, 1986; Sheng & Greenberg, 1990; Herdegen & Leah, 1998). The proteins encoded by IEGs have a wide variety of function from secreted cytokines to cytoplasmic enzymes, however c-fos belongs to a subset of IEGs which includes c-jun, c-myc, krox-20 and krox-24, that encode transcription factors (Herdegen & Leah, 1998). Expression of the c-fos gene produces the Fos protein, which translocates from the cytoplasm to the nucleus where it forms a heterodimer with Jun family proteins (c-Jun, JunB or JunD). The dimerisation of the Fos / Jun proteins is facilitated through the interaction of a conserved series of leucine residues located on a α-helical domain within the Fos and Jun proteins called the “leucine zipper”. Once dimerised the Fos/Jun dimer binds specifically to a consensus recognition sequence on the AP-1 DNA binding site were it regulates the transcription of other genes (Morgan & Curran, 1989; Sheng & Greenberg, 1990; Herdegen & Leah, 1998).

The expression of the c-fos gene, is itself regulated by the constitutive transcription factors CREB and serum response factor (SRF), which, when activated dimerise with themselves allowing them to bind to specific binding elements upstream from the mRNA initiation site of the c-fos gene. The dimerisation of these constitutive transcription factors is in turn initiated by 2nd messenger systems such as cAMP / PKA, DAG / PKC and Ca$^{2+}$ / calmodulin protein kinase which induce dimerisation by phosphorylating the constitutive transcription factors (Sheng & Greenberg, 1990; Herdegen & Leah, 1998).
In *vivo* the enhanced expression of *c-fos* within the CNS has been demonstrated in response to electrical stimulation of peripheral nerves (Erickson & Millhorn, 1994; Gestreau *et al*., 1997; Harrison, 2001; Malakhova & Davenport, 2001) and CNS structures (Krukoff *et al*., 1992, 1994; Petrov *et al*., 1996). Physiological stimuli such as changes in auditory stimuli (Rouiller *et al*., 1992; Friauf, 1995), blood pressure (Murphy *et al*., 1994; Li & Dampney, 1994) and the application of noxious stimuli (Hunt *et al*., 1987; Bullitt, 1990) have also been shown to elicit *c-fos*.

The expression of *c-fos* can also be induced by the direct application of neurotransmitters or agonists e.g. acetylcholine (Greenberg *et al*., 1986; Ennis & Shipley, 1992) and NMDA (Szekely *et al*., 1989; Lerea & McNamara, 1993), to neurones *in vivo* or in culture, or by direct depolarisation induced by elevating extracellular K⁺ concentration (Greenberg *et al*., 1986; Herdegen *et al*., 1993).

The pattern of *c-fos* expression has been shown to be specific for the particular stimuli employed e.g. auditory vs noxious, activating pathways consistent with existing electrophysiological or tracing studies (Hunt *et al*., 1987; Bullitt, 1990; Rouiller *et al*., 1992; Friauf, 1995).

An increase in the expression of *c-fos* has been demonstrated previously in response to electrical stimulation of the vagus nerve within the NTS and other pontine and medullary structures which receive strong afferent projections from the NTS (Gieroba & Blessing, 1994; Yousfi-Malki & Puizillout, 1994). Furthermore the distubution of Fos positive neurones within the NTS and VLM has been described following stimulation of the Bezold-Jarisch reflex by intravenous injections of PBG in the rat and rabbit (Gieroba *et al*., 1995). Although it was suggested that *c-fos* expression was elicited by the combined stimulation of cardiac and pulmonary vagal afferent fibres the contribution of cardiac vagal afferent input to the pattern of Fos labelling in this study remains unclear. In addition intravenous injection of PBG is a relatively non specific stimulus and the contribution of other afferent fibres which could be stimulated by PBG to the observed Fos labelling is as yet undetermined.

The aim of experiments in this chapter was to clarify the distribution of Fos labelling induced by cardiac vagal afferent input. Subsequently the expression of *c-fos* within
sub-cortical areas of the CNS was examined in response to electrical stimulation of the cardiac branch of the vagus.
Materials and methods
All experiments were performed in accordance with the Animals Scientific Procedures Act 1986.

3.2 Anaesthesia and femoral cannulation
Male Sprague Dawley rats (300-375g) were prepared for surgery as previously described (section 2.2.). Femoral arterial and venous cannulations were made on the right side in all animals.

3.3 Surgery
The trachea was cannulated below the larynx and the animal ventilated artificially with 40% oxygen / nitrogen mixture using a positive pressure ventilator (Harvard rodent ventilator, model 683) with 1 cm H\textsubscript{2}O positive end-expiratory pressure (tidal volume 1.5 - 2.0 ml, frequency 50-60 Hz), and the animal placed in a stereotaxic frame (Kopf, Tujunga, Ca., U.S.A.).

The right clavicle was reflected laterally and the 2\textsuperscript{nd} right rib removed to allow access to the superior vena cava and bronchus rostral to the right atrium. The right cardiac branch of the vagus was located anatomically, travelling from the vagus below the origin of the recurrent laryngeal nerve behind the azygos vein through a fat pad located between the vena cava and trachea to the right atrium (Figure 8). Correct identification of the cardiac branch of the vagus was confirmed physiologically by a brief 1 s electrical stimulation (15 V, 1 ms, 50 Hz; Digitimer D4030 stimulus generator and Digitimer DS2A isolated stimulator) of the isolated branch using bipolar silver wire electrodes, which produced an immediate and pronounced bradycardia (Figure 8). The cardiac branch was isolated, cut peripherally and electrically insulated using dental impression material (President, Coltene AG, Switzerland) in the stimulated group of animals. In the control group of animals the cardiac branch was isolated and covered with dental impression material.

The right cervical vagus was also isolated and prepared for whole nerve recording with bipolar silver wire electrodes insulated using dental impression material (whole nerve recordings were made as described in section 2.4.).
3.4 Stimulation protocol

On completion of surgery cardiovascular and respiratory variables were stabilised over a 30 min period.

In the stimulated group of rats (n = 4) the central cut end of the right cardiac branch of the vagus was stimulated (12.5 V, 1 ms pulse, 1 Hz) for 2 h, a stimulus intensity supra-maximal for C-fibre activation that had been determined previously in separate series of rats (n = 3) by measuring the compound action potential (Figure 9) evoked in the cervical vagus following electrical stimulation of the cardiac branch of the vagus (1 Hz, 1 ms pulse, 5 – 12.5 V for 500s). The control group of rats (n = 4) was maintained under anaesthesia for a control 2 h period of sham stimulation.

Animals were perfused through the left ventricle 1 h later with 300ml 0.9% w/v saline followed by 500ml 4 % paraformaldehyde in 0.1M phosphate buffer (pH 7.4). The brain was removed and stored overnight in a 2 % paraformaldehyde and 15 % sucrose solution in 0.1M phosphate buffer (pH 7.4) before being transferred into 30% sucrose solution in 0.1M phosphate buffer (pH 7.4).

3.5 c-fos immunohistochemistry

Coronal sections (40μm) were cut throughout the brain on a freezing microtome (Leica Microsystems Ltd, Milton Keynes, U.K) and placed in gauze bottomed trays bathed in 0.1M phosphate buffer pH 7.4 (PB). Sections were transferred to a rotatory shaker (Heidolph, Titramax 100, Germany) and incubated for 30 min in 50% ethanol followed by 3% hydrogen peroxide in 50% ethanol for a further 30 min. Sections were then washed in 0.1M PB (3 times, 10 min) and incubated in 10% normal goat serum (Sigma) diluted in 0.1M PB for 30 min. Sections were then transferred into glass vials containing a primary rabbit polyclonal antibody to Fos protein (a gift from Dr Gerrard Evan, ICRF, London, U.K.) diluted 1:5000 with 1% phosphate buffered goat serum, containing 0.1% bovine serum albumin and 0.2% Triton X-100 (PBGS) and incubated for 36h at 4 °C.

Following the primary incubation sections were washed in 0.1M PB (3 times, 10 min) and incubated in a 1:200 dilution of secondary biotinylated goat anti-rabbit IgG (Vector Laboratories Ltd., Peterborough, U.K.) in PBGS for 2 h at room temperature. After a further 0.1M PB wash (3 times, 10 min) sections were incubated for 2.5 h, at room
temperature in a 1:1000 dilution of ExtrAvidin-peroxidase (Sigma) in PBGS. Sections were then washed in 0.1M PB (3 times, 10 min) and incubated in 0.05% diaminobenzidine (DAB) solution containing 0.004% ammonium chloride, 0.001% nickel ammonium (II) sulphate and 0.2% D-glucose in 0.1M PB for 20 min. Sections were further incubated for another 20 min in fresh DAB solution containing a 1:1000 dilution of glucose oxidase to initiate the peroxidase reaction.

Fos antibody labelling could then be visualised as a black / brown precipitate formed by the nickel intensified chromogenic DAB reaction product. The reaction was terminated by repeated washes with 0.1M PB.

Sections were mounted on gelatin subbed slides, allowed to air dry and dehydrated in ethanol (70%, 90%, 2 x 100%), cleared using histoclear and coverslipped in DPX (BDH, Lutterworth, Dorset, U.K.).

Control immunohistochemical experiments were performed on randomly selected sections, which involved incubation of sections in the absence of primary Fos antibody or the absence of the secondary antibody. No DAB labelling was observed in any of these sections.

3.6 Analysis of c-fos labelling

Sections were examined under bright field illumination using an Olympus BH2 microscope (Olympus, London, U.K.), Fos positive neurones were identified by their black/brown nuclear staining (Figure 10).

3.6.1 Central autonomic areas examined

Fos labelling was examined within the nucleus tractus solitarii (NTS), area postrema (AP), ventrolateral medulla (VLM), nucleus raphe magnus (NRM), locus coeruleus (LC), an area consistent with the A5 noradrenergic cell group (A5), the parabrachial nucleus (PBN), periaqueductal grey (PAG), median and paramedian raphe nucleus (MRN + PMR), dorsal raphe nucleus (DRN), medial hypothalamus (MH), paraventricular hypothalamus (PVN) and the central nuclei of the amygdala (CNA). Areas were identified using the rat brain stereotaxic atlas of Paxinos and Watson (1992).
The NTS, VLM, PBN and PAG were further sub-divided along their rostrocaudal axis based on easily identifiable anatomical landmarks. The NTS was divided into 5 rostrocaudal levels, centred approximately 0.3mm caudal to obex, at obex (calamus scriptorius) and 0.5mm, 1.0mm and 1.5mm rostral to obex. The VLM was divided into 3 rostrocaudal levels referred to as the caudal VLM (CVLM), the intermediate VLM (IVLM) and the rostral (RVLM). The CVLM referred to the VLM in sections from the level of obex and caudal to obex. The IVLM referred to the VLM in sections containing the area postrema and up to 1.0mm rostral to obex. The RVLM referred to the VLM within sections rostral to the IVLM. The PBN was divided into two rostrocaudal levels, the pontine PBN which referred to the PBN caudal to the inferior colliculi and the mesencephalic PBN which referred to the PBN at the level of the inferior colliculi. The Kölliker-Fuse nucleus of the PBN was examined separately to the PBN in the present study. The PAG was divided into 3 rostrocaudal levels, centred approximately 8.40mm, 8.00mm, and 7.60mm caudal to bregma which were identified by clear changes in the size of the central aqueduct and surrounding PAG at these levels.

3.6.2 Quantification of Fos labelling

In each animal the mean number of Fos positive cells per section was calculated for each area of the CNS examined from a minimum of 3 sections and maximum of 10 sections. Fos labelling was counted separately on sections taken both ipsilateral and contralateral to the side of peripheral stimulation. In areas with a high amount of Fos labelling camera lucida drawings were made of sections using a microscope fitted with a drawing tube (Leica Microsystems Ltd, Milton Keynes, U.K) to enable an accurate count of the Fos labelling present to be made. From these values mean and standard error of the mean for control and stimulated groups were derived. For areas of the CNS divided into different rostrocaudal levels, each level was treated as an individual area and Fos labelling counted as described above.

3.6.3 Statistical analysis of data

The difference in the mean number of Fos labelled cells between the control and the stimulated group of animals was compared statistically using a unpaired Student’ t-test, significance was set at P< 0.05. Statistical comparisons were made between the sides of sections ipsilateral and contralateral to cardiac vagal stimulation or the isolated cardiac
branch of the vagus, using a paired Student's t-test of the mean values for each animal, significance was set at $P< 0.05$. All values are expressed as mean ± sem unless otherwise stated.
Figure 8 Identification of the cardiac branch of the vagus.

A Schematic illustration of the position of the right cardiac branch of the vagus with respect to the surrounding structures as viewed following a right sided thoractomy.

B Raw data illustrating the bradycardia and hypotension following brief electrical stimulation of the cardiac branch of the vagus. Stimulation period indicated by bar above raw data.
(a) cardiac branch of the vagus

(b) 50 Hz, 2 v, 1 ms

- E.C.G. (μV)
- Blood Pressure (mmHg)
Figure 9  Illustration of the compound action potential recorded in the cervical vagus following electrical stimulation of the cardiac vagus.

Stimulus artefact indicated by closed circle.

Responses illustrate the waveform average of 500 stimulations at intensities indicated adjacent to the illustration and at a duration of 1ms and frequency of 1 Hz. C-fibre component of the compound action potential indicated by bracket. Note supra-maximal stimulus intensity for C-fibre activation at 12.5 V.
Figure 10  Digitised photomicrograph illustrating Fos positive labelling.

Top panel: digitised photomicrograph illustrating Fos positive labelling in the lateral PBN from a section taken from a stimulated group animal.

Bottom panel: digitised photomicrograph taken at higher magnification of the area enclosed within the rectangle within the top panel. Arrows indicate examples of Fos positive labelling.

Abbreviations

BC  brachium conjunctiva
LPB  lateral parabrachial nucleus
Me5  mesencephalic trigeminal nucleus
MPB  medial parabrachial nucleus
3.7 Cardiovascular and respiratory variables

The cardiac branch of the vagus was stimulated at a low frequency (1Hz) to prevent stimulation inducing changes in heart rate and blood pressure elicited by cardiac vagal afferent activation. The heart rate and mean arterial blood pressure recorded during cardiac vagal stimulation was $332 \pm 12$ b.p.m. and $85 \pm 5$ mmHg respectively, which was not found to be significantly different to values recorded from control group animals during sham stimulation ($319 \pm 5$ b.p.m. and $93 \pm 2$ mmHg). Furthermore no change in heart rate or blood pressure was observed in the stimulated group animals on commencing stimulation.

Blood gas values obtained from experimental animals were also similar for the control ($pH 7.40 \pm 0.03$, $pCO_2 36 \pm 3$ mmHg, $pO_2 109 \pm 8$ mmHg) and the stimulated ($pH 7.45 \pm 0.01$, $pCO_2 31 \pm 3$ mmHg, $pO_2 110 \pm 1$ mmHg) group animals.

3.8 Distribution of Fos-labelled neurones: control vs stimulated group animals.

3.8.1 Overall

The pattern of distribution of Fos labelling was similar in both the control and the stimulated group of animals where Fos labelling was observed bilaterally in all the areas examined (Figures 11 to 16). However significantly greater ($p < 0.05$) amounts of Fos positive neurones were observed within the NTS, VLM, NRM, PBN and PAG of the stimulated group of animals compared to the control group of animals (Figures 11, 12, 13, 14 & 15). By contrast no significant difference ($p > 0.05$) was observed in the amount of Fos positive neurones observed within the AP, A5, LC, PMR + MRN, DRN, MH, PVN and CNA of the stimulated group of animals compared to the control group of animals (Figures 11, 13, 15, & 16). The mean values of Fos positive neurones per section in each area examined ipsilateral and contralateral to stimulation of the cardiac branch of the vagus are illustrated in Tables 1 & 2 for the control and the stimulated group of animals. Digitised photomicrographs of Fos labelling within each area examined are found in the appendix.
Table 1  Mean ± sem values of Fos positive neurones per hemisection within the brainstem for control and stimulated group animals.

Column labelled p shows the probability value following a 2 way unpaired Student’s t-tested comparision between control and stimulated group values.

Abbreviations

NTS  nucleus tractus solitarii
AP    area postrema
CVLM caudal ventrolateral medulla
IVLM intermediate ventrolateral medulla
RVLM rostral ventrolateral medulla
<table>
<thead>
<tr>
<th>NTS position relative to obex (mm)</th>
<th>Left</th>
<th>Right</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>stimulated</td>
</tr>
<tr>
<td>-0.3</td>
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</tr>
<tr>
<td>0.0</td>
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<td>0.5</td>
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<tr>
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</tr>
<tr>
<td>AP</td>
<td>20 ± 5</td>
<td>12 ± 7</td>
</tr>
<tr>
<td>CVLM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVLM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RVLM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2  Mean ± sem values of Fos positive neurones per hemisection within supramedullary areas of the CNS examined for control and stimulated group animals.

Column labelled p shows the probability value following a 2 way unpaired Student's t-tested comparision between control and stimulated group values.

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>A5</td>
<td>A5 noradrenergic cell area</td>
</tr>
<tr>
<td>NRM</td>
<td>nucleus raphe magnus</td>
</tr>
<tr>
<td>LC</td>
<td>locus coeruleus</td>
</tr>
<tr>
<td>pPBN</td>
<td>pontine parabrachial nucleus</td>
</tr>
<tr>
<td>mPBN</td>
<td>mesencephalic parabrachial nucleus</td>
</tr>
<tr>
<td>KF</td>
<td>kolliker-fuse nucleus</td>
</tr>
<tr>
<td>PAG (a)</td>
<td>periaqueductal grey matter 8.30mm caudal to bregma</td>
</tr>
<tr>
<td>PAG (b)</td>
<td>periaqueductal grey matter 8.00mm caudal to bregma</td>
</tr>
<tr>
<td>PAG (c)</td>
<td>periaqueductal grey matter 7.60mm caudal to bregma</td>
</tr>
<tr>
<td>MRN</td>
<td>median raphe nucleus</td>
</tr>
<tr>
<td>PMR</td>
<td>paramedian raphe nucleus</td>
</tr>
<tr>
<td>DRN</td>
<td>dorsal raphe nucleus</td>
</tr>
<tr>
<td>CNA</td>
<td>central amygdaloid nucleus</td>
</tr>
<tr>
<td>MH</td>
<td>medial hypothalamus</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus of the hypothalamus</td>
</tr>
<tr>
<td></td>
<td>Left</td>
</tr>
<tr>
<td>-------</td>
<td>---------------------</td>
</tr>
<tr>
<td></td>
<td>control</td>
</tr>
<tr>
<td>NRM</td>
<td>3 ± 0.5</td>
</tr>
<tr>
<td>A5</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>LC</td>
<td>15 ± 1</td>
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</tr>
<tr>
<td>mPBN</td>
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</tr>
<tr>
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</tr>
<tr>
<td>DRN</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>CNA</td>
<td>16 ± 4</td>
</tr>
<tr>
<td>MH</td>
<td>24 ± 1</td>
</tr>
<tr>
<td>PVN</td>
<td>98 ± 13</td>
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</table>
3.8.2 Nucleus Tractus Solitarii

Fos labelling was examined within 5 rostrocaudal levels of the NTS. In the control group of animals the greatest amount of Fos labelling was found in sections taken approximately 0.5mm rostral of obex (Figures 11 & Table 1). Within these sections Fos labelling was predominantly found within the intermediate, dorsal and medial subnuclei of the NTS.

In the stimulated group of animals the number of Fos positive neurones were significantly greater bilaterally compared with the control group of animals within the NTS of sections taken approximately 0.3mm caudal to obex (left p = 0.0003; right p = 0.01), at the level of obex (left p = 0.0001; right p = 0.006) and approximately 0.5mm rostral to obex (left p = 0.02; right p = 0.009) (Figure 11 & Table 1). However no significant difference was observed in the number of Fos positive neurones observed in the control and stimulated group of animals within the NTS of sections taken approximately 1.0 mm rostral to obex (left p = 0.68; right p = 0.43) and 1.5mm rostral to obex (left p = 0.67; right p = 0.35)(Figure 11 & Table 1). Within the stimulated group of animals Fos labelling was predominantly observed in the commissural, intermediate, dorsal and medial subnuclei of the NTS (Figure 11).

No Fos positive labelling was observed within the dorsal vagal motor nucleus within sections taken rostral to obex, although occasional Fos labelling was observed within sections at the level of obex and caudal to obex. These labelled nuclei were however small in size and easily distinguishable from the larger motor neurones of the dorsal vagal motor nucleus.

3.8.3 Ventrolateral medulla

Fos positive neurones were examined within 3 rostrocaudal levels of the VLM, the CVLM, IVLM and RVLM. A similar pattern of distribution of Fos positive neurones was observed bilaterally within the CVLM, IVLM and RVLM in the control group of animals and the stimulated group of animals (Figure 12). Labelling was predominantly located ventral to the nucleus retroambiguus in the CVLM and extended laterally towards the spinal trigeminal tract. In the IVLM, the pattern of Fos labelling was
confined to an area between the nucleus ambiguus and the lateral reticular nuclei. In the RVLM, Fos positive neurones were observed below the nucleus ambiguus, extending medially into the lateral paragigantocellular nucleus.

Despite the bilateral distribution of Fos labelling within the VLM it was only possible to quantify the amount of Fos positive neurones in the right VLM ipsilateral on stimulation of the cardiac branch of the vagus. Fos labelling was greatest within the CVLM, and decreased in a caudal to rostral direction through the VLM in both control and stimulated group of animals. A significant increase in the number of Fos positive neurones was observed within the RVLM (p = 0.05) and IVLM (p = 0.05) in the stimulated group of animals compared to the control group animals (Figure 12 & Table 1). However, in the CVLM, although the mean number of Fos positive was greater in the stimulated group of animals, this apparent increase in Fos labelling was not found to be significantly different (p = 0.07) from the level of Fos labelling in the control group of animals (Figure 12 & Table 1).

3.8.4 Parabrachial nucleus

A similar pattern of Fos labelling was observed bilaterally within the pontine PBN, mesencephalic PBN and the Kölliker-Fuse nucleus of both the stimulated and the control group of animals (Figure 14). In the pontine PBN and the mesencephalic PBN Fos labelling was restricted to the lateral PBN with only occasional labelling being observed in the medial PBN. Within the lateral pontine PBN Fos positive neurones were observed predominantly in two clusters, one consistent with the central lateral PBN and the second in an area extending from the external medial to the external lateral PBN. Within the lateral mesencephalic PBN, Fos positive neurones were located in the central lateral PBN nucleus with only the occasional labelling extending further ventral into the external lateral PBN (Figure 14).

Significant bilateral (left p = 0.001; right p = 0.01) increases in the amount of Fos positive neurones within the pontine PBN were observed in the stimulated group of animals compared with the control group of animals (Figure 14 & Table 2). However no significant differences were observed between the amount of Fos labelling observed within the mesencephalic PBN (left p = 0.12; right p = 0.13) and Kölliker-Fuse nucleus
3.8.5 Periaqueductal grey

A similar pattern of Fos labelling was observed within the 3 rostrocaudal levels of the PAG examined in both the control and stimulated group of animals. In sections approximately 8.40mm and 8.00mm caudal to bregma, bilateral Fos labelling was observed in two clusters within the PAG, one in the ventrolateral region of the PAG and the second distributed throughout the dorsolateral region of the PAG. Further rostral at a level approximately 7.60mm caudal to bregma the pattern of bilateral Fos labelling was predominantly located within the ventrolateral subdivision of the PAG (Figures 15).

A statistically significant increase in the number of Fos positive neurones was observed bilaterally (left p = 0.03; right p = 0.003) within the PAG at the rostrocaudal level approximately 8.40 mm caudal to bregma in the stimulated group of animals compared to the control group of animals (Figure 15 & Table 2). However no significant difference was observed in the number of Fos positive neurones within the PAG at the rostrocaudal levels approximately 8.00mm caudal to bregma (left p = 0.34; right p = 0.07) and 7.60mm caudal to bregma (left p = 0.33; right p = 0.60) in the stimulated group of animals compared with the control group of animals (Figure 15 & Table 2).

3.9 Distribution of Fos-labelled neurones: ipsilateral vs contralateral to stimulation

With the exception of one rostrocaudal level of the NTS and one rostrocaudal level of the PAG no significant difference (p > 0.05) was observed between the amount of Fos labelling observed within each area examined, when comparing ipsilateral to contralateral distribution on cardiac vagus stimulation. Within the NTS, 0.5mm rostral of obex the amount of Fos labelling observed on the right side, ipsilateral to cardiac vagus stimulation was found to be significantly greater (p = 0.05) than the amount of Fos labelling observed on the left side, contralateral to cardiac vagal stimulation in the
stimulated group of animals. No significant difference ($p = 0.43$) was observed between the amount of Fos labelling observed ipsilateral compared to contralateral to the exposed cardiac branch of the vagus within the control group of animals at this rostrocaudal level of the NTS. Within the PAG at a rostrocaudal level approximately 7.60 mm caudal of bregma the amount of Fos labelling observed on the left side, contralateral to cardiac vagus stimulation was found to be significantly greater ($p = 0.04$) than the amount of Fos labelling observed on the right side. No significant difference ($p = 0.06$) was observed between each side of the PAG in the control group of animal at this rostrocaudal level of the PAG, although this value was close to significance. Furthermore no significant difference was observed between the amount of Fos labelling observed in the stimulated group of animals compared with the control group of animals for either side of the PAG at this rostrocaudal level.
Figure 11 Illustration of Fos labelling within the NTS and area postrema from a stimulated group animal (a). Histogram of the mean number Fos positive neurones observed within the NTS and area postrema of control and stimulated group animals (b) and (c).

(a) Fos labelling (closed circles) plotted on representative sections adapted from the rat brain stereotaxic atlas of Paxinos and Watson (1992).

(b) and (c) Columns represent mean Fos labelling per section and bars s.e.m.
Closed columns indicate labelling in stimulated group animals (n = 4).
Open columns indicate labelling in control group animals (n = 4).

Statistical comparisons made between stimulated and control group animals using 2 way unpaired Student's t-test.
* denotes p < 0.05, ** denotes p < 0.01, *** denotes p < 0.005.

Statistical comparisons made between left and right side of sections a paired Student's t-test.
# denotes p < 0.05.

Abbreviations
AP area postrema
CVLM caudal ventrolateral medulla
DVN dorsal vagal motor nucleus
IVLM intermediate ventrolateral medulla
LPGi lateral paragigantocellular nucleus
LRt lateral reticular nucleus
NA nucleus ambiguus
NTS nucleus tractus solitarii
rNA retroambiguus nucleus
RVLM rostral ventrolateral medulla
Ts solitary tract
(a)

0.3mm caudal to obex

1.0mm rostral to obex

0.5mm rostral to obex

1.5mm rostral to obex

(b)

Fos positive cells per section

NTS

(c)

Fos positive cells per section

AP

Position relative to obex (mm)

LEFT

RIGHT

108
Figure 12  Illustration of Fos labelling within the ventrolateral medulla from a stimulated group animal (a). Histogram of the mean number Fos positive neurones observed within the ventrolateral medulla of control and stimulated group animals (b).

(a) Fos labelling (closed circles) plotted on representative sections adapted from the rat brain stereotaxic atlas of (Paxinos & Watson, 1992).

(b) Columns represent mean Fos labelling per section and bars s.e.m. Closed columns indicate labelling in stimulated group animals (n = 4). Open columns indicate labelling in control group animals (n = 4).

Statistical comparisons made between stimulated and control group animals using 2 way unpaired Student’s t-test. * denotes p < 0.05.

Abbreviations

AP area postrema
CVLM caudal ventrolateral medulla
DVN dorsal vagal motor nucleus
IVLM intermediate ventrolateral medulla
LPGi lateral nucleus
LRt lateral reticular nucleus
NA nucleus ambiguus
NTS nucleus tractus solitarii
rNA retroambiguus nucleus
RVLM rostral ventrolateral medulla
Ts solitary tract

109
(a) 0.3mm caudal to obex

Obex 1.0mm rostral to obex

Obex 1.5mm rostral to obex

Obex 0.5mm rostral to obex

Obex 2.0mm rostral to obex

1 mm

(b) Fos positive cells per section

P = 0.07

CVLM IVLM RVLM
Figure 13 Illustration of Fos labelling within the nucleus raphe magnus, locus coeruleus and the A5 area of the pontine medulla from a stimulated group animal (a). Histogram of the mean number Fos positive neurones observed within (b) the nucleus raphe magnus, (c) locus coeruleus and (d) the A5 area of the pontine medulla of control and stimulated group animals.

(a) Fos labelling (closed circles) plotted on representative sections adapted from the rat brain stereotaxic atlas of Paxinos and Watson (1992).

(b), (c) and (d)
Columns represent mean Fos labelling per section and bars s.e.m.
Closed columns indicate labelling in stimulated group animals (n = 4).
Open columns indicate labelling in control group animals (n = 4).
Statistical comparisons made between stimulated and control group animals using 2 way unpaired Student's t-test.

* denotes p < 0.05

Abbreviations

7n facial nerve or its root
A5 A5 noradrenaline cells
BC brachium conjunctiva
LC locus coeruleus
MPB medial parabrachial nucleus
NRM nucleus raphe magnus
sp5 spinal trigeminal tract
(a) 10.30mm caudal to bregma

(b) NRM

Fos positive cells per section

LEFT  RIGHT

(c) LC

Fos positive cells per section

LEFT  RIGHT

(d) A5

Fos positive cells per section

RIGHT
Figure 14: Illustration of Fos labelling within the PBN from a stimulated group animal (a) and (c). Histogram of the mean number Fos positive neurones observed within the PBN of control and stimulated group animals (b) and (d).

(a) and (c) Fos labelling (closed circles) plotted on representative sections adapted from the rat brain stereotaxic atlas of Paxinos and Watson (1992) and (Herbert et al., 1990).

(b) and (d)
Columns represent mean c-fos labelling per section and bars s.e.m.
Closed columns indicate labelling in stimulated group animals (n = 4).
Open columns indicate labelling in control group animals (n = 4).
Statistical comparisons made between stimulated and control group animals using 2 way unpaired Student's t-test.
* denotes p < 0.05, ** denotes p < 0.01, *** denotes p < 0.005.

Abbreviations
BC brachium conjunctiva
KF kölliker-fuse nucleus
LC locus coeruleus
LPBC central lateral parabrachial nucleus
LPBD dorsal lateral parabrachial nucleus
LPBE external lateral parabrachial nucleus
LPBI internal lateral parabrachial nucleus
Me5 mesencephalic trigeminal nucleus
MPB medial parabrachial nucleus
MPBE external medial parabrachial nucleus
py pyramidal tract
s5 sensory root trigeminal nerve
Fos positive cells per section

(a) Pontine PBN

(b) Fos positive cells per section

(c) Mesencephalic PBN

(d) Fos positive cells per section
Figure 15  Illustration of Fos labelling within the periaqueductal grey and midbrain raphe nuclei from a stimulated group animal (a). Histogram of the mean number Fos positive neurones observed within (b) the periaqueductal grey, (c) nucleus raphe magnus and (d) median / paramedian raphe nuclei of control and stimulated group animals.

(a) Fos labelling (closed circles) plotted on representative sections adapted from the rat brain stereotaxic atlas of Paxinos and Watson (1992).

(b), (c) and (d)

Columns represent mean Fos labelling per section and bars s.e.m.
Closed columns indicate labelling in stimulated group animals (n = 4).
Open columns indicate labelling in control group animals (n = 4).
Statistical comparisons made between stimulated and control group animals using 2 way unpaired Student's t-test.
* denotes p < 0.05, ** denotes p < 0.01, *** denotes p < 0.005.
Statistical comparisons made between left and right side of sections a paired Student's t-test.
* denotes p < 0.05.

Abbreviations

DRN dorsal raphe nucleus
MRN median raphe nucleus
PAG periaqueductal grey
PMR paramedian raphe nucleus
(a) Bregma - 8.30mm  
Bregma - 8.00mm  
Bregma - 7.60mm

(b) Fos positive cells per section

PAG

-8.40 -8.00 -7.60 -8.40 -8.00 -7.60
LEFT RIGHT
Position relative to bregma (mm)

(c) Fos positive cells per section

DRN

LEFT  RIGHT

(d) Fos positive cells per section

MRN + PMR

LEFT  RIGHT
Figure 16  Illustration of Fos labelling within (a) the amygdala and (c) hypothalamus from a stimulated group animal. Histogram of the mean number Fos positive neurones observed within (b) the amygdala and (d) hypothalamus of control and stimulated group animals.

(a) and (c) Fos labelling (closed circles) plotted on representative sections adapted from the rat brain stereotaxic atlas of Paxinos and Watson (1992).

(b) and (d)

Columns represent mean c-fos labelling per section and bars s.e.m.
Closed columns indicate labelling in stimulated group animals (n = 3).
Open columns indicate labelling in control group animals (n = 3).

Statistical comparisons made between stimulated and control group animals using 2 way unpaired Student's t-test.

* denotes p < 0.05.

Abbreviations
Arc  arcuate hypothalamic nucleus
CNA  central amygdaloid nucleus
DMH  dorsomedial hypothalamic nucleus
opt  optic tract
PVN  paraventricular hypothalamic nucleus
VMH  ventromedial hypothalamic nucleus
VL  ventrolateral thalamic nucleus
VM  ventromedial thalamic nucleus
VPL  ventroposterolateral thalamic nucleus
VPM  ventroposteromedial thalamic nucleus
ZI  zona incerta
Discussion

The results of this study demonstrated a significant bilateral increase in Fos labelling within the nucleus tractus solitarii, ventrolateral medulla, nucleus raphe magnus, parabrachial nucleus and the periaqueductal grey following the electrical stimulation of the right cardiac branch of the vagus. Fos labelling was also examined with the area postrema, the A5 area of the pons, locus coeruleus, paramedian and dorsal raphe nuclei, central nuclei of the amygdala and the paraventricular and medial hypothalamus. However, no significant difference was found between the amount of Fos labelling in the control group of animals or the stimulated group of animals within these areas.

3.10 Methodological considerations

3.10.1 Control conditions

The neuronal expression of \( c-fos \) can be elicited in response to a variety of stimuli (Herdegen & Leah, 1998), although the basal expression of \( c-fos \) is sparse or absent within the majority of the CNS (Bullitt, 1990; Li & Dampney, 1994). In order to use \( c-fos \) expression to detect neuronal activity in response to specific stimuli the upmost care must be taken to minimise the activation in response to other stimuli.

Within this study the presence of Fos labelling in the control group of animals illustrates the expression of \( c-fos \) elicited by non-specific stimuli or by ongoing afferent input. The use of invasive surgical procedures, in the isolation of the cardiac and cervical vagus, the cannulation of blood vessels and the intubation of the trachea, will have activated both cutaneous and visceral afferent fibres and will have contributed to the majority \( c-fos \) expression observed in the control group of animals.

Anaesthesia alone has also been shown to elicit differing patterns of \( c-fos \) expression based on the anaesthetic agent used (Bullitt, 1990; Murphy et al., 1994; Krukoff et al., 1992; Bellavance & Beitz, 1996; Clement et al., 1996). In the present study sodium pentobarbitone was used as the anaesthetic agent. Previous studies have demonstrated that with the exception of the habenula nucleus, sodium pentobarbitone does not appear to elicit the expression of \( c-fos \) within the CNS (Krukoff et al., 1992). Furthermore sodium pentobarbitone may also reduce the expression of \( c-fos \) within the CNS.
(Dragunow & Faull, 1989) and so it is unlikely to have contributed to the Fos labelling observed in control animals in this study.

It is also possible that the brief high frequency stimulation used to identify the cardiac branch may have resulted in \textit{c-fos} expression. This is however unlikely, as previous studies have demonstrated that a sustained period of stimulation is required for \textit{c-fos} expression (Dragunow & Faull, 1989; Li & Dampney, 1994). Considering the brief nature of the stimulation period used to identify the cardiac branch of the vagus (maximum 5s), it would be unlikely that this contributed to the expression of \textit{c-fos} in this study.

3.10.2 Cardiac vagal stimulation

The expression of \textit{c-fos} can be used to determine the pattern of activation within the CNS, in response to a specific stimulus down to the level of a single neurone. Due to the comparatively high amount of Fos labelling observed within the control group of animals it was not possible to make a detailed analysis of the distribution of Fos positive neurones elicited by electrical stimulation of the cardiac branch of the vagus. Instead the response of defined CNS areas or nuclei to cardiac vagal stimulation was determined by the statistical comparison of the number of Fos positive neurones present within these areas in the stimulated group of animals compared to the control group of animals. Within some structures eg. NTS and PAG, it was, possible to further subdivide these structures along their rostro-caudal axis and so provide some limited topographical information on the response to cardiac vagal stimulation within these areas. The high degree of \textit{c-fos} expression in response to non-specific stimuli, as observed within the control group of animals, represented a major limitation of the present experiments.

The cardiac branch of the vagus has been shown in the cat to contain afferent fibres that terminate within areas outside the heart such as the lungs and oesophagus (Bennett \textit{et al.}, 1985). Whether this is true of the rat is unknown. In the rat the cardiac vagus does not send any obvious branches in the direction of the lungs (personal observations) as observed in the cat (Bennett \textit{et al.}, 1985). However, without detailed study into the termination of the cardiac vagus in the rat the possibility that the cardiac vagus in the rat also contains afferent fibres that terminate outside the heart cannot be excluded. Furthermore it is possible that sympathetic afferent fibres may also travel with the
cardiac branch of the vagus, however again no obvious branching of the cardiac vagus was observed between the point of stimulation and the main vagal trunk. The use of electrical stimulation of the cardiac branch of the vagus as a means of specifically activating cardiac vagal afferent fibres is therefore limited by the contribution of other afferent fibres of extracardiac or non-vagal origin to the cardiac branch of the vagus. However the majority of afferent fibres stimulated will be of cardiac vagal origin. Care was also taken to isolate the cardiac vagus from surrounding structures in order to avoid any possible activation of non-cardiac afferent fibres due to current spread. Stimulation of connective tissue surrounding the cardiac vagus did not produce any cardiac slowing indicating that current spread was not a problem.

As an alternative to electrical stimulation of cardiac vagal afferent fibres the use of chemical mediators or distension of the cardiac chambers have been employed to stimulate cardiac vagal afferent fibres. However both these methods would also activate sympathetic cardiac afferent fibres and would still require the use of invasive surgical procedure to limit their effects on extracardiac sites.

Electrical stimulation of the central cut end of the cardiac branch of the vagus in the cat at intensities sufficient to activate unmyelinated fibres has been shown to produce a frequency dependent bradycardia and a decrease in blood pressure (Oberg & White, 1970; Oberg & Thoren, 1973b). Within this study, electrical stimulation of the cardiac branch of the vagus was performed at high intensity, sufficient to stimulate C-fibres supra-maximal, but at low frequency (1 Hz) to avoid secondary changes in c-fos expression caused by sustained hypotension (Murphy et al., 1994; Li & Dampney, 1994). At these parameters no significant difference was observed between cardiovascular variables measured in the control group of animals compared to the stimulated group of animals.

The results of this study indicate that low frequency stimulation (1Hz) of the cardiac vagus was sufficient to elicit c-fos expression. In agreement with this, other studies have also demonstrated an enhanced expression of c-fos within the CNS following low frequency stimulation of peripheral nerves (Hoskin et al., 1996; Gestreau et al., 1997; Malakhova & Davenport, 2001; Sang & Goyal, 2001). The possibility that stimulation of the cardiac vagus at higher frequencies may have elicited a greater expression of c-
fos was not however explored in the present study and represents a limitation of this study.

In order to avoid changes in cardiovascular variables elicited by the activation of efferent cardiac vagal fibres the cardiac vagus was sectioned in the stimulated group of animals and only the central cut end electrically stimulated. It is therefore plausible that the removal of tonic afferent input by sectioning the cardiac vagus may have contributed to the Fos labelling seen in the stimulated group of animals.

The duration of stimulation of the cardiac vagus (2 hr) and subsequent time following stimulation before perfusion (1hr) was selected based on existing experimental data for the transient accumulation of c-fos mRNA within the cytoplasm and the approximate half life of the Fos protein (Morgan & Curran, 1989).

3.10.3 c-fos expression as a marker of neuronal activity

Although c-fos has been shown be a reliable marker of neuronal activity, not all neurones responding to a specific stimulus will express c-fos, for example, the ventrolateral thalamus has not yet been shown to express c-fos in response to somatic stimuli (Morgan & Curran, 1989; Bullitt, 1990; Herdegen & Leah, 1998). Comparisons of the patterns of c-fos expression have been shown to be similar to those obtained by metabolic mapping studies using 2-deoxyglucose, although this is not always the case (Dragunow & Faull, 1989; Morgan & Curran, 1989; Sharp et al., 1993). Furthermore the expression of c-fos does not occur in response to inhibitory GABAergic input mediated by increased Chloride ion influx (Sheng & Greenberg, 1990). Observations from studies using c-fos as a marker of neuronal activity must therefore be restricted to the population of neurones expressing c-fos as the absence of expression is not necessarily an indication of inactivity.
3.11  *c-fos* expression following electrical stimulation of the cardiac branch of the vagus

3.11.1 Nucleus tractus solitarii

Consistent with existing anatomical (Kalia & Mesulam, 1980; Xie *et al.*, 1999) and electrophysiological studies which have demonstrated the termination of cardiac vagal afferent fibres input within the NTS (Bennett *et al.*, 1985; Paton, 1998a; Silva-Carvalho *et al.*, 1998; Seagard *et al.*, 1999), statistically significant increases in the number of Fos positive neurones were observed within the NTS following cardiac vagal stimulation compared to control treated animals. The significant increases in Fos labelling were only observed within sections of the NTS from 0.3mm caudal to obex through to 0.5mm rostral of obex which correlate with the previous described caudal and intermediate divisions of the NTS (Loewy, 1990; Saper, 1995). No statistically significant increases in Fos labelling were observed following cardiac vagal stimulation in more rostral sections of the NTS. This topographical distribution of the increased Fos labelling in response to cardiac vagal stimulation within the NTS is in agreement with previously reported distribution of cardiac vagal afferent fibres within the NTS (Kalia & Mesulam, 1980; Xie *et al.*, 1999).

The number of Fos positive neurones present within the intermediate NTS (sections approximately 0.5mm rostral to obex) ipsilateral to cardiac vagal stimulation was significantly greater than that observed on the contralateral side. A similar distribution was not observed in the control group of animals indicating that the stimulation of the cardiac vagus was responsible for the greater Fos labelling on the ipsilateral side. This is in agreement with previous studies, which have demonstrated a greater distribution of Fos labelling within the NTS ipsilateral to the side of electrical stimulation of the cervical (Yousfi-Malki & Puizillout, 1994) and the sub-diaphragmatic vagus (Gieroba & Blessing, 1994).

Within the other subdivisions of the NTS at the level of obex and caudal to obex which demonstrated a bilateral increase in *c-fos* expression in response to cardiac vagal stimulation no significant difference was observed in the amount of Fos labelling ipsilateral to cardiac vagal stimulation compared with contralateral. The absence of an ipsilateral dominance in the amount of Fos labelling following unilateral cardiac vagal
stimulation within the NTS at the level of obex and caudal to obex does not necessarily reflect the distribution of the fibre terminations from the right cardiac branch of the vagus within the NTS. Tracing studies have demonstrated that approximately a 2/3 of vagal afferent fibres terminate within the NTS ipsilateral to tracer injections within paired visceral organs (Kalia & Mesulam, 1980). A similar distribution could be expected for the termination of vagal afferent fibres within the right cardiac branch of the vagus. The bilateral distribution of Fos labelling seen within the NTS at the level of obex and caudal to obex following cardiac vagal stimulation most probably reflects the bilateral distribution of cardiac vagal input along a polysynaptic pathway within the NTS. Indeed extensive projections have been shown between the two sides of the NTS (Norgren, 1978; Otake et al., 1992). Furthermore the expression of c-fos will not differentiate between the activation of NTS neurones in response to afferent input compared with efferent input from other CNS areas.

An ipsilateral predominance in the distribution of Fos labelling following unilateral cardiac vagal stimulation was not observed within any of the other CNS areas examined suggesting that the processing of cardiac vagal afferent input from the right cardiac branch of the vagus occurred bilaterally beyond the NTS.

3.11.2 Ventrolateral medulla

Consistent with previous studies, which have demonstrated an enhanced expression of c-fos within the RVLM and IVLM following stimulation of mechanosensitive cardiac afferent fibres or chemosensitive combined cardiac and pulmonary afferent fibres (Gieroba et al., 1995; Cunningham et al., 2002a). An enhanced expression of c-fos was observed within the RVLM and IVLM following electrical stimulation of the cardiac branch of the vagus indicating that cardiac vagal input is further relayed from the NTS to both the RVLM and IVLM. Within the CVLM an apparent increase in Fos labelling was observed within the stimulated group of animals although this did not reach significance (p=0.07, Student’s t-test). However, previous studies have demonstrated that neurones within the CVLM respond to chemosensitive combined cardiac and pulmonary vagal afferent or mechanosensitive cardiac afferent input (Verberne & Guyenet, 1992; Gieroba et al., 1995; Vayssettes-Courchay et al., 1997; Cunningham et
Consequently the failure to observe a significant increase in Fos labelling within the CVLM in the present study would appear to be due to the high level of Fos labelling caused by non-specific stimuli preventing any further increase in c-fos expression to cardiac vagal stimulation from being observed, rather than an indication that the CVLM does not receive cardiac vagal input.

The enhanced expression of c-fos within the VLM is consistent with the proposed role of this area in mediating the cardiovascular responses elicited following the stimulation of both chemosensitive and mechanosensitive cardiac vagal afferent fibres (Lee et al., 1972; Burkhart & Ledsome, 1977; Verberne et al., 1989; Verberne & Guyenet, 1992; Vayssettes-Courchay et al., 1997). Although the sympathoinhibitory responses elicited by chemosensitive cardiac vagal afferent stimulation may not have contributed to the c-fos expression within the RVLM as this is dependent upon GABA_A receptor activation, which does not elicit c-fos expression (Sheng & Greenberg, 1990; Verberne & Guyenet, 1992).

In addition to a possible role in mediating the reflex cardiovascular effects elicited by cardiac vagal afferent stimulation the VLM has been demonstrated to send efferent projects to numerous regions of the CNS including prominent projections to the PAG and PBN (Loewy et al., 1981; Van Bockstaele et al., 1989). It is therefore possible that the increased Fos labelling observed in the VLM following cardiac vagal stimulation, may reflect a role in the relay of cardiac vagal input to supermedullary areas. Conversely the VLM also receives descending inputs from the PAG, PBN and NRM and so the increase in Fos labelling within the VLM may be due to the activation of descending pathways from higher centres in response to cardiac vagal input (Connelly et al., 1989; Herbert et al., 1990; Carrive & Bandler, 1991).

### 3.11.3 Supra-medullary areas

Significant bilateral increases in Fos labelling were observed within the NRM, PAG and PBN following cardiac vagal stimulation compared to control animals. The significant increases in Fos labelling observed within the PBN and PAG were restricted to the pontine PBN and the caudal most subdivision of the PAG examined.
Previous studies have suggested the involvement of these areas in the processing of cardiac vagal input although the precise nature of the stimulus used in these studies was not well defined and often involved the activation of both sympathetic and vagal afferent fibres from the heart and lungs. Within the NRM electrophysiological studies have demonstrated both excitatory and inhibitory responses following stimulation of chemosensitive cardiac and pulmonary vagal afferent fibres (Evans & Blair, 1993). Whilst occlusion of the left coronary artery has been shown to decrease serotonergic activity within the NRM through the activation of vagal afferent fibres (Sole et al., 1983). Within the PAG enhanced expression of c-fos has been previously demonstrated in response to combined cardiac and pulmonary afferent stimulation following the i.v. injection of 5-HT in the rat (Clement et al., 1996). The expression of c-fos following cardiac and pulmonary afferent stimulation was greatest within the caudal PAG, which is consistent with the observation of significant increases in Fos labelling within sections approximately 8.30mm caudal to bregma in the present study. Within the PBN excitation of recorded neurones has been reported in response to atrial distension in the rat (Jhamandas et al., 1991a). However in the majority of neurones recorded atrial distension had no effect leading the authors to conclude that the PBN was not prominently involved in the processing of input from atrial stretch receptors. By contrast significant bilateral increases in Fos labelling were observed with the pontine PBN within this study indicating that the PBN is involved in the processing of cardiac vagal input.

The role of the NRM, PAG and PBN in the processing of cardiac vagal input is unclear, although transection studies indicate that supramedullary areas are not important in mediating the cardiovascular responses to cardiac vagal afferent stimulation (Lee et al., 1972; Burkhart & Ledsome, 1977). However both the PBN and the PAG have been shown to modulate the baroreflex (Nosaka et al., 1993; Inui et al., 1994; Saleh & Connell, 1997; Saleh & Connell, 1998), and may therefore be involved in the depression of the baroreflex elicited by chemosensitive cardiac vagal afferent stimulation. Furthermore the PBN and the PAG have been demonstrated to send extensive projection to higher centres in the CNS such as the hypothalamus, central nucleus of the amygdala, ventrobasal and intralaminar thalamus and hence may be involved in the further relay of cardiac vagal input within the CNS (Loewy, 1990; Saper, 1995).
In addition to eliciting cardiovascular reflex responses, stimulation of cardiac and pulmonary afferent fibres has been reported to have an antinociceptive effect, increasing the latency of withdrawal reflexes and inhibiting the response of thoracic dorsal horn neurones to nociceptive input, including cardiac and pulmonary sympathetic afferent fibres (Ammons et al., 1983; Hobbs et al., 1989; Meller et al., 1992). The NRM, PAG and the PBN have been implicated in the descending modulation of nociceptive inputs and may be important in mediating the antinociceptive effects elicited by cardiac and pulmonary vagal afferent activation, consistent with the enhanced expression of c-fos within these areas (Fields et al., 1977; Katayama et al., 1984; Sandkuhler, 1996). In particular, stimulation of the PAG, NRM and the PBN have been shown to inhibit spinothalamic neurones within the thoracic spinal cord that receive putative nociceptive sympathetic cardiac input (Brennan et al., 1987; Girardot et al., 1987; Chandler et al., 1989; Ammons et al., 1984). The processing of cardiac vagal afferent input within the PBN, NRM and PAG may therefore form part of a vagally mediated antinociceptive pathway designed to balance nociceptive input from the heart relayed by sympathetic cardiac afferent fibres.

**Conclusion**

The results of this study indicate an involvement of both medullary and supramedullary structures in the central processing of cardiac vagal input within the rat. It is proposed that the activation of these areas contributes to the generation of the cardiovascular and sympathoinhibitory reflex effects of cardiac vagal afferent activation. Furthermore these areas may also be involved in the ascending relay of cardiac vagal input to higher centres within the CNS and in the descending modulation of nociceptive inputs into the CNS.
Chapter 4 Projection of NTS neurones which receive cardiac vagal afferent inputs to the parabrachial nucleus

Introduction
The results presented in chapter 3 showed an enhanced expression of c-fos within the NTS, VLM, NRM, PAG and PBN following electrical stimulation of the cardiac branch of the vagus indicating that neurones within these areas were activated in response to cardiac vagal input. The pathway by which cardiac vagal input activated neurones within these areas was not determined in the Fos study. However as discussed within the introduction to this thesis cardiac vagal afferent input has been demonstrated to terminate within the NTS and is further relayed from the NTS to the VLM via direct projections from the NTS and also via a possible di-synaptic connection from the CVLM to the RVLM. There is however only a sparse literature concerning the supramedullary processing of cardiac vagal input within the NRM, PAG and PBN, and so the pathway by which cardiac vagal afferent input elicits an enhanced expression of c-fos with these areas is unclear.

Anatomical studies have demonstrated a strong efferent projection from areas of the NTS which receive cardiac vagal input to the PBN and to a lesser extent the PAG, indicating that cardiac vagal afferent input may be relayed via ascending projections from the NTS to the PBN or PAG (Loewy & Burton, 1978; Norgren, 1978; Ricardo & Koh, 1978; Kapp et al., 1989; Herbert et al., 1990; Herbert & Saper, 1992; Otake et al., 1992). By contrast, although the NRM has been demonstrated to receive afferent projections from the NTS, the NTS is not a major source of afferent input to the NRM (Beitz, 1982a; Beitz, 1982b; Hermann et al., 1997), consequently the NRM would be likely to receive cardiac vagal input via an indirect pathway from the NTS.

One study by Ward et al. (1977), demonstrated that neurones within the dorsal medulla, including the NTS, which respond to volume pulsations of the right atrium could be antidromically activated by stimulation of the locus subcoeruleus. Since previous studies have not demonstrated an appreciable efferent projection from the NTS to the LC (Ennis & Aston-Jones, 1989; Aston-Jones et al., 1991). It is probable that the dorsal medulla neurones antidromically activated by locus subcoeruleus stimulation in the study by Ward responded to the activation of fibre terminations within the adjacent
PBN or fibres which traversed the locus subcoeruleus before terminating within the PAG (Herbert & Saper, 1992; Otake et al., 1992).

Consequently the aim of experiments within this chapter was to clarify whether NTS neurones, which receive cardiac vagal input, project directly to the PBN. Furthermore considering a previous study by Jhamandas et al. (1991a) concluded that the PBN was not an important structure in the integration of atrial stretch receptor input, the second aim of this study was to provide information on the modality of the cardiac vagal input received by the PBN by examining the response of investigated neurones to left ventricular injection of PBG.

The PBN is an attractive site for further study. Previous studies have demonstrated that the PBN receives a variety of visceral afferent input from pulmonary and gastrointestinal vagal afferent fibres, as well as baroreceptor, chemoreceptor, laryngeal and gustatory afferent fibres via direct projections from the NTS (Norgren & Pfaffmann, 1975; Cechetto & Calaresu, 1983; Hermann & Rogers, 1985; Kobashi & Adachi, 1986; Jhamandas et al., 1991a; Yuan & Barber, 1991; Saleh & Cechetto, 1996; Ezure et al., 1998a; Miyaoka et al., 1998). Furthermore the PBN sends strong ascending projections to many areas which receive only weak afferent input from the NTS such as the PVN, SON, CNA and BNST, providing an alternative pathway by which visceral inputs into the NTS are relayed to these higher CNS areas (Ricardo & Koh, 1978; Saper & Loewy, 1980; Fulwiler & Saper, 1984; Kapp et al., 1989; Cunningham, Jr. et al., 1990; Jhamandas et al., 1991b; Krukoff et al., 1993; Jhamandas et al., 1996; Bester et al., 1997; Bianchi et al., 1998). In addition the PBN sends extensive projections to the thalamus and via a thalamic relay or directly to the insular cortex and represents a major relay for vagal and other visceral input received by the NTS to the thalamus and insular cortex (Saper, 1982; Cechetto & Saper, 1987; Bester et al., 1999; Krout & Loewy, 2000a). The PBN also receives strong inputs from spinal cord and has been shown to receive both cutaneous and visceral spinal afferent input (Cechetto et al., 1985; Hylden et al., 1985; McMahon & Wall, 1985; Hylden et al., 1986; Bernard et al., 1989; Berkley & Scofield, 1990; Bernard et al., 1994; Bester et al., 1995; Menendez et al., 1996).

Subsequently the PBN is regarded after the NTS as the second major site for the integration and relay of vagal and other visceral afferent input within the CNS.
Furthermore the PBN is also regarded as a major integrative of both visceral and cutaneous spinal afferent input to the CNS. The further relay of cardiac vagal input from the NTS to the PBN would consequently be very important in the processing of cardiac vagal input in higher areas of the CNS and also in the integration with spinal afferent input.
**Materials and Methods**

All experiments were performed in accordance with the Animals Scientific Procedures Act 1986.

### 4.2 Anaesthesia and femoral cannulation

Male Sprague Dawley rats (300-385g) were prepared for surgery as previously described (section 2.2).

### 4.3 Cannulation of the chambers of the heart

Double lumen cannula (Harvard apparatus Ltd.) pre-filled with phenylbiguanide (PBG; 200µg/ml) and saline were advanced into the right atrium and the left ventricle via the right external jugular vein and right carotid artery respectively.

Correct placement of the right atrial cannula was assessed by :-

- The presence of a dropped heartbeat observed on the blood pressure recording due to the cannula entering the right atrium.

- Strong and short latency pulmonary chemoreflex following the injection of 4 – 6 µg PBG (Figure 17a).

Correct placement of the left ventricular cannula was determined by :-

- Marking the appropriate distance from the right carotid artery to the heart on the cannula.

- Characteristic tapping of the cannula on entering the ventricle.

- Strong and short latency cardiac chemoreflex following the injection of 10µg PBG from the cannula (Figure 17b).

Correct placement of the both cannula was verified post-mortem.
4.4 Surgery

The trachea was cannulated below the larynx and the animal was ventilated artificially with 40% oxygen / nitrogen mixture using a positive pressure ventilator (Harvard rodent ventilator, model 683) with 1 cm H$_2$O positive end-expiratory pressure (tidal volume 1.5 - 2.0 ml, frequency 50-60 Hz).

The animal was placed in a stereotaxic frame and a mid-line incision was made from the front of the skull to the level of the 8$^{th}$ rib allowing the skin to be removed to expose the underlying musculature. A pneumothorax was made between the fifth and sixth right rib and both thoracic vagi crushed below the level of the heart. The right cardiac branch of the vagus was isolated and placed on stimulating electrodes as previously described in section 3.3.

The right phrenic nerve was exposed following lateral reflection of the clavicle and the cervical vagus exposed by blunt dissection of connective tissue above the brachial plexus. The phrenic nerve was isolated, cut peripherally and its central cut end placed on bipolar silver wire recording electrodes. The cervical vagus was then isolated and placed on bipolar silver wire stimulating electrodes. Both nerves were insulated electrically using paraffin and dental impression material. Percutaneous lacquer insulated copper wire electrodes, were placed in the right fore limb and left hind limb to record ECG.

The skull was opened caudal to lambda on the right side to allow a concentric bipolar stimulating electrode (SNEX-100, Clark Electromedical Instruments, Reading, U.K.: tip diameter 100$\mu$m.) to be inserted into the right parabrachial nucleus. The placement of the stimulating electrode within the parabrachial nucleus was varied between experiments using stereotaxic co-ordinates from 9.10 to 9.30mm caudal to bregma and 2.00 to 2.20mm lateral to bregma. Stimulating electrodes were lowered to a depth between 5.50 and 6.00mm below the surface of the skull. The correct placement of the stimulating electrode within the parabrachial nucleus was determined by the presence of a rise in blood pressure and tachycardia following a brief 5 - 10 s stimulation at 200$\mu$A, 0.1ms 50 Hz (Figure 18).

The muscles overlying the occipital bone and atlanto-occipital membrane were removed and the dura and pia mater carefully removed to expose the dorsal surface of the
brainstem around obex. In some animals the cerebellum obscured obex. In these animals the cerebellum overlying obex was carefully removed using suction.

Animals were then paralysed using gallamine triethiodide (12mg/kg i.v.) (Concord Pharmaceuticals Ltd, Dunmow, Essex, U.K.). Following neuromuscular blockade the level of anaesthesia was monitored by the stability of cardiovascular and respiratory variables and the absence of any change in them following noxious toe pinch.

4.5 Extracellular single unit recording

Extracellular recordings were made using single borosilicate glass capillary electrodes, tip diameter 1 – 3 μm (GC150F-10, Clark Electromedical Instruments) connected to a AxoClamp 2B via HS-2A head-stage (gain x 10). Electrodes were filled with 1.5% biocytin (Sigma) in 0.5M sodium chloride.

The recorded potential was further amplified using a home made AC/DC amplifier (gain x 200) and filtered (frequency band 600 – 5000Hz) before being passed into digital recorder from which data was sent to a VCR for storage. The analogue output from the digital recorder was connected via a patch panel to the analogue to digital converter of the 1401 plus interface. The signal from the patch panel was distributed to a storage scope (Model 204 – A, Nicolet Instrument Corporation, Warwick, U.K.) and spike processor (Digitimer D. 130) where spikes were counted using the window discriminator and visualised on a further oscilloscope (V-555, Hitachi, Maidenhead, U.K.). The output from the spike processor was sent to the digital input on the 1401 plus interface. Both the extracellular potential and spike processor output were displayed continuously on a computer monitor in a chart recorder style and simultaneously recorded on a computer hard disk using the Spike 2 on line data capture programme. Extracellular potentials were sampled at a rate of 10kHz.

4.6 Recording protocol

Recordings were taken preferentially from NTS neurones activated synaptically on stimulation of the cervical vagus (1Hz, 1ms, 1 - 15V).
Following examination of the effect of cervical vagal stimulation the effect of electrical stimulation of the PBN (1Hz, 1ms, 50 – 1000 μA) was assessed. Antidromic activation of recorded neurones following electrical stimulation of the PBN was confirmed using 3 criteria (Lipski, 1981) (Figure 19)

- Constant latency of the evoked response.

- The ability of the evoked response to follow high frequency paired pulse stimulation (i.e. 5 ms separation: 200Hz).

- Collision of the evoked response with a spontaneous action potential within the latency of the evoked response.

Finally the response of recorded neurones to cardiac and pulmonary afferent fibre input was examined following a bolus injection of PBG (2 - 20 μg; maximum volume 100μl) into the right atrium or left ventricle. A short latency excitation (1s or less from completion of injection) was judged to be a response to the activation of either cardiac or pulmonary afferent fibres endings. Repeated PBG injections were given at a minimum interval of 5 min to prevent receptor desensitisation (Whalen et al., 2000).

Control responses to equivalent, or greater, volumes of saline were assessed in neurones that responded to PBG injections. The further possibility that neurones activated by injections of PBG might be responding secondarily to the hypotension elicited by the activation of cardiac and pulmonary afferent endings was examined by replicating the hypotension using intra-venous injections of sodium nitroprusside (SNP; 250 – 500ng).

Recording sites were marked with biocytin using the juxtacellular labelling technique (Pinault, 1996). This involved entrainment of the neurone to positive current pulses (2 – 8 nA, 200 ms, 2.5 Hz) applied using an AxoClamp 2B (Axon Instruments, Inc, C.A., U.S.A.) driven by a Digitimer D4030 pulse generator (see Figure 20 for an illustration of the entrainment).

At the end of each experiments the PBN stimulating site was marked by electrolytic lesion 50μA and animals were perfused through the left ventricle with 300ml 0.9 % saline followed by 500ml 4 % paraformaldehyde in 0.1M phosphate buffer (PB) (pH 7.4). Brains were removed and sectioned at the level of the cervical spinal cord, the
facial nucleus and the superior colliculi. The caudal block containing the NTS was stored overnight in 2% paraformaldehyde and 15% sucrose in 0.1M phosphate buffer before being stored in 30% sucrose containing 0.05% sodium azide in 0.1M PB prior to processing. The rostral block containing the PBN was stored in a saturated solution of potassium ferro-cyanide and potassium ferric-cyanide in 2% paraformaldehyde and 15% sucrose in 0.1M phosphate buffer.

4.7 Location of recording sites

Neurones labelled with biocytin were visualised using standard avidin-biotin peroxidase techniques. Serial coronal sections were cut (50 μm) on a freezing microtome and incubated on a rotatory shaker in 50% ethanol for 30 min, followed by 50% ethanol and 3% hydrogen peroxide for 30 min before being washed in 0.1M PB (3 x 10 min). Washed sections were then incubated for 2.5 h at room temperature in a 1:1000 dilution of ExtrAvidin peroxidase in 1% phosphate buffered goat serum, containing 0.1% bovine serum albumin and 0.2% Triton X-100. Sections were then washed in 0.1M PB (3 times 10 min) and incubated in 0.05% diaminobenzidine (DAB) solution containing 0.004% ammonium chloride, 0.001% nickel ammonium (II) sulphate and 0.2% D-glucose in 0.1M PB for 20 min. Sections were further incubated for a further 20 min in fresh DAB solution containing a 1:1000 dilution of glucose oxidase to initiate the peroxidase reaction. Labelling could then be visualised as a black/brown precipitate formed by the nickel intensified chromogenic DAB reaction product. The DAB reaction was terminated by repeated washes with 0.1M PB. Sections were mounted on gelatin subbed slides, allowed to air dry, dehydrated in ethanol (70%, 90%, 2 x 100%), cleared using histoclear and coverslipped in DPX. Filled neurones could then be reconstructed using a camera lucida.

4.8 Location of stimulating sites

Stimulating sites in the PBN were marked as described above and visualised following the sectioning of the brain on a freezing microtome (50 μm) by the presence of Prussian blue stain at the lesion site. Sections were then counter stained with neutral red and dehydrated and mounted as above.
4.9 Measurement of cardiovascular and respiratory variables

Cardiovascular and respiratory variables were recorded as previously described in section 2.4.

4.10 Analysis of recorded neurones.

The response of recorded neurones to right atrial and left ventricle injection of PBG and electrical stimulation of the cardiac branch of the vagus, cervical vagus and parabrachial were quantified off-line using Spike2 software (CED).

The latency of antidromic and synaptically evoked responses were determined from the peaks in post-stimulus triggered histograms (PSTH) (50 sweeps, 1ms bin width) constructed for evoked spikes following electrical stimulation of the cardiac branch of the vagus, cervical vagus and parabrachial nucleus. The presence and duration of a suppression in ongoing activity was also determined from the PSTH in neurones with sufficient ongoing activity to allow a cessation of activity to be determined.

Excitatory and inhibitory responses elicited by right atrial or left ventricular injection of PBG were quantified as the number of spikes within the first second following injection of PBG. Statistical comparisons were made with the mean pre-injection firing frequency calculated from the 10 s period prior to PBG injection using Student’s paired t-test (significance set at p < 0.05). The onset latency of excitatory responses was measured from the completion of PBG injection to the first spike of the bin demonstrating a two fold increase in firing frequency compared to the 10 s period prior to PBG injection from rate histogram plots of firing frequency constructed with 0.25 s bins. The onset latency of inhibitory responses was measured from the completion of PBG injection to the last spike before the cessation in ongoing activity. The duration of excitatory responses was measured from the time of onset (as described above) to first bin of the firing frequency histogram (0.25 s bin width), which had returned to the pre injection firing frequency. In some cases baseline firing frequency remained slightly elevated following PBG injection and so the duration of excitatory responses were determined to a return of activity to this new elevated firing frequency. The duration of inhibitory responses was measured as the duration of the cessation of ongoing activity.
The firing frequency of neurones with ongoing activity was calculated from a 50 s period at the start of recording using rate histogram plots constructed with the use of 1 s bins. Statistical comparisons were made between the ongoing firing frequency of investigated neurones using a one way ANOVA with Fisher’s LSD (significance set at p < 0.05).

Additional histograms were made of ongoing activity over this 50s period, triggered by the R wave of the ECG (10 ms histogram bin width) and the peak of the integrated phrenic nerve activity (50 ms integration time constant, 50 and 100 ms histogram bin width) to assess cardiac or respiratory modulation of ongoing activity.

Stimulation sites with the PBN were categorised into four groups based on the placement of the electrode within the pontine or mesencephalic PBN and centred approximately within the central lateral subnucleus (cIPBN) or the external lateral subnucleus (eIPBN). The threshold stimulation intensity required to elicit antidromic or synaptically evoked responses in NTS neurones from each of these four sites was compared statistically using a one way ANOVA with Fisher’s LSD (significance set at p < 0.05).

4.11 Experimental protocol

The results of this chapter were obtained from two experimental protocols. In initial studies referred to as experiment A, the response of investigated neurones to electrical stimulation of the cervical vagus and the parabrachial nucleus were examined in addition to stimulating combined cardiac and pulmonary afferent endings by right atrial injection of PBG. In these experiments the method described above was followed except the cardiac branch of the vagus was not isolated or stimulated, the thoracic vagi were not crushed and no cannula was placed in the left ventricle. In latter studies referred to as experiment B the response of investigated neurones to electrical stimulation of the cardiac branch of the vagus and left ventricular injection of PBG were also examined in addition to electrical stimulation of the cervical vagus stimulation and parabrachial nucleus, and right atrial injection of PBG. Subsequently in experiment B the complete method described above was followed.
Figure 17  Raw data illustrating the cardiovascular and respiratory effects following (a) right atrial and (b) left ventricle injections of PBG.

A Illustration of the pulmonary chemoreflex (bradycardia, hypotension and apnoea) elicited by right atrial injection of PBG (6 µg). Injection duration indicated by bar above the raw trace.

B Illustration of the coronary chemoreflex (bradycardia and hypotension), and increase in breath-breath interval elicited by left ventricle injection of PBG (10 µg). Injection duration indicated by bar above the raw trace.

Note the reduced magnitude of the coronary chemoreflex compared to the pulmonary chemoreflex.
(a) Integrated phrenic nerve activity

Heart rate (b.p.m.)

Blood Pressure (mmHg)

(b) Integrated phrenic nerve activity

Heart rate (b.p.m.)

Blood Pressure (mmHg)
Figure 18  Raw data illustrating the cardiovascular response to electrical stimulation of the Parabrachial nucleus

Correct placement of the stimulating electrodes within the parabrachial nucleus was confirmed by the presence of a tachycardia and increase in blood pressure elicited following a brief period of stimulation at 200μA, 0.1ms 50Hz. An example of this response to correct placement of the stimulating electrode within the parabrachial nucleus is shown on the opposing page. The stimulation period is indicated by the bar above the raw data.
Heart rate (b.p.m.)

Blood Pressure (mmHg)

200 μA, 0.1 ms, 50 Hz

5 s
Figure 19  Raw data illustration of the criteria used to identify antidromic activation of recorded neurones.

A  Constant latency of evoked response (5 superimposed sweeps)
B  Ability to follow high frequency paired pulse stimuli
C  Collision of evoked response with spontaneous action potential. Note the cancellation of an evoked spike when the separation of the spontaneous action potential and the stimulus artefact is less than the latency of the evoked response compared with greater than the latency of the evoked response.

Stimulus artefact indicted by closed circle, spontaneous action potential indicated by open circle.
Figure 20  Raw data illustrating the entrainment of a neurone during positive current pulse ejection.

A  Break through of entrainment indicated by arrow.
B  Entrainment of neurone

Before entrainment spontaneous activity is observed both during and between positive current pulses, however following the break through of entrainment (indicated by arrow) action potentials are only observed during the period of the positive current pulses.
Results

4.12 Cardiovascular and respiratory variables

The heart rate and mean arterial blood pressure at the onset of electrophysiological recordings were $327 \pm 5.8$ b.p.m. and $105 \pm 3.2$ mmHg in experiment A and $310 \pm 6.5$ b.p.m. and $91 \pm 3.8$ mmHg in experiment B. Blood gas values obtained at the onset of electrophysiological recordings were pH $7.35 \pm 0.01$, $pCO_2 33 \pm 1.2$ mmHg, $pO_2 153 \pm 6.4$ mmHg in experiment A and pH $7.35 \pm 0.01$, $pCO_2 39 \pm 1.3$ mmHg, $pO_2 140 \pm 5.2$ mmHg in experiment B.

4.13 Overall classification of investigated neurones

The response to cervical vagus stimulation was examined in single unit extracellular recordings taken from a total of 220 neurones located in the dorsal vagal complex during two experimental protocols, experiment A and experiment B. Twenty one of these neurones were antidromically activated following stimulation of the cervical vagus and were classified as dorsal vagal motor (DVN) neurones of which 11 were studied in experiment A and 10 were identified in experiment B (Figure 21). The remaining 199 neurones, were synaptically activated following cervical vagal stimulation and were classified as NTS neurones, of which 123 were observed in experiment A, and 76 in experiment B (Figure 21).

4.14 NTS neurones

4.14.1 Response to cervical vagus stimulation

Stimulation of the cervical vagus elicited 377 evoked responses in 199 NTS neurones, at both short and long latencies seen as two distinct peaks in the frequency distribution histogram (Figure 22). Long latency responses (20ms or greater) had a mean latency of $37.1 \pm 0.1$ ms (range 20 to 88 ms: n = 291). For an estimated distance of 20mm between the cervical vagus stimulating electrode and the recording site within the NTS, this gave a mean conduction velocity of $0.57 \pm 0.01$ m/s that was consistent with the activation of unmyelinated afferent vagal fibres. Short latency responses (less than 19 ms) had a mean latency of $8.5 \pm 0.5$ ms (range 1 to 19ms: n = 86) giving a mean conduction
velocity of $3.2 \pm 0.3$ m/s, which was consistent with the activation of myelinated vagal afferent fibres. Long latency responses were observed in 178 NTS neurones of which 38 also had a short latency response (Figure 22). In the remaining 21 neurones only short latency evoked responses were observed following cervical vagus stimulation (Figure 22).

4.14.2 Response to activation of the Parabrachial nucleus

Stimulation of the PBN produced excitatory evoked response in 147 / 199 (74%) of NTS neurones studied of which 83 (42%) were antidromically activated and 64 (32%) were synaptically activated following PBN stimulation (Figure 23). The mean latency of the antidromic response was $23.5 \pm 0.6$ ms and the synaptic response was $26.8 \pm 1.1$ ms (Figure 24). For an estimated distance of 10 mm between the stimulating site in the PBN and the recording site in the NTS, the conduction velocity of the antidromic evoked responses was calculated to be $0.45 \pm 0.01$ m/s and synaptic evoked responses calculated to be $0.45 \pm 0.03$ m/s, which were consistent with the activation of unmyelinated fibres. The intensity of the stimulation threshold required to elicit an evoked response was similar for antidromic evoked responses ($401 \pm 28$ μA) and synaptically evoked responses ($400 \pm 34$ μA).

The majority of NTS neurones excited by PBN stimulation responded to cervical vagal stimulation with at least one long latency evoked responses (antidromic 75 / 83 and synaptic 61 / 64) although short latency evoked responses were also observed (antidromic 29/ 83 and synaptic 10 / 64).

Ongoing activity was observed in 49 / 64 (75%) of NTS neurones synaptically activated following PBN stimulation compared with 37 / 83 (45%) of NTS neurones antidromically activated and 27 / 52 NTS (52%) neurones not excited following PBN stimulation. Furthermore the ongoing activity was significantly less in NTS neurones antidromically activated by PBN stimulation ($1.55 \pm 0.24$ Hz) compared with neurones that were synaptically activated ($3.33 \pm 0.5$ Hz: $p < 0.01$) or not excited ($3.33 \pm 0.67$ Hz: $p < 0.01$) following PBN stimulation (Figure 24).
Figure 21  Raw data illustrating the classification of NTS and DVN neurones.

A  NTS neurone: cervical vagus stimulation (closed circle), 5 superimposed sweeps.

B  NTS neurone: collision test, spontaneous action potential indicated by open circle, cervical vagus stimulation (closed circle). Note no cancellation of the evoked spike.

C  DVN neurone: cervical vagus stimulation (closed circle), 5 superimposed sweeps.

D  DVN neurone: collision test, spontaneous action potential (open circle), cervical vagus stimulation (closed circle). Note, cancellation of the evoked spike.
Figure 22  Frequency distribution histogram and raw data illustrating the latency of the evoked responses to cervical vagus stimulation in NTS neurones.

A  Frequency distribution histogram of the latency of 377 evoked responses in 199 NTS neurones following cervical vagus stimulation.

B  Raw data illustrating a long latency response to cervical vagus stimulation consistent with the activation of unmyelinated afferent fibres (closed circle: stimulus artefact).

C  Raw data illustrating a short latency response to cervical vagus stimulation consistent with the activation of myelinated afferent fibres (closed circle: stimulus artefact).

D  Raw data illustrating a long and short latency response to cervical vagus stimulation (closed circle: stimulus artefact)
(a) Number of evoked response

(b) myelinated

(c) unmyelinated

(d)
Figure 23  Raw data illustrating antidromic and synaptic activation of NTS neurones following PBN stimulation.

A  Antidromic; PBN Stimulation (closed circle), 5 superimposed sweeps.

B  Antidromic; collision test, spontaneous action potential indicated by open circle, PBN stimulation (closed circle). Note, cancellation of the evoked spike.

C  Synaptic; PBN Stimulation (closed circle), 5 superimposed sweeps.

D  Synaptic; collision test, spontaneous action potential indicated by open circle, PBN stimulation (closed circle). Note no cancellation of the evoked spikes.
(a) PBN antidromic

(b) PBN synaptic

(d)
Figure 24  Frequency distribution histogram of the latency of evoked responses in NTS neurones following PBN stimulation (a & b). Histogram comparing the ongoing activity in NTS neurones classified by their response to PBN stimulation (c).

A  Frequency distribution histogram of the latency of evoked responses in 83 NTS neurones following antidromic PBN stimulation.

B  Frequency distribution histogram of the latency of 88 evoked responses in 64 NTS neurones following synaptic PBN stimulation.

C  Histogram of ongoing activity in NTS neurones that were antidromically (n = 37), synaptically (n = 49) and not excited (n = 27) following PBN stimulation

** denotes p< 0.01 following comparison of ongoing activity between groups using one way ANOVA with Fisher's LSD (protected t).
(a) PBN antidromic

Number of evoked response

(b) PBN synaptic

Number of evoked response

(c) Ongoing activity (Hz)

Antidromic (n = 37) Synaptic (n = 49) Not excited (n = 27)
4.15 Experiment (A) combined cardiac and pulmonary afferent fibre stimulation

4.15.1 Response to right atrial injection of PBG

Right atrial injection of PBG produced a significant increase in ongoing firing frequency from $0.7 \pm 0.3$ Hz to $25.2 \pm 6.8$ Hz ($P<0.005$), for a duration of $13.1 \pm 5.3$ s in $22/102$ NTS neurones tested with an onset latency of $1$ s or less ($0.4 \pm 0.1$ s). A cessation of ongoing activity (duration $5.1 \pm 0.8$ s), with onset latency of $1$ s or less ($0.4 \pm 0.1$ s) was observed in a further $10/102$ NTS neurones tested, following right atrial injection of PBG.

NTS neurones excited by right atrial PBG injection were classified as $eCP$-NTS neurones and neurones inhibited by PBG injection were classified as $iCP$-NTS neurones (Figure 25).

4.15.2 Control responses

Injection into the right atrium of a similar or greater volume of saline (20-100 µl) compared with volume of PBG injected, produced no change in ongoing activity in $7/7$ $eCP$-NTS neurones and $1/1$ $iCP$-NTS neurones tested (Figure 26). Furthermore eliciting a comparable systemic hypotension to that observed following right atrial injection of PBG using sodium nitroprusside also produced no change in ongoing activity in $2/2$ $eCP$-NTS neurones and $1/1$ $iCP$-NTS neurones (Figure 26).

4.15.3 Response of CP-NTS neurones to cervical vagus stimulation

Stimulation of the cervical vagus elicited long latency ($35.5 \pm 1.2$ ms) evoked responses consistent with the activation of unmyelinated afferent fibres in $18/22$ (82%) of $eCP$-NTS neurones recorded. Short latency ($7.0 \pm 0.7$ ms) evoked responses consistent with the activation of myelinated afferent fibre were also observed in $9/18$ $eCP$-NTS with long latency synaptic vagal input and a further $4$ $eCP$-NTS in which only short latency evoked responses were observed.

Stimulation of the cervical vagus only produced long latency ($34.4 \pm 2.3$ ms) evoked responses in $iCP$-NTS neurones.
4.15.4 Response of CP-NTS neurones to Parabrachial nucleus stimulation

A total of 12/22 (54%) eCP-NTS neurones were antidromically activated following PBN stimulation. A further 3 (14%) eCP-NTS neurones were synaptically activated and 7 (32%) were not excited by PBN stimulation.

By contrast 3/10 iCP-NTS neurones recorded were antidromically activated and 5/10 iCP-NTS neurones were synaptically activated following PBN stimulation.

Stimulation of the cervical vagus produced both long and short latency evoked responses in eCP-NTS neurones antidromically activated and synaptically activated following PBN stimulation.

4.15.5 Ongoing activity: correlation with phrenic nerve activity and ECG

Ongoing activity was observed in 10/22 (45%) eCP-NTS and all iCP-NTS neurones with a mean firing rate of 1.64 ± 0.63 Hz and 2.16 ± 0.23 Hz respectively. This ongoing activity was found to be closely correlated with phrenic nerve activity (PNA) in 1/6 (17%) eCP-NTS neurones tested and ECG in 3/10 (30%) eCP-NTS (Figure 27). In 2/10 (20%) iCP-NTS neurones tested ongoing activity was found to be weakly correlated with ECG (Figure 27c). No correlation in ongoing activity with PNA was observed in iCP-NTS neurones.

Ongoing activity (3.14 ± 0.44 Hz) was also observed in 42 of the remaining NTS neurones investigated in experiment A, of which 1/10 NTS (10%) neurones had discharge correlated with PNA and 13/42 (31%) had activity correlated with ECG.
Figure 25  Raw data illustrating excitatory and inhibitory responses following right atrial injection of PBG.

A  example of the excitation seen following PBG injection in a eCP-NTS neurone.

B  example of the cessation of ongoing activity following PBG injection in a iCP-NTS neurone.

Injection of PBG denoted by the bar above raw data.
Figure 26  Raw data illustrating the response to right atrial control volume injection of saline and sodium nitroprusside induced hypotension compared with the response to right atrial injection of PBG.

A  Example of the response to right atrial injection of saline (50 μl) compared with the response to right atrial injection of PBG (8 μg; volume 40 μl) in a eCP-NTS neurone.

B  Example of the response to sodium nitroprusside (SNP) induced hypotension compared with the response to right atrial injection of PBG (8 μg) in a eCP-NTS neurone.

Injection of drugs or saline denoted by the bar above raw data. Note the absence of a neuronal response to either saline or SNP compared to that elicited by PBG.
(a)

Spike bin^{-1}

BP (mmHg)

Saline 50 \mu l

PBG 8 \mu g

(b)

SNP 500 ng

PBG 8 \mu g

BP (mmHg)
Figure 27 Correlograms illustrating the correlation of ongoing activity with integrated phrenic nerve activity and ECG in a eCP-NTS neurone (a & b), and ECG in a iCP-NTS neurone (c).

A Correlation of ongoing activity with integrated phrenic nerve activity in a eCP-NTS neurone.
B Correlation of ongoing activity with ECG in a eCP-NTS neurone.
C Correlation of ongoing activity with ECG in a iCP-NTS neurone.

Histograms of ongoing activity triggered by peak integrated phrenic nerve activity (50 ms bin width) and R-wave of the ECG (10ms bin width) respectively over a 50s period.
(a) eCP-NTS

Integrated phrenic nerve activity

Spikes \( \text{bin}^{-1} \)

(b) ECG

Spikes \( \text{bin}^{-1} \)

(c) iCP-NTS

ECG

Spikes \( \text{bin}^{-1} \)
4.16 Experiment (B) cardiac vagal afferent fibre stimulation

4.16.1 Response to electrical stimulation of the cardiac branch of the vagus

Excitatory synaptically evoked responses were recorded in 25/68 (37%) NTS neurones following stimulation of the cardiac branch of the vagus (Figure 28). These neurones were classified as cardiac-NTS neurones.

The latency of 38 evoked responses recorded from 24/25 cardiac-NTS neurones were consistent with C-fibre activation (latency $62.3 \pm 1.3$ ms, range 37 to 80 ms: conduction velocity calculated for an estimated distance of 45 mm of $0.74 \pm 0.02$ m/s). In a remaining cardiac-NTS neurone the evoked response had a latency of 18 ms (calculated conduction velocity of 2.5 m/s) consistent with the activation of myelinated cardiac vagal fibres (Figures 28).

The variability of the latency of the evoked response to electrical stimulation of the cardiac branch of the vagus was indicative of a relatively direct input (less than 3 ms) in 11/25 (44%) cardiac-NTS neurones (see Figure 28a for example). The majority of these neurones 8/11 (73%) were also excited following left ventricular injection of PBG (see below). The remaining 14 cardiac-NTS neurones had latency variability greater than 3 ms indicating that they were activated by mainly polysynaptic cardiac vagal input.

4.16.2 Response of cardiac-NTS neurones to left ventricle injection of PBG

Left ventricular injection of PBG produced an increase in ongoing activity (from $1.0 \pm 0.5$ Hz to $15.3 \pm 3.2$ Hz ($p < 0.001$) for a duration of $26.3 \pm 9.9$ s) in 15/22 cardiac-NTS neurones tested with an onset latency of 1s or less (mean latency $0.1 \pm 0.1$ s) (Figure 29). These neurones were subsequently classified as chemosensitive cardiac-NTS neurones.

In 7 cardiac-NTS no change in ongoing activity was observed in response to left ventricular injection of PBG and these neurones were therefore classified as non-chemosensitive cardiac-NTS neurones (Figure 29). This included the cardiac-NTS neurone activated by myelinated cardiac vagal afferent fibres.

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The remaining 3 cardiac-NTS were not tested for left ventricular injection of PBG.

4.16.3 Response of cardiac-NTS neurones to right atrial injection of PBG

The response to right atrial injection of PBG was examined in 9 chemosensitive cardiac-NTS neurones. An increase in ongoing activity (from $0.2 \pm 0.2$ Hz to $16.0 \pm 3.0$ Hz ($p < 0.005$) with a duration $27.8 \pm 12.3$ s) was observed in 7 / 9 chemosensitive cardiac-NTS neurones tested with an onset latency of 1s or less (mean latency $0.3 \pm 0.1$ s) following right atrial injection of PBG. In the remaining 2 chemosensitive cardiac-NTS neurones an increase in ongoing activity from $4.2 \pm 1.7$ Hz to $12.5 \pm 1.5$ Hz of duration $3.0 \pm 0.9$s was observed at a latency of $1.7 \pm 0.1$s following right atrial injection of PBG.

By contrast only 2 / 6 non-chemosensitive cardiac-NTS neurones demonstrated an increase in ongoing activity from $0.4 \pm 0.3$ Hz to $37.0 \pm 9.0$ Hz of a duration of 1.3 s and 73 s, with a onset latency of 1s or less (mean latency $0.1 \pm 0.1$s) following right atrial injection of PBG.

4.16.4 Control responses

Injection into the left ventricle of a similar or greater volume of saline to that injected of PBG produced no change in ongoing activity in 6 / 6 chemosensitive cardiac-NTS neurones tested (Figure 30a). Similar injections of saline into the right atrium also produced no change in ongoing activity in 7 / 7 cardiac-NTS that were excited by right atrial injection of PBG (Figure 30b). Furthermore eliciting a comparable fall in blood pressure to that observed following right atrial or left ventricular injections of PBG using sodium nitroprusside also produced no change in ongoing activity in 3 / 3 cardiac-NTS neurones tested (Figure 30c).

4.16.5 Response of cardiac-NTS neurones to Parabrachial nucleus stimulation

Stimulation of the PBN produced excitatory evoked responses in 24 / 25 (96%) cardiac-NTS neurones recorded. Of which 18 (72%) were antidromically activated and 6 (24%) were synaptically activated following PBN stimulation.

Of the cardiac-NTS neurones further classified as chemosensitive cardiac-NTS neurones 13 / 15 (87%) were antidromically activated, and 2 / 15 (13%) were
synaptically activated, following PBN stimulation. Similarly 4 / 7 (57%) of the non-
chemosensitive cardiac-NTS neurones were antidromically activated and 2 / 7 (29%)
synaptically activated following PBN stimulation.

4.16.6 Ongoing activity: correlation with phrenic nerve activity and ECG

Ongoing activity was observed in 8 / 15 (53%) chemosensitive cardiac-NTS neurones,
6 / 7 (86%) non-chemosensitive cardiac-NTS neurones and 1 cardiac-NTS neurone that
was not further classified. The mean firing rate was 3.29 ± 1.30 Hz in neurones
classified as non-chemosensitive cardiac-NTS neurones and 1.69 ± 0.66 Hz in neurones
classified as chemosensitive cardiac-NTS neurones.

Ongoing activity was correlated with PNA in 2 / 8 (25%) of chemosensitive cardiac-
NTS neurones examined and 1 / 6 (17%) of non-chemosensitive cardiac-NTS neurones
examined (Figures 31 & 32). By contrast ongoing activity was correlated with ECG in 5
/ 7 (71%) of non-chemosensitive cardiac-NTS neurones examined (including the
cardiac-NTS neurone activated by myelinated cardiac vagal afferent fibres) and 2 / 8
(25%) of chemosensitive cardiac-NTS neurones examined (Figures 31 & 32). This
included one non-chemosensitive cardiac-NTS neurones that had ongoing activity that
was correlated with both ECG and PNA. In three of the non-chemosensitive-NTS
neurones ongoing activity was only weakly correlated with ECG (Figure 32c).

In the two chemosensitive cardiac-NTS neurones with ongoing activity correlated with
PNA the peak in the PNA triggered PSTH occurred during the inspiratory phase of PNA
(Figure 31a) whilst in the non-chemosensitive cardiac-NTS neurones the peak in the
PSTH occurred during late expiration (Figure 32a). The peak in the R wave triggered
PSTH were compared with the ECG adjusted for an estimated conduction time from the
heart to the NTS (derived from the estimated conduction velocity of each neurone and
an estimated distance of 55 mm). Peaks were observed during or just after the P wave
of the ECG consistent with maximal ventricular filling and the "a" wave of the venous
pressure pulse in 1 chemosensitive cardiac-NTS neurone and 2 non-chemosensitive
cardiac-NTS neurones (Figures 31b & 32c). Whilst peaks in the R wave triggered
PSTH were observed during or close to the T wave of the ECG consistent with maximal
aortic pressure in 1 chemosensitive cardiac-NTS neurone and 3 non-chemosensitive
cardiac-NTS neurones (Figure 32b).

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4.17 Experiment (B) CP-NTS neurones

The response to right atrial injection of PBG was examined in a further 36 neurones which were not synaptically activated following stimulation of the cardiac branch of the vagus. Ten neurones exhibited an increase in ongoing activity from 1.0 ± 0.4 Hz to 14.3 ± 2.4 Hz (p < 0.001), of duration 14.3 ± 5.7 s with an onset latency of 1s or less (0.3 ± 0.1 s). A further 3 NTS neurones demonstrated a cessation in ongoing activity (duration 10.1 ± 8.1 s) with an onset latency of 1s or less (0.33 ± 0.29 s) following right atrial injection of PBG. Neurones excited by PBG were classified as eCP-NTS neurones and those inhibited by PBG injection were classified as iCP-NTS.

4.17.1 Response of CP-NTS neurones to cervical vagus stimulation

Stimulation of the cervical vagus elicited long latency (38.5 ± 2.1 ms) evoked responses consistent with the activation of unmyelinated afferent fibres in 9 / 10 (90%) of eCP-NTS neurones recorded. Short latency (6.7 ± 1.5 ms) evoked responses consistent with the activation of myelinated afferent fibre were also observed in 2 / 9 eCP-NTS with long latency synaptic vagal input and a further 1 eCP-NTS in which only short latency evoked responses were observed.

Stimulation of the cervical vagus only produced long latency (35.0 ± 3.5 ms) evoked responses in 2 / 3 iCP-NTS neurones and only a short latency (4 ms) evoked response in the remaining 1 iCP-NTS neurone.

4.17.2 Response of CP-NTS neurones to PBN stimulation

Stimulation of the PBN antidromically activated 6 / 10 (60%) eCP-NTS neurones recorded and synaptically activated a further 1/10 (10%) of eCP-NTS neurones. However only 1 / 3 (33%) iCP-NTS neurones recorded were antidromically activated following PBN stimulation. The remaining 2 / 3 iCP-NTS neurones were synaptically activated following PBN stimulation.

Cervical vagal stimulation produced both long and short latency evoked responses in CP-NTS neurones antidromically activated by PBN stimulation. Only long latency evoked responses were observed in CP-NTS neurones synaptically activated by PBN stimulation, following stimulation of the cervical vagus.

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4.17.3 Ongoing activity: correlation with phrenic nerve activity and ECG

Ongoing activity was observed in 7 / 10 (70%) of eCP-NTS neurones (2.22 ± 0.58 Hz) and all 3 iCP-NTS neurones recorded (3.00 ± 0.54 Hz). This was shown to be correlated with PNA in a total of 3 / 7 (42%) and ECG in 2 / 7 (29%) of eCP-NTS neurones examined, including 1 eCP-NTS neurone that had ongoing activity correlated with both ECG and PNA (Figure 33). No correlation of ongoing activity with ECG was observed in the three iCP-NTS neurones examined, however one iCP-NTS neurones showed a correlation in ongoing activity with PNA. Interestingly the iCP-NTS neurones with ongoing activity correlated with PNA also demonstrated a correlation with an event in-between the phrenic nerve discharges (Figure 33c). A similar observation was also observed in two of the eCP-NTS neurones. The time interval between all peaks in the PNA triggered PSTH was similar to ventilation pump cycle suggesting that ongoing activity was also correlated with the pump cycle.

Ongoing activity (3.46 ± 0.70 Hz) was observed in 23 of the remaining NTS neurones recorded and was found to be correlated with PNA in 4 / 23 (17%) and ECG in 7 / 23 (30%) of neurones examined. This included three NTS neurones that demonstrated a correlation between ongoing activity and both ECG and PNA.
Figure 28 Raw data illustrating the synaptic response to stimulation of the cardiac branch of the vagus in NTS neurones (a & b). Raw data and Frequency distribution histogram illustrating the latency of the evoked responses to stimulation of the cardiac branch of the vagus in NTS neurones (c, d & e).

A Stimulation of the cardiac branch of the vagus (closed circle), 5 superimposed sweeps.

B Collision test, spontaneous action potential indicated by open circle, stimulation of the cardiac branch of the vagus (closed circle). Note no cancellation of evoked spike.

C Raw data illustrating a long latency response to stimulation of the cardiac branch of the vagus consistent with the activation of unmyelinated afferent fibres (closed circle: stimulus artefact).

D Raw data illustrating a short latency response to stimulation of the cardiac branch of the vagus consistent with the activation of myelinated afferent fibres (closed circle: stimulus artefact).

E Frequency distribution histogram of the latency of 39 evoked responses in 25 cardiac-NTS neurones following stimulation of the cardiac branch of the vagus.
(a)  

(b)  

(c)  

(d)  

(e) Cardiac evoked response

Number of evoked response

0 to 9  10 to 19  20 to 29  30 to 39  40 to 49  50 to 59  60 to 69  70 to 79  80 to 89

200 μV 10 ms
Figure 29  Raw data illustrating the response to left ventricular injection of PBG in chemosensitive and non chemosensitive cardiac-NTS neurones.

A  chemosensitive cardiac-NTS neurone.
B  non chemosensitive cardiac-NTS neurone.

Injection of PBG denoted by the bar above raw data. Note the absence of a neuronal response to PBG in the non-chemosensitive cardiac-NTS neurones compared to that elicited by PBG in the chemosensitive cardiac-NTS neurone.
(a) chemosensitive cardiac-NTS

PBG (10 µg)

Ev (mV)

Spike bin⁻¹

Blood pressure (mmHg)

(b) non chemosensitive cardiac-NTS

PBG (20 µg)

Ev (mV)

Spike bin⁻¹

Blood pressure (mmHg)
Figure 30  Raw data illustrating the response to right atrial and left ventricular control volume injections of saline and sodium nitroprusside induced hypotension compared with the response to right atrial and left ventricular injections of PBG in *chemosensitive cardiac-NTS* neurones.

A  Example of the response to left ventricular injection of saline (150 μl) compared with the response to left ventricular injection of PBG (20 μg: volume 100 μl) in a *chemosensitive cardiac-NTS* neurone.

B  Example of the response to a further left ventricular injection of saline (150 μl) and a right atrial injection of saline (50 μl) compared with the response to right atrial injection of PBG (4 μg: volume 20 μl) in a the same *chemosensitive cardiac-NTS* neurone.

C  Example of the response to sodium nitroprusside (SNP) induced hypotension compared with the response to left ventricular injection of PBG (10 μg) in a *chemosensitive cardiac-NTS* neurone.

Injection of drugs or saline denoted by the bar above raw data. Note the absence of a neuronal response to either saline or SNP compared to that elicited by PBG.
(a) Blood pressure (mmHg)

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(c) Blood pressure (mmHg)

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Correlograms illustrating the correlation of ongoing activity with integrated phrenic nerve activity and ECG in chemosensitive cardiac-NTS neurones.

A Correlation of ongoing activity with integrated phrenic nerve activity.

B Correlation of ongoing activity with ECG. Dotted lines represent the position relative to the ECG adjusted for the estimated conduction time from the heart to the NTS.

Histograms of ongoing activity triggered by peak integrated phrenic nerve activity (50 ms bin width) and R-wave of the ECG (10ms bin width) respectively over a 50s period.
chemosensitive cardiac-NTS

(a)

Integrated phrenic nerve activity

Spikes bin^1

Time (s)

(b)

ECG

Spikes bin^1

Time (s)
Figure 32  Correlograms illustrating the correlation of ongoing activity with integrated phrenic nerve activity and ECG in non chemosensitive cardiac-NTS neurones.

A  Correlation of ongoing activity with integrated phrenic nerve activity.

B  Correlation of ongoing activity with ECG.

C  Weak correlation of ongoing activity with ECG.

Histograms of ongoing activity triggered by peak integrated phrenic nerve activity (50 ms bin width) and R-wave of the ECG (10 ms bin width) respectively over a 50s period.
(a) *non chemosensitive cardiac-NTS*

Integrated phrenic nerve activity

Spikes bin'

![Graph showing phrenic nerve activity](image)

(b) ECG

Spikes bin'

![Graph showing ECG activity](image)

(c) ECG

Spikes bin'

![Graph showing ECG activity](image)
Figure 33 Correlograms illustrating the correlation of ongoing activity with (a & b) integrated phrenic nerve activity and ECG in a eCP-NTS neurone, and (c) phrenic nerve activity in a iCP-NTS neurone.

A Correlation of ongoing activity with integrated phrenic nerve activity in a eCP-NTS neurone.

B Correlation of ongoing activity with ECG in a eCP-NTS neurone.

C Correlation of ongoing activity with integrated phrenic nerve activity in a iCP-NTS neurone.

Histograms of ongoing activity triggered by peak integrated phrenic nerve activity (50 ms bin width) and R-wave of the ECG (10ms bin width) respectively over a 50s period.
4.18 Location of recording and stimulating sites

Recordings were preferentially made within the commissural NTS from 0.3mm caudal to obex through to 0.3mm rostral to obex and from 0.05mm to 0.40mm lateral of the midline and at a depth from 100 to 900 µm from the dorsal surface of the brainstem in experiment A and experiment B. No difference was observed between the position relative to obex or the depth of recording sites between the different classes of neurones recorded in both experiment A and experiment B. In experiment A, 7 neurones were successfully juxtacellularly labelled of which 3 were classified as eCP-NTS neurones and 1 was classified as a iCP-NTS neurones (Figures 34 & 35). In experiment B 7 neurones were successfully juxtacellularly labelled of which 4 were classified as cardiac-NTS neurones and 3 were classified as eCP-NTS neurones (Figure 34 & 35).

Stimulating electrode placement was identified in the lateral PBN of the pontine and mesencephalic PBN, in positions extending from the central lateral subnucleus of the PBN (cIPBN) to the external lateral subnucleus of the PBN (eIPBN) (Figure 36). No difference was observed between the position of the stimulating electrode in the PBN and the evoked response (antidromic or synaptic) elicited in NTS neurones following PBN stimulation (Figures 37 & 38). However when the mean stimulation intensity thresholds were compared for stimulating sites classified as being located in the pontine or mesencephalic PBN, and centred approximately within the cIPBN or eIPBN, significant differences were observed in the intensity of stimulation required to elicit antidromically or synthaptically evoked responses. The mean stimulation intensity threshold required to evoke antidromic responses in NTS neurones was significantly greater (P < 0.05) following electrode placement in the mesencephalic PBN centred within the eIPBN (mean intensity 526 ± 61 μA) compared with pontine electrode placement centred on the cIPBN (mean intensity 332 ± 51 μA) and eIPBN (mean intensity 368 ± 36 μA) (Figure 39). Whilst, the mean stimulation intensity threshold required to evoke synaptic responses in NTS neurones was significantly greater (P < 0.05) following electrode placement in the mesencephalic PBN centred within the cIPBN (mean intensity 690 ± 98 μA) compared with pontine electrode placement centred on the cIPBN (mean intensity 350 ± 72 μA) and eIPBN (mean intensity 357 ± 65 μA) (Figure 39).
Figure 34 Digitised photomicrograph illustrating eCP-NTS, chemosensitive cardiac-NTS and NTS neurones visualised using the juxtacellular labelling technique.

A Example of a eCP-NTS neurone.

B Example of a chemosensitive cardiac-NTS neurone

C Example of a NTS neurone.
Figure 35  Schematic illustration of recording sites within the NTS of neurones labelled using the juxtacellular technique plotted on a representative camera lucida drawings of the dorsal vagal complex.

A  Distribution of labelled eCP-NTS neurones
B  Distribution of labelled cardiac-NTS neurones
C  Distribution of labelled NTS neurones.
(a) eCP-NTS

- Experiment A: eCP-NTS neurone antidromically activated by PBN stimulation
- Experiment A: eCP-NTS neurone synaptically activated by PBN stimulation
- Experiment B: eCP-NTS neurone antidromically activated by PBN stimulation
- Experiment B: eECP-NTS neurone synaptically activated by PBN stimulation

(b) cardiac-NTS

- non chemosensitive cardiac-NTS neurone antidromically activated by PBN stimulation
- non chemosensitive cardiac-NTS neurone synaptically activated by PBN stimulation
- chemosensitive cardiac-NTS neurone antidromically activated by PBN stimulation

(c) NTS

- Experiment A: NTS neurone antidromically activated by PBN stimulation
- Experiment A: NTS neurone synaptically activated by PBN stimulation
- Experiment A: NTS neurone not excited by PBN stimulation
- Experiment B: eECP-NTS neurone synaptically activated by PBN stimulation
Figure 36 Digitised photomicrograph illustrating the lesion sites of stimulating electrodes placed within the parabrachial nucleus.

A Example of lesion sites within the mesencephalic external lateral parabrachial nucleus.

B Example of lesion sites within the mesencephalic central lateral parabrachial nucleus.

C Example of lesion sites within the pontine external lateral parabrachial nucleus.

D Example of lesion sites within the pontine central lateral parabrachial nucleus.

E & F Example of lesion sites following electrode placement outside the parabrachial nucleus.

Abbreviation

BC brachium conjunctiva
Figure 37 Schematic illustration of the location of stimulating sites within the parabrachial nucleus in experiment A and experiment B and their evoked responses elicited in recorded neurones.

Stimulating sites are plotted on illustrations of the pontine and mesencephalic PBN adapted from the stereotaxic atlas of Paxinos and Watson (1992).

Key:
- Closed diamond: indicates sites in which stimulation elicited antidromic and synaptically evoked responses in recorded neurones.
- Closed circle: indicates sites in which stimulation only elicited antidromically evoked responses in recorded neurones.
- Closed square: indicates sites in which stimulation only elicited synaptically evoked responses in recorded neurones.
- Closed pentagon: indicates sites outside the PBN in which stimulation did not elicit evoked responses in recorded neurones.

Abbreviations
- BC: brachium conjunctiva
- LC: locus coeruleus
- Me5: mesencephalic trigeminal nucleus
Experiment A

Experiment B

KEY: ♦ Antidromic and synaptic
• Antidromic only
■ Synaptic only
* No response
Figure 38  Schematic illustration of the location of stimulating sites within the parabrachial nucleus which elicited evoked responses in eCP-NTS neurones recorded in experiment A and cardiac-NTS neurones in experiment B.

Stimulating sites are plotted on illustrations of the pontine and mesencephalic PBN adapted from the stereotaxic atlas of Paxinos and Watson (1992).

Key:
- Closed diamond: indicates sites in which stimulation elicited antidromic and synaptically evoked responses in recorded neurones
- Closed circle: indicates sites in which stimulation only elicited antidromically evoked responses in recorded neurones.
- Closed square: indicates sites in which stimulation only elicited synaptically evoked responses in recorded neurones.

Abbreviations
- BC  brachium conjunctiva
- LC  locus coeruleus
- Me5  mesencephalic trigeminal nucleus
Experiment A: eCP-NTS

Experiment B: cardiac-NTS

KEY:
• Antidromic and synaptic
• Antidromic only
■ Synaptic only
Figure 39  Histograms comparing the threshold stimulation intensity required to elicit
(a) antidromic and (b) synaptic evoked responses in NTS neurones following electrode
placement in the central lateral or external lateral subnuclei of the pontine or
mesencephalic PBN.

Each column represents the mean threshold stimulation intensity required to elicit an evoked
response and bars show s.e.m.

* denotes p < 0.05 following comparison of threshold stimulation intensity between groups using
one way ANOVA with Fisher's LSD (protected t).
(a) Antidromic

Intensity (μA)

* * *

external lateral (n = 28)  central lateral (n = 17)  external lateral (n = 19)  central lateral (n = 6)

pontine  mesencephalic

(b) Synaptic

Intensity (μA)

* * *

external lateral (n = 14)  central lateral (n = 10)  external lateral (n = 6)  central lateral (n = 5)

pontine  mesencephalic
4.19 Inhibitory responses

In both experiment A and experiment B, a long duration post excitatory suppression of ongoing activity was observed in 92 / 96 neurones activated synaptically following cervical vagal stimulation with sufficient ongoing activity to determine inhibitory responses (Figures 40 & 41).

Similarly a post excitatory depression of ongoing activity was also observed in all of these neurones antidromically activated following PBN stimulation and in all but 1 of these neurones synaptically activated following PBN stimulation (Figures 40 & 41). The mean latency of the depression was 37 ± 4.2 ms and 51 ± 2.7 ms with a mean duration of 260 ± 24.9 ms and 239 ± 25.8 ms for neurones antidromically activated and synaptically activated by PBN stimulation respectively. A depression of ongoing activity was also observed in 14 NTS neurones (including 1 cardiac-NTS neurone, 2 eCP-NTS neurones and 1 iCP-NTS neurones) not excited by PBN stimulation the mean latency of this response was 48 ± 6.4 ms with a duration of 209 ± 44.8 ms.

In cardiac-NTS neurones a post excitatory depression in ongoing activity was observed following stimulation of the cardiac branch of the vagus in neurones that were antidromically activated by PBN stimulation (latency 93 ± 12.6 ms: duration 132 ± 24.9 ms: Figure 40). No post excitatory depression in ongoing activity was observed in response to stimulation of the cardiac branch of the vagus in cardiac-NTS neurones that were synaptically activated following PBN stimulation. Stimulation of the cervical vagus and PBN did however produce a post excitatory depression in ongoing activity in these neurones (Figure 41). A post excitatory depression of ongoing activity was also observed in the one cardiac-NTS neurone, which was not excited by PBN stimulation although the latency of this response was long (latency 311 ms: duration 208 ms).
Figure 40 Post stimulus triggered histogram illustrating the presence of a post excitatory depression of ongoing activity in a cardiac-NTS neurone activated antidromically following PBN stimulation.

A PSTH (1ms bin width, 50 sweeps) of ongoing activity following cervical vagus stimulation.

B PSTH (1ms bin width, 50 sweeps) of ongoing activity following stimulation of the cardiac branch of the vagus.

C PSTH (1ms bin width, 50 sweeps) of ongoing activity following PBN stimulation.

Stimulus artifact is indicated by the closed circle above the PSTH. Whilst peaks in the PSTH represent the excitatory evoked response to respective stimulation and are followed by a period of post excitatory depression in ongoing activity as indicated by the bracket in figure (a).
(a) Vagus

Post excitatory depression

Spikes $\text{bin}^{-1}$

(b) Cardiac

Spikes $\text{bin}^{-1}$

(c) PBN antidromic

Spikes $\text{bin}^{-1}$
Figure 41 Post stimulus triggered histogram illustrating the absence of a post excitatory depression of ongoing activity following the stimulation of the cardiac branch of the vagus in a cardiac-NTS neurones activated synaptically following PBN stimulation.

A  Cervical vagus stimulation.

B  Stimulation of the cardiac branch of the vagus.

C  PBN stimulation.

Stimulus artifact is indicated by the closed circle above the PSTH. Peaks in the PSTH represent the excitatory evoked response to respective stimulation and are followed by a period of post excitatory depression in ongoing activity as indicated by the bracket in figure (a). Note the absence of a post excitatory depression of ongoing activity following stimulation of the cardiac branch of the vagus in cardiac-NTS neurones synaptically activated by PBN stimulation.
Post excitatory depression

(a) Vagus

Spikes bin$^{-1}$

(b) Cardiac

(c) PBN synaptic

Spikes bin$^{-1}$
4.20 DVN neurones

4.20.1 Response of DVN neurones to cervical vagus and PBN stimulation

Twenty one DVN neurones were antidromically activated by cervical vagal stimulation with a mean latency of 31.1 ± 0.9 ms (calculated conduction velocity of 0.65 ± 0.2 m/s), which was consistent with the activation of unmyelinated vagal fibres (Figure 42). Stimulation of the PBN synaptically activated 14 / 19 DVN neurones tested. The mean latency of these evoked responses was 32.2 ± 1.7 ms with a calculated conduction velocity of 0.26 ± 0.01 m/s, which was consistent with the activation of C-fibre input (Figure 42).

A post excitatory depression of ongoing activity (latency 35.2 ± 1.9 ms: duration 588 ± 53 ms) was also observed following cervical vagus stimulation in all 18 DVN neurones, which had a reasonable level of ongoing activity. Similarly a post excitatory depression of ongoing activity (latency 42.6 ± 2.1 ms: duration 328 ± 54 ms) was also observed in all 14 DVN neurones synaptically activated following PBN stimulation. A depression of ongoing activity (latency 41.5 ± 3.5 ms: duration 311 ± 55 ms) was also observed in 2 DVN neurones that were not synaptically activated following PBN stimulation.

4.20.2 Response of DVN neurones to cardiac and pulmonary afferent stimulation

No excitatory evoked responses were observed in DVN neurones following electrical stimulation of the cardiac branch of the vagus. However a depression of ongoing activity was observed in 1 / 5 DVN (latency 21 ms: duration 316 ms) following cardiac vagal stimulation (Figure 43). In addition a cessation in ongoing activity (latency 0.3 ± 0.3 s, duration 2.3 ± 0.8 s) was also observed in 2 / 16 DVN neurones following right atrial injection of PBG (Figure 43).

4.20.3 Ongoing activity: correlation with phrenic nerve activity and ECG

Ongoing activity was observed in all 21 DVN neurones recorded with a mean baseline firing rate of 1.95 ± 0.25 Hz which was significantly less (p < 0.05) than that observed in NTS neurones (2.80 ± 0.25 Hz n = 110).
Ongoing activity was found to be correlated with ECG in 4/21 (19%) of DVN neurones and PNA in 1/14 (7%) of DVN neurones.
Figure 42  Frequency distribution histogram of the latency of evoked responses in DVN neurones following cervical vagus and PBN stimulation.

A  Latency of evoked responses in 21 DVN neurones following cervical vagus stimulation.

B  Latency of 15 evoked responses in 14 DVN neurones following synaptic PBN stimulation.
(a) VAGUS

Number of evoked response

Latency (ms)

(b) PBN synaptic

Number of evoked response

Latency (ms)
Figure 43  Post stimulus triggered histogram illustrating the depression of ongoing activity following the stimulation of the cardiac branch of the vagus in a DVN neurone (a). Raw data illustrating the cessation of ongoing activity following right atrial injection of PBG in a DVN neurone (b).

A  PSTH (1ms bin width, 50 sweeps) of ongoing activity following stimulation of the cardiac branch of the vagus. Stimulus artifact is indicated by the closed circle above the PSTH. Note the absence of an excitatory evoked response to stimulation of the cardiac branch of the vagus, however a post stimulus period of depression in ongoing activity is observed as indicated by the bracket in figure (a).

B  example of the cessation of ongoing activity following right atrial injection of PBG injection in a DVN neurone. Injection of PBG denoted by the bar above raw data.
(a) Cardiac vagus stimulation

Depression of ongoing activity

Spikes bin\(^{-1}\)

Time (s)

(b) Atrial

PBG 10 \(\mu\)g

Ev (mV)

Spike bin\(^{-1}\)

Blood pressure (mmHg)

4 s
Discussion

4.21 Methodological considerations

4.21.1 PBN stimulation

The use of electrical stimulation to determine synaptic or antidromic connections between specific regions of the CNS is limited by the specificity of the electrical stimulus for the activation of neuronal cell bodies or afferent fibre terminations within the stimulated region. In the present study the activation of fibres of passage travelling through the PBN from other CNS areas to the NTS or vice versa will also result in antidromically or synaptically evoked responses in NTS neurones and is a major limitation of the technique. The PBN is the major recipient of ascending efferent projections from the NTS, whilst with the exception of the PAG, projections to other supra-pontine CNS structures from the NTS are relatively sparse (Loewy & Burton, 1978; Norgren, 1978; Ricardo & Koh, 1978; Kapp et al., 1989; Herbert et al., 1990; Herbert & Saper, 1992; Otake et al., 1992). Therefore with the exception of the PAG the probability of activating ascending fibres from the NTS to other supra-pontine CNS areas, which pass through the PBN, following PBN stimulation is low compared to the probability of activating afferent fibres from the NTS, which terminate within the PBN. The PAG does however receive a strong afferent projection from the NTS which has been shown to travel from the NTS to the PAG in part via a projection through the dorsolateral medulla / pons and that may enter the PAG after traversing the mesencephalic trigeminal nucleus and locus coeruleus to enter the PAG (Herbert & Saper, 1992; Otake et al., 1992). It is therefore possible that antidromic responses elicited in the present study may be due to the activation of afferent fibres travelling from the NTS through or near to the PBN, which terminated in the PAG.

Areas outside the PBN may have also been activated due to the spread of current following stimulation of the PBN. In the present study the mean threshold current used to elicit antidromically or synaptically evoked responses in NTS neurones was 400µA. Based on the observations of previous studies (Jhamandas et al., 1991b; Bester et al., 1995; Jhamandas et al., 1996; Ezure et al., 1998a) and personal observations made in the present study stimulation at an intensity of 400µA would have activated an area of approximately 0.5mm from the site of stimulation. For stimulating sites centred on the
elPBN this would still encompass areas within the PBN medial to the site of stimulation but would have extended beyond the ventrolateral border of the PBN. In two animals where the placement of the stimulating electrode was found to be beyond the ventrolateral extent of the PBN no excitatory evoked responses were observed in NTS neurones recorded indicating that responses observed following the stimulation of electrodes placed within the elPBN nucleus were not due to current spread beyond the PBN. For stimulating sites centred on the eIPBN although the spread of current lateral to the site of stimulation would still be within the PBN there is a possibility that stimulation may have also activated neuronal elements medial to the PBN within the locus ceruleus and surrounding pontine grey matter. The activation of cell bodies within the locus coeruleus may therefore have contributed to the synaptically evoked responses. However, it is unlikely that antidromically evoked responses elicited in NTS neurones following PBN stimulation were due to the activation of afferent fibres terminating within the locus coeruleus as no direct afferent projection has been reported between the locus ceruleus and the NTS (Ennis & Aston-Jones, 1989; Aston-Jones et al., 1991).

4.21.2 Right atrial injection of PBG: combined cardiac and pulmonary afferent activation

In the present studies right atrial injection of PBG was used to stimulate cardiac and pulmonary afferent fibres. The circulation time between the right atrium and left ventricle / aortic arch is approximately 1 - 2 s in the rat (Hines et al., 1994; Brophy et al., 1999). Consequently by restricting observations to responses that occurred with an onset latency of 1s or less following right atrial injection of PBG it is possible to discriminate neurones, which responded to cardiac and pulmonary afferent activation. Neurones, which responded to right atrial injection of PBG at latencies greater than 1s may have responded to the activation of afferent fibres located outside the cardiopulmonary circulation eg. peripheral chemoreceptors or vascular afferent fibres and so were not classified as CP-NTS neurones.

In many studies excitatory responses following right atrial injection of PBG occurring within the circulation time between the right and left sides of the heart have been considered to be due to the selective activation of pulmonary afferent fibres (Daly, 1991; Jones et al., 1998; Paton, 1998a, 1998c; Silva-Carvalho et al., 1998). No
excitation was deemed to have occurred due to the activation of endocardial receptors in the heart. This assertion is supported by the preferentially epicardial distribution of chemosensitive ventricular cardiac vagal afferent fibres determined by probing both endocardial and epicardial surfaces of the dissected heart (Coleridge et al., 1964; Muers & Sleight, 1972). The observation that excitatory responses within both atrial and ventricular cardiac vagal afferent fibres following left atrium or left ventricle injection of chemical mediators are of a long latency (3 – 15 s), is consistent with the activation of fibres situated in the epicardium and myocardium accessed through the coronary arteries rather than a direct activation of afferent fibres located on the endocardial surface (Paintal, 1955; Coleridge et al., 1964; Kaufman et al., 1980). Furthermore ventricular cardiac vagal afferent fibres whose fibre ending located on the endocardial surface of the heart were not chemosensitive (Coleridge et al., 1964).

However, the experiments outlined above were performed in either the cat or dog, whether a similar preferential epicardial distribution of chemosensitive cardiac vagal afferent fibres exists in the rat is unclear. Initial studies conducted by Thoren (1979) reported the presence of only atrial cardiac vagal afferent fibres in the rat approximately half of these located on the endocardial surface. However Thoren did not examine the response to chemical stimuli. More recent studies have demonstrated the presence of both atrial and ventricular chemosensitive cardiac vagal afferent fibres in the rat (Ustinova & Schultz, 1994a). The receptive fields of these fibres were mapped with "gentle probing" of the heart suggesting a preferentially epicardial distribution, however, the latency of excitatory responses to epicardial application of capsaicin was long (approximately 6 s) suggesting the location of these fibres was deep below the epicardial surface. In the cat and dog, the onset latency of the coronary chemoreflex elicited by left atrial injection of chemical mediators is approximately 3 – 4 s (Zucker & Cornish, 1981; Daly et al., 1988) consistent with the activation of fibres following the distribution of mediators within the coronary circulation opposed to via the endocardial surface. In the rat the onset latency of the coronary chemoreflex elicited by left ventricular injection of PBG was short (approximately 1 s, see Chapter 2), which does not allow the possible activation of endocardial cardiac vagal afferent fibres compared with fibres activated via the coronary circulation to be temporally resolved. Therefore until such data becomes available to exclude the presence of endocardial chemosensitive cardiac vagal afferent fibres in the rat it was considered prudent to refer
to responses to right atrial injection of PBG as due to combined cardiac and pulmonary afferent fibre stimulation.

4.21.3 Cardiac vagal stimulation

The use of electrical stimulation of the cardiac branch of the vagus as a selective means of activating cardiac vagal afferent fibres has already been considered in detail in chapter 3 and so will not be repeated again. In addition, left ventricular injection of PBG was used to define the sensory modality of cardiac afferent input onto NTS neurones identified as receiving cardiac vagal input by electrical stimulation of the cardiac vagus. As demonstrated in chapter 2, injection of PBG into the left ventricle will activate chemosensitive cardiac vagal afferent fibres eliciting the coronary chemoreflex. However as previously considered in chapter 2, left ventricular injection of PBG will also activate other afferent fibres e.g. peripheral chemoreceptors (Brophy et al., 1999) and vascular afferent fibres (Coleridge et al., 1973) within the onset latency used to determine the chemical activation of cardiac vagal afferent fibres in the present study. It is therefore possible that some neurones classified as chemosensitive cardiac-NTS neurones may not have responded to the chemical stimulation of cardiac vagal afferent fibres but to the chemical activation of non-cardiac afferent fibres. The impact of these false positive results is diminished when one considers the high degree of convergence between peripheral chemoreceptors and chemosensitive cardiac vagal input on NTS neurones (Paton, 1998a). However the possibility remains that a small percentage of NTS neurones classified as chemosensitive cardiac-NTS neurones may not have responded to chemosensitive cardiac vagal afferent fibre activation but rather the chemical activation of non-cardiac afferent fibres.

4.21.4 Activation of cardiac sympathetic afferent fibres

Although PBG is routinely used as a chemical stimulant of cardiorespiratory vagal afferent fibres, PBG has also been shown to stimulate sympathetic afferent fibres both in the heart and viscera (Blair et al., 1992; Fu & Longhurst, 1998a, b). It is therefore plausible that responses elicited following right atrial or left ventricular injection of PBG, which have been attributed to the activation of vagal cardiac, or pulmonary afferent fibres may have been due to the activation of sympathetic afferent fibres. The reported incidence of convergence between sympathetic and vagal cardiac afferent
fibres is low (approximately 15%) within the NTS (Tjen et al., 1997). Considering all NTS neurones classified as cardiac-NTS neurones demonstrated a synaptic vagal input following electrical stimulation of the cardiac branch of the vagus the probability that reported neurones were excited by chemosensitive sympathetic cardiac afferent activation opposed to cardiac vagal afferent activation would be equally as low (15%).

4.22 Responses of investigated neurones

In agreement with previous studies which have documented the presence of reciprocal connections between the NTS and the PBN (Norgren, 1978; Ricardo & Koh, 1978; Saper & Loewy, 1980; Herbert et al., 1990; Krukoff et al., 1992), stimulation of the PBN elicited both antidromic and synaptic responses in NTS neurones in the present study. Whilst no difference was observed in the location of stimulating sites within the lateral PBN compared with type of evoked response (antidromic or synaptic) elicited within recorded NTS neurones. The threshold stimulus intensity required to elicit antidromic evoked responses was found to be significantly greater following electrode placement in the mesencephalic elPBN compared with electrodes placed in the pontine elPBN or cIPBN, indicating that that fibre terminations or fibres of passage activated were at a greater distance from the mesencephalic elPBN. Consistent with this observation, injections of anterograde tracer into the commissural NTS have been demonstrated to produce dense labelling within the pontine elPBN and cIPBN but only weak labelling in the mesencephalic PBN found dorsal to the cIPBN (Berkley & Scofield, 1990; Herbert et al., 1990; Otake et al., 1992). Similarly, a significantly greater stimulus threshold was required to elicit synaptically evoked responses in NTS neurones following electrode placement in the mesencephalic cIPBN compared to placement in the pontine cIPBN and elPBN indicating that efferent projections from the PBN or travelling through the PBN to the NTS were located at a greater distance from the mesencephalic cIPBN than the pontine cIPBN and elPBN. Consistent with the smaller stimulation intensities required to elicit synaptic evoked responses within NTS neurones following electrode placement within the pontine cIPBN and elPBN previous electrophysiological studies have demonstrated that stimulation of pontine cIPBN and elPBN elicits synaptically evoked responses within the NTS which receive carotid sinus input (Felder & Mifflin, 1988).
4.23 Experiment A: Combined cardiac and pulmonary afferent fibre stimulation

The result of this study demonstrates the presence of both excitatory and inhibitory responses in NTS neurones following combined cardiac and pulmonary afferent stimulation. Control volume injections of saline failed to produce excitatory or inhibitory responses indicating that the observed responses were due to the activation of chemosensitive cardiac and pulmonary vagal afferent fibres by PBG rather than the activation of atrial stretch receptors or ventricular mechanosensitive afferent fibres. Indeed only injection of saline at volumes greater than that used in the present study have been reported to activate atrial stretch afferent fibres in the rat (Hines et al., 1994). Similar responses to right atrial injection of PBG were not thought to be a consequence of the evoked fall in blood pressure as a similar fall in blood pressure following systemic injection of SNP did not elicit any change in ongoing activity.

Following PBN stimulation antidromic and synaptically evoked responses were observed in majority of eCP-NTS (68%) and iCP-NTS (80%) neurones investigated. The presence of antidromically evoked response in CP-NTS neurones following PBN stimulation demonstrates that cardiac and pulmonary vagal afferent input can be relayed from the NTS to the PBN via a direct projection between the two areas. Furthermore the presence of a population of CP-NTS synaptically excited by PBN stimulation also demonstrates that descending input from the PBN modulates NTS neurones receiving vagal cardiac and pulmonary input. Interestingly a greater percentage of eCP-NTS was antidromically activated (54%) compared to synaptically activated (14%) following PBN stimulation. Whereas a greater percentage of iCP-NTS neurones were synaptically activated (50%) compared to antidromically activated (30%) by PBN stimulation. These results would suggest that excitatory cardiac and pulmonary afferent input was preferentially relayed via an ascending projection to the PBN compared to inhibitory cardiac and pulmonary input which was predominately modulated by a descending input from the PBN.

4.24 Experiment B: Cardiac vagal afferent stimulation

Cardiac-NTS neurones could be further subdivided into two populations chemosensitive and non-chemosensitive based on the response of recorded neurones to left ventricular injection of PBG. Previous electrophysiological studies have also demonstrated the
existence of two populations of cardiac vagal afferent fibres based on their response to mechanical or chemical stimulation of afferent endings. A small percentage (12%) of these neurones were suggested to respond to both stimuli (Armour, 1994) although as discussed in the introduction this will depend on the type of chemical stimulus employed and the magnitude of the mechanical stimulus. It is therefore proposed that the population of cardiac-NTS neurones classified as non-chemosensitive probably received mechanosensitive cardiac vagal input. In support of this assertion the ongoing discharge in non-chemosensitive cardiac-NTS was observed to be correlated with ECG in 5 / 7 neurones examined. A similarly high proportion of mechanosensitive cardiac vagal afferent fibres have been reported previously to have ongoing activity correlated with ECG in the rat (Thoren et al., 1979; Ustinova & Schultz, 1994a).

Further investigation of correlation between ongoing activity and ECG indicated that the peak in the R wave triggered PSTH occurred during or just after the P wave of the ECG adjusted to account for the approximate conduction time between the heart and the NTS in two non-chemosensitive cardiac-NTS neurones. This was consistent with maximal ventricular filling and the “a” wave of the atrial pressure pulse indicating that the ECG related input was derived from mechanosensitive cardiac vagal input. In the remaining three non-chemosensitive cardiac-NTS neurones the peaks within the R wave triggered PSTH occurred during or close to the T wave of the ECG consistent with maximal aortic pressure. The cardiac modulation of ongoing activity in these neurones may be derived from baroreceptor input and would be consistent with the demonstration of a high proportion of convergent baroreceptor input onto non-chemosensitive cardiac-NTS neurones in previous studies (Paton, 1998a; Silva-Carvalho et al., 1998). A correlation of ongoing discharge with ECG was also observed within 2 / 8 chemosensitive cardiac-NTS neurones suggesting that these neurones also received mechanosensitive cardiac vagal or baroreceptor afferent input.

A correlation between ongoing activity and PNA was also observed in two chemosensitive cardiac-NTS neurones in phase with inspiration and one non-chemosensitive cardiac-NTS neurones in phase with late expiration. Although previous studies have not reported a respiratory related discharge in NTS neurones receiving cardiac vagal input (Bennett et al., 1985; Paton, 1998a, c). Respiratory modulated discharge had been reported in recordings made from mechanosensitive but not
chemosensitive cardiac vagal afferent fibres and may account for the respiratory modulation in the non-chemosensitive cardiac-NTS neurone (Coleridge et al., 1964; Baertschi & Gann, 1977; Thames et al., 1977; Kaufman et al., 1980; Ustinova & Schultz, 1994a). The presence of respiratory related activity in NTS neurones within the commissural NTS of the rat has been reported previously in neurones that received input from lung stretch afferent fibres eg. pulmonary slowly adapting receptors (SARs) and pulmonary rapidly adapting receptors (RARs) (Ezure & Tanaka, 1996; Ezure et al., 1998b). Consistent with the correlation of ongoing activity with the inspiratory phase of PNA in chemosensitive cardiac-NTS neurones both SARs and RARs have been shown to be activated by lung inflation. As an index of lung inflation was not recorded during the present study it is not possible to determine the relationship of ongoing activity with lung inflation. However the phrenic interval was approximately twice that of the ventilator, or of similar interval, suggesting some entrainment of PNA with lung inflation. The respiratory related discharge observed in cardiac-NTS neurones may therefore reflect a convergence of cardiac vagal and lung stretch afferent input on these neurones. Respiratory modulation of NTS neurones has also been reported in response to central respiratory inputs (Ezure et al., 1998b; Miyazaki et al., 1998).

Control experiments demonstrated that the excitatory response elicited in cardiac-NTS neurones following left ventricular injection of PBG were not due to the volume of the injectate nor due to the fall in blood pressure elicited by the activation of the coronary chemoreflex. Furthermore excitatory responses were also observed following right atrial injection of PBG in 7/9 chemosensitive cardiac-NTS neurones and 2/6 non-chemosensitive cardiac-NTS neurones. It is proposed that these responses represent the convergence of pulmonary afferent input onto NTS neurones receiving cardiac vagal input, however as discussed earlier further investigation is required to confirm this in the rat. The predominance of convergence of chemosensitive pulmonary afferent input with chemosensitive cardiac input compared to non-chemosensitive cardiac vagal input is consistent with previous reports in the mouse (Paton, 1998a, c) and the cat (Silva-Carvalho et al., 1998).

The majority of cardiac-NTS neurones recorded were antidromically activated (72%) by PBN stimulation suggesting a major role for the PBN in the ascending integration of cardiac vagal input received via a direct projection from the NTS. A further 24% of
cardiac-NTS neurones investigated were synaptically activated by PBN stimulation demonstrating that the PBN is also involved in the descending modulation of cardiac vagal input received by the NTS. Antidromic evoked responses were observed in 87% of the chemosensitive cardiac-NTS neurones investigated following PBN stimulation, indicating that the vast majority of chemosensitive cardiac vagal input received in the NTS can be relayed via a direct projection to the PBN. By contrast only 57% of non-chemosensitive cardiac-NTS neurones studied were antidromically activated by PBN stimulation indicating that although the PBN is an important relay of mechanosensitive cardiac vagal afferent input within the CNS, other relays possibly within the VLM are also important.

A further population of NTS neurones, which did not respond to electrical stimulation of the cardiac branch of the vagus, but responded to right atrial injection of PBG were investigated in experiment B. Based on their response (excitatory or inhibitory) to right atrial injection of PBG these neurones classified as either eCP-NTS or iCP-NTS neurones respectively. Although similar to the eCP-NTS neurones investigated in experiment A, the population of eCP-NTS neurones studied in experiment B did not include cardiac-NTS neurones which responded to right atrial injections of PBG, that may have been present in the population eCP-NTS neurones studied in experiment A. However, a similar percentage of eCP-NTS neurones studied in experiment B were antidromically (60%) or synaptically (10%) activated following PBN stimulation as observed previously in experiment A, suggesting the number of cardiac-NTS neurones present in the population of eCP-NTS neurones studied in experiment A was low.

The percentage eCP-NTS antidromically activated by PBN stimulation was smaller than for chemosensitive cardiac-NTS neurones indicating that compared to chemosensitive cardiac vagal afferent input a greater proportion of chemosensitive pulmonary afferent input is relayed from the NTS to CNS structures other than the PBN. The VLM receives a strong afferent projection from the NTS and may receive inputs from these eCP-NTS neurones which did not project directly to the PBN (Ross et al., 1985; Otake et al., 1992; Verberne & Guyenet, 1992). Considering that supramedullary structures are not considered important in mediating the Bezold-Jarisch reflex (Lee et al., 1972) the high proportion of chemosensitive cardiac-NTS neurones which project to the PBN compared to eCP-NTS neurones may explain the smaller depressor response elicited by
the coronary chemoreflex in the rat compared to the Bezold-Jarisch reflex. (see Figure 17).

Both ECG and respiratory modulated ongoing activity was observed CP-NTS neurones investigated in experiment A and experiment B. The presence of ECG modulated ongoing activity in CP-NTS neurones suggests that these neurones also received convergent baroreceptor or mechanosensitive cardiac vagal afferent input. Similarly the presence of a respiratory modulation of ongoing activity suggests that these CP-NTS neurones received convergent central respiratory or lung stretch afferent input. Although lung inflation was not measured, the time interval between peaks in the PNA triggered PSTH was consistent with that of the ventilator in the majority of cases, suggesting that responses were due to lung inflation and that PNA had become entrained with lung inflation in a 1:1 or 2:1 ratio.

4.25 DVN neurones

In agreement with previously reported findings demonstrating an efferent projection from the PBN to the DVN, synaptically evoked responses were observed in DVN neurones following PBN stimulation (Luiten et al., 1987). However in the present study cardiac and pulmonary afferent stimulation did not produce a response in the majority of DVN neurones investigated in contrast with previous studies which have shown that combined cardiac and pulmonary afferent stimulation produces excitatory responses in DVN neurones (Jones et al., 1998).

4.26 Inhibitory responses

A common feature of the majority of all neurones investigated, including DVN neurones, was the presence of a long duration post excitatory suppression of ongoing activity following stimulation of the cervical vagus, cardiac branch of the vagus, and PBN. Similar effects have been documented previously in NTS neurones (Kidd, 1987; Silva-Carvalho et al., 1998) and are believed to due to both pre- and post synaptic inhibitory responses involving GABAergic transmission (McWilliam, 1987). In the present study post excitatory depression of ongoing activity was observed following the
antidromic activation of NTS neurones by PBN stimulation, and in DVN neurones by 
cervical vagus stimulation, suggesting the suppression was mediated by a post synaptic 
mechanism. However no post excitatory suppression of ongoing activity was observed 
following the electrical stimulation of the cardiac vagus in cardiac-NTS neurones 
synaptically excited by PBN stimulation. Interestingly both PBN and cervical vagus 
stimulation did produce a post excitatory suppression of ongoing activity in these 
neurones, which might suggest the mechanism involved, was presynaptic. The post 
excitatory suppression of ongoing activity would act to control the maximal firing 
frequency. The absence of a post excitatory suppression of ongoing activity following 
cardiac vagal stimulation in non-chemosensitive cardiac-NTS synaptically activated by 
PBN stimulation would indicate that such a control of firing rate does not occur in 
response to cardiac vagal input in these neurones.

Conclusion

The present study provides evidence, which demonstrates that NTS neurones, which 
receive cardiac vagal afferent input, send direct projections to or through the PBN. 
Furthermore, the proportion cardiac-NTS neurones which send projections to or 
through the PBN was far greater for cardiac-NTS neurones which received 
chemosensitive cardiac vagal afferent input, compared with cardiac-NTS neurones 
which were proposed to receive mechanosensitive cardiac vagal afferent input, 
indicating that the modality of the afferent input was important in determining the relay 
of cardiac vagal inputs from the NTS to the PBN. A smaller population of cardiac-NTS 
neurones were also demonstrated to receive descending inputs from the PBN.

The results of this chapter demonstrate that the enhanced expression of c-fos within the 
PBN observed following stimulation of the cardiac branch of the vagus may have been 
mediated by the direct projection of cardiac vagal afferent input from the NTS to the 
PBN. The potential role of the PBN in the central processing of cardiac vagal afferent 
input has been discussed previously in chapter 3 and it is suggested the PBN may be 
involved in the further relay of cardiac vagal afferent input to higher centres within the 
CNS, the antinociceptive effects elicited by cardiac and pulmonary vagal afferent 
stimulation and in the suppression of the baroreflex by cardiac and pulmonary vagal 
afferent fibres.
Despite the numerous investigations examining the properties of cardiac vagal afferent fibres and their reflex effects relatively little is known about the central processing of cardiac vagal inputs beyond the NTS. The present understanding is limited further when one considers that the majority of information is derived from studies in which the activation of other afferent fibres will have occurred in addition to stimulating cardiac vagal afferent fibres, for example intravenous or right atrial injection of chemical mediators will activate both pulmonary and cardiac vagal afferent fibres and so the precise contribution of cardiac vagal afferent fibres is often unclear. The aim of the present thesis was therefore to further investigate the central pathways involved in the processing of cardiac vagal input with particular emphasis placed on supramedullary areas that received ascending inputs from NTS neurones activated by chemosensitive cardiac vagal afferent fibres.

Integration of cardiac vagal afferent input within the NTS

In the present thesis through the use of c-fos gene expression and extracellular single unit recordings the activation of neurones within the NTS in response to cardiac vagal afferent input was confirmed in the rat in agreement with existing anatomical and electrophysiological studies which have also demonstrated that the NTS receives cardiac vagal afferent input in the cat, dog and mouse (Kalia & Mesulam, 1980; Bennett et al., 1985; Paton, 1998a; Silva-Carvalho et al., 1998; Seagard et al., 1999). The increased c-fos expression following cardiac vagal stimulation was observed within the NTS caudal of obex through to the rostral pole of the area postrema consistent with the recently described distribution of cardiac vagal afferent fibre terminations in the rat (Xie et al., 1999). Whilst, electrophysiological studies demonstrated the presence of two populations of cardiac-NTS neurones one which received chemosensitive cardiac vagal afferent input and a second, which did not respond to left ventricle injections of PBG and is proposed to receive mechanosensitive cardiac vagal afferent fibres. This was consistent with previous observations of a modality coding in cardiac vagal afferent fibres recorded in the rat and also in the cat and dog and indicated that this coding of information based on stimulus was preserved at the level of the NTS (Baker et al., 1979;
Coleridge et al., 1964; Kaufman et al., 1980; Armour et al., 1994; Ustinova & Schultz, 1994a).

In addition a high proportion of chemosensitive cardiac-NTS neurones were also excited by right atrial injection of PBG at onset latencies within the pulmonary circulation time whilst only a small proportion of proposed mechanosensitive cardiac-NTS neurones responded to PBG. This suggested that a high degree of convergence occurred between chemosensitive inputs from cardiac and pulmonary vagal afferent fibres onto NTS neurones, but only a small degree of convergence occurred between chemosensitive pulmonary vagal input and mechanosensitive cardiac vagal inputs onto NTS neurones similar to observations made in the cat and mouse (Paton, 1998a, c; Silva-Carvalho et al., 1998). However further investigation is required to establish that right atrial injection of PBG did not also activate chemosensitive cardiac vagal afferent fibres within the 1s onset latency used to restrict observations to the pulmonary circulation.

Central processing of cardiac vagal afferent input from the NTS

An enhanced expression of c-fos was observed in the IVLM, RVLM, NRM, PBN and PAG following electrical stimulation of the cardiac branch of the vagus indicating that neurones within these areas were activated in response to cardiac vagal afferent input. The pathway by which an enhanced c-fos expression was elicited in response to cardiac vagal stimulation was not investigated in the Fos study, however previous anatomical studies indicate the IVLM, RVLM, NRM, PBN and PAG could receive cardiac vagal afferent input via a direct projection from the NTS (Norgren, 1978; Ricardo & Koh, 1978; Beitz, 1982a, b; Ross et al., 1985; Van Bockstaele et al., 1989; Herbert et al., 1990; Herbert & Saper, 1992; Otake et al., 1992) (Figure 44).

The NTS also receives efferent projections from the IVLM, RVLM, NRM, PBN and PAG and so the increased c-fos expression in the NTS following cardiac vagal stimulation may also be due to efferent input from these CNS structures in addition to afferent input from cardiac vagal afferent fibres (Saper & Loewy, 1980; Fulwiler & Saper, 1984; Guyenet & Young, 1987; Thor & Helke, 1987; Herbert et al., 1990; Roder & Ciriello, 1992; Krukoff et al., 1993; Farkas et al., 1997; Bianchi et al., 1998; Odeh & Antal, 2001). Furthermore reciprocal connections have also been demonstrated between
the IVLM, RVLM, NRM, PBN and PAG and so cardiac vagal input may also be received in these areas via both monosynaptic and polysynaptic pathways originating from the NTS (Saper & Loewy, 1980; Beitz, 1982a, b, c; Marchand & Hagino, 1983; Fulwiler & Saper, 1984; Ross et al., 1985; Guyenet & Young, 1987; Carrive et al., 1988; Van Bockstaele et al., 1989; Herbert et al., 1990; Nicholas & Hancock, 1990; Carrive & Bandler, 1991; Van Bockstaele et al., 1991; Herbert & Saper, 1992; Sim & Joseph, 1992; Krukoff et al., 1993; Cameron et al., 1995; Chen & Aston-Jones, 1996; Hermann et al., 1997; Bianchi et al., 1998; Henderson et al., 1998; Krout et al., 1998; Odeh & Antal, 2001).

The involvement of monosynaptic compared with polysynaptic pathways in the relay of cardiac vagal input from the NTS would be expected to vary between the IVLM, RVLM, NRM, PBN and PAG. For example the PBN receives the largest ascending projection from the NTS (Norgren, 1978; Ricardo & Koh, 1978; Saper, 1995), whilst the NRM is only reported to receive a weak afferent projection from the NTS (Hermann et al., 1997). Consequently the contribution of a monosynaptic compared to polysynaptic pathway in the relay of cardiac vagal input from the NTS to the PBN would be expected to be greater than for the relay of cardiac vagal afferent input from the NTS to the NRM, which would be expected to be predominantly polysynaptic (Figure 44).

**Relay of cardiac vagal afferent input from the NTS to the PBN**

The direct projection of NTS neurones which receive cardiac vagal afferent input to the PBN was examined in electrophysiological studies which demonstrated that electrical stimulation of the PBN elicited antidromic responses in the vast majority of the *chemosensitive cardiac*-NTS neurones investigated although only approximately half of the *non-chemosensitive cardiac*-NTS neurones studied (Figure 45). It would therefore appear that not only does the PBN receive ascending cardiac vagal input but that the modality of the cardiac vagal afferent input received by *cardiac*-NTS neurones is important in determining whether this input is relayed to or through the PBN. It should be stressed however that recordings were predominantly made from the commissural NTS in the present thesis, whether NTS neurones which receive cardiac vagal afferent input located in more rostral or lateral areas of the NTS, send projections towards the PBN was not examined and future studies should be undertaken to examine this.
possibility. Furthermore the present study did not intend to determine the afferent terminations of projections from the NTS within the PBN merely to demonstrate existence of projections to that area. Consequently, NTS neurones antidromically activated by PBN stimulation may send projections through the PBN, which terminate in other areas of the CNS rostral to the PBN. Indeed a strong ascending projection has been demonstrated from the NTS to the PAG in which some afferent fibres enter the PAG after traversing the PBN and hence would be activated by electrical stimulation of the PBN (Herbert & Saper, 1992; Otake et al., 1992). A more detailed antidromic mapping study (see Lipski, 1981) would be required to demonstrate the termination of the ascending projections from cardiac-NTS within the PBN although the strong efferent projection from the NTS to the PBN demonstrated in tracing studies would indicate this was likely to be the case (Loewy & Burton, 1978; Norgren, 1978; Ricardo & Koh, 1978; Kapp et al., 1989; Herbert et al., 1990; Otake et al., 1992).

**Descending modulation of cardiac vagal afferent input within the NTS**

PBN stimulation also elicited synaptic excitatory responses in cardiac-NTS neurones demonstrating that NTS neurones, which received cardiac vagal afferent input, could also be modulated by descending projections from the PBN or from higher centres whose efferent projections passed through the PBN. Unlike cardiac-NTS neurones, which projected towards the PBN, a greater proportion of non-chemosensitive cardiac-NTS neurones than chemosensitive cardiac-NTS neurones were synaptically activated by PBN stimulation (Figure 45). However the number of neurones investigated was low and further study would be required to establish whether this trend represented a reproducible difference. Furthermore the proportion of non-chemosensitive cardiac-NTS neurones, which were synaptically modulated by PBN stimulation was still less than those which sent ascending projections to the PBN, indicating that the predominant function of the PBN was the ascending processing of cardiac vagal input received from the NTS, compared with the descending modulation of cardiac vagal input at the level of the NTS.
Inhibitory responses: Further evidence for a differential integration of cardiac vagal input within the NTS

Electrical stimulation of the cardiac branch of the vagus produced a post excitatory depression of ongoing activity in cardiac-NTS, which sent direct projections to the PBN, but not cardiac-NTS neurones, which received synaptic input from the PBN (Figure 46). In contrast stimulation of the cervical vagus or PBN elicited a post excitatory depression of ongoing activity in all cardiac-NTS neurones examined indicating the absence of an inhibitory response was specific for cardiac vagal input. Consequently, it would appear that the processing cardiac vagal input within NTS neurones that project to the PBN is different to the processing of cardiac vagal input by NTS neurones, which received descending inputs from the PBN. The post excitatory depression of ongoing activity would act to control the maximum firing frequency of NTS neurones as well as to reduce the temporal summation of excitatory convergent inputs. It is noticeable that the absence of a post excitatory depression of ongoing activity was not determined by the modality of input but appeared to by related to the presence of a descending synaptic input from the PBN. Although by virtue of the high percentage of chemosensitive cardiac-NTS neurones, which send direct projections to the PBN the effect of this observation, is likely to be greater for mechanosensitive cardiac vagal input. Whether the presence of a post excitatory depression only occurs in cardiac-NTS neurones which project to the PBN following electrical stimulation of the cardiac branch of the vagus would require further investigation as only one cardiac-NTS neurone was recorded that was not excited by PBN stimulation.

Role of central areas in the processing of cardiac vagal afferent input

Existing evidence in the literature indicates that the cardiovascular reflex effects elicited by cardiac vagal afferent stimulation are mediated within medullary relays. Whilst the supramedullary processing of cardiac vagal inputs identified within the NRM, PBN and PAG in the present study could be involved either in the further relay of cardiac vagal input to higher areas of the CNS as part of a sensory pathway for visceral input, the initiation or relay of descending antinociceptive projections to the spinal cord or the suppression of the baroreflex control of heart rate and sympathetic nerve activity (Figure 47).
Previous studies have demonstrated that the VLM is involved in the generation of reflex depressor, sympathoinhibitory responses elicited by stimulating chemosensitive cardiorespiratory vagal afferent fibres and the release of vasopressin following atrial vagal stimulation (Harris & Loewy, 1990; Verberne & Guyenet, 1992; Vayssettes-Courchay et al., 1997; Grindstaff & Cunningham, 2001a). Supramedullary areas do not appear to be important in the cardiovascular responses elicited by cardiac vagal afferent stimulation as lower pontine transection did not affect the magnitude of Bezold-Jarisch reflex elicited by intravenous injection of veratridine or the tachycardia elicited by atrial distension (Lee et al., 1972; Burkhart & Ledsome, 1977). However, although the PAG and PBN may not be directly involved in the reflex cardiovascular responses elicited by the activation of cardiac vagal afferent fibres stimulation both the PAG and PBN has been shown to modulate baroreflex control of heart rate and sympathetic nerve activity (Nosaka et al., 1993; Inui et al., 1994; Saleh & Connell, 1997; Saleh & Connell, 1998). Consequently relays within these areas may represent a pathway by which chemosensitive cardiac vagal afferent fibres reduce baroreflex sensitivity (Chen, 1979; Holmberg et al., 1983).

In addition to eliciting cardiovascular and endocrine reflex responses, cardiac vagal afferent fibres have also been suggested to be involved in the relay of nociceptive visceral information from the heart (Meller & Gebhart, 1992). Both the PAG, PBN and VLM have been implicated in the relay of nociceptive visceral input to the CNS and hence the activation of these areas by cardiac vagal afferent fibres could represent a possible pathway by which nociceptive input is relayed by cardiac vagal afferent to higher centres of the CNS (Bernard et al., 1989; Lovick, 1993; Bernard et al., 1994; Keay et al., 1997; Krout & Loewy, 2000b; Gauriau & Bernard, 2002; Lima et al., 2002). By contrast there is evidence in the literature which indicates that the stimulation of cardiac and pulmonary vagal afferent fibres has antinociceptive effects increasing the latency of withdrawal reflexes to noxious stimuli and inhibiting the activation of dorsal horn neurones in response to sympathetic cardiac afferent input via central relays (Ammons et al., 1983; Hobbs et al., 1989; Meller et al., 1992). The VLM, NRM, PBN and PAG have all been demonstrated to be involved in the generation of descending antinociceptive inputs and in particular the inhibition of dorsal horn neurones which receive sympathetic cardiac input (Ammons et al., 1984; Brennan et al., 1987; Girardot et al., 1987; Chandler et al., 1989; Ren et al., 1990; Randich et al.,
1990). The activation of these areas may therefore also be involved in the generation of an antinociceptive response following cardiac vagal afferent activation, which in particular may act to reduce nociceptive inputs received from sympathetic cardiac afferent fibres.

Conclusions
The results of this thesis provide evidence that demonstrates that:

- The NTS, IVLM, RVLM, NRM, PBN and PAG are involved in the central processing of cardiac vagal afferent input.

- The separation of chemosensitive and mechanosensitive input with cardiac vagal afferent fibres is preserved in the processing of cardiac vagal input within the NTS.

- The majority of cardiac-NTS neurones send direct projections to or through the PBN whilst another smaller population of cardiac-NTS neurones was shown to receive descending synaptic inputs from the PBN or fibres travelling through the PBN.

- The relay of cardiac vagal input from the NTS to the PBN is influenced by the modality of the cardiac vagal input received in the NTS and the PBN and appears to be integral in the central processing of chemosensitive cardiac vagal afferent input received by NTS neurones.

- The descending modulation of cardiac vagal input by synaptic inputs from or travelling through the PBN was greater in NTS neurones, which are proposed to receive mechanosensitive cardiac vagal input.

- A post excitatory suppression of ongoing activity occurs following electrical stimulation of the cardiac branch of the vagus in cardiac-NTS neurones, which projected towards the PBN, but not in cardiac-NTS neurones, which received descending input from the PBN.
The suppression of ongoing activity appeared to be independent of the modality of the cardiac vagal input received and may represent a fundamental difference in the processing of cardiac vagal input which is relayed towards the PBN compared with cardiac vagal input which is modulated by the PBN or descending inputs which pass through the PBN.
Figure 44 Schematic Illustration of the central projections of cardiac vagal afferent input

Red boxes illustrate central structures, which demonstrated increased activity in response to stimulation of the cardiac branch of the vagus.

Red lines illustrate the direct projection of cardiac vagal input to the NTS and the further direct relay of cardiac vagal input from the NTS to the PBN demonstrated within the present thesis.

Solid blue lines illustrate the suggested projection of cardiac vagal input based on existing anatomical studies, whilst the dotted blue line illustrates the weak projection from the NTS to the NRM.
Cardiac vagal afferent input
Percentages refer to the proportion of NTS neurones, which received chemosensitive or mechanosensitive cardiac vagal afferent input and sent direct projections to the PBN or received synaptic input from the PBN respectively.

NTS neurones, which received synaptic input from the PBN are suggested to project to either the PAG or VLM.

Note that the vast majority of NTS neurones which received chemosensitive cardiac vagal afferent input sent direct projections to the PBN, whilst a greater percentage of NTS neurones which received mechanosensitive compared to chemosensitive cardiac vagal afferent input where modulated by decending input from the PBN.
Chemosensitive cardiac vagal afferent input

Mechanosensitive cardiac vagal afferent input

87% 13% 57% 29%

PBN

VLM PAG?

NTS

VLM PAG?

227
Cardiac vagal afferent input is illustrated in red, whilst suggested inhibitory interneurones are illustrated in blue. All other neurones are illustrated in black.

A post excitatory depression of ongoing activity following electrical stimulation of the cardiac branch of the vagus was observed in NTS neurones that projected directly to the PBN but not NTS neurones, which received synaptic input from the PBN. Following PBN stimulation a post excitatory depression of ongoing activity was observed in both types of NTS neurones suggesting a differential processing of cardiac vagal input in NTS neurones which projected to the PBN compared to those which received synaptic input from the PBN.

Note in the present illustration the depression of NTS activity is suggested to be due to local interneurone pathways. However this was not investigated, other pathways may be involved.
Figure 47  A schematic illustration of the role of central areas in the processing of cardiac vagal afferent input

Areas activated by cardiac vagal afferent input relayed from the NTS are illustrated in red boxes.

Green boxes and arrows illustrate the suggested responses elicited by respective areas following activation by cardiac vagal afferent input.
HIGHER CENTRES

PAG
Baroreflex modulation

NRM
Descending antinociception

PBN

VLM
Coronary chemoreflex
References


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255


259


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Table 1  Changes (Δ) and onset latencies of changes in (a) heart rate, (b) MABP and (c) breath-breath interval, following intraventricular injection of saline (0.9% w/v) pH 7 or pH 10.

Each data column shows mean values ± s.e.m (n = 4).

Column labelled p shows the statistical probability of the change in respective variables following paired Student's t-test of absolute values before and after saline administration.

Column labelled p (II) shows the statistical comparison of the change in respective variables following saline administration vs respective drug administration (eg 50 & 100 μl saline pH7 vs veratridine in group I; 200 & 500 μl saline pH7 vs PGE₂; 100 & 200 μl saline pH 10 vs PBG) using an unpaired Student's t-test.

Column labelled p (III) shows the statistical comparison of the change in respective variables following saline administration vs veratridine administration in group II using an unpaired Student's t-test.
### (a) Δ heart rate (b.p.m.)

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<th>Volume (μl)</th>
<th>Baseline</th>
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<th>P</th>
<th>P (II)</th>
<th>P (III)</th>
<th>Latency (s)</th>
<th>Baseline</th>
<th>pH 10</th>
<th>P</th>
<th>P (II)</th>
<th>Latency (s)</th>
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<td>0.24</td>
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<td>0.03</td>
<td>0.0 ± 0.0</td>
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<td>0.12</td>
<td>0.03</td>
<td>0.0 ± 0.0</td>
<td>362.25 ± 21.92</td>
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### (b) Δ MABP (mmHg)

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<th>P</th>
<th>P (II)</th>
<th>P (III)</th>
<th>Latency (s)</th>
<th>Baseline</th>
<th>pH 10</th>
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<th>P (II)</th>
<th>Latency (s)</th>
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<td>92.20 ± 3.36</td>
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<td>0.03</td>
<td>0.16</td>
<td>0.0 ± 0.0</td>
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<td>0.01</td>
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<td>-6.17 ± 2.74</td>
<td>0.11</td>
<td>0.03</td>
<td>0.0 ± 0.0</td>
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<td>500</td>
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<td>0.01</td>
<td>0.0 ± 0.0</td>
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### (c) Δ breath-breath interval (s)

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<th>P</th>
<th>P (II)</th>
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<th>Latency (s)</th>
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<td>0.16</td>
<td>2.1 ± 0.2</td>
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Table ii  Changes (Δ) in (a) heart rate, (b) MABP and (c) breath-breath interval, following intraventricular injection of PBG, before vagotomy, after bilateral cervical vagotomy and after intra arterial injection of PBG.

Each data column shows mean values ± s.e.m (n = 4).

Column labelled p shows the statistical probability of the change in respective variables following paired Student's t-test of absolute values before and after PBG administration.

Column labelled p(II) shows the statistical probability of the change in respective variables compared before and after bilateral cervical vagotomy using an unpaired Student's t-test.
### (a) Δ heart rate (b.p.m.)

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<th>PBG (ug)</th>
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<th>Baseline</th>
<th>Change</th>
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<th>Baseline</th>
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### (b) Δ MABP (mmHg)

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### (c) Δ breath-breath interval (s)

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<th>Baseline</th>
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<td>3.79 ± 1.41</td>
<td>1.33 ± 0.13</td>
<td>2.66 ± 1.21</td>
<td>0.15</td>
<td>0.57</td>
<td>1.00 ± 0.05</td>
<td>-0.38 ± 0.11</td>
</tr>
</tbody>
</table>
Table iii  Onset latencies of changes (Δ) in (a) heart rate, (b) MABP and (c) breath-breath interval, following intraventricular injection of PBG, before vagotomy, after bilateral cervical vagotomy and after intra arterial injection of PBG.

Each data column shows mean values ± s.e.m (n = 4).
### (a) Δ heart rate

<table>
<thead>
<tr>
<th>PBG (µg)</th>
<th>Before Vagotomy</th>
<th>After Vagotomy</th>
<th>Systemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.3 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>5</td>
<td>0.5 ± 0.3</td>
<td>0.2 ± 0.2</td>
<td>1.9 ± 1.9</td>
</tr>
<tr>
<td>10</td>
<td>0.0 ± 0.0</td>
<td>0.8 ± 0.5</td>
<td>7.4 ± 1.1</td>
</tr>
<tr>
<td>20</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>4.5 ± 0.8</td>
</tr>
<tr>
<td>40</td>
<td>0.0 ± 0.0</td>
<td>0.2 ± 0.2</td>
<td>4.2 ± 0.7</td>
</tr>
</tbody>
</table>

### (b) Δ MABP

<table>
<thead>
<tr>
<th>PBG (µg)</th>
<th>Before Vagotomy</th>
<th>After Vagotomy</th>
<th>Systemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1.3 ± 0.4</td>
<td>0.8 ± 0.3</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>5</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.2</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>10</td>
<td>0.8 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>5.0 ± 1.9</td>
</tr>
<tr>
<td>20</td>
<td>0.7 ± 0.4</td>
<td>0.3 ± 0.3</td>
<td>5.5 ± 0.6</td>
</tr>
<tr>
<td>40</td>
<td>0.4 ± 0.4</td>
<td>0.0 ± 0.0</td>
<td>4.2 ± 0.4</td>
</tr>
</tbody>
</table>

### (c) Δ breath-breath interval

<table>
<thead>
<tr>
<th>PBG (µg)</th>
<th>Before Vagotomy</th>
<th>After Vagotomy</th>
<th>Systemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.8 ± 0.3</td>
<td>1.3 ± 0.9</td>
<td>7.3 ± 1.2</td>
</tr>
<tr>
<td>5</td>
<td>1.9 ± 1.1</td>
<td>1.9 ± 1.1</td>
<td>5.4 ± 1.1</td>
</tr>
<tr>
<td>10</td>
<td>3.0 ± 1.6</td>
<td>0.7 ± 0.4</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>20</td>
<td>1.9 ± 1.5</td>
<td>0.2 ± 0.2</td>
<td>4.1 ± 0.7</td>
</tr>
<tr>
<td>40</td>
<td>2.0 ± 1.2</td>
<td>0.2 ± 0.2</td>
<td>2.7 ± 0.4</td>
</tr>
</tbody>
</table>
Table iv Changes (Δ) in (a) heart rate, (b) MABP and (c) breath-breath interval, following intraventricular injection of PGE₂, before vagotomy, after bilateral cervical vagotomy and after intra arterial injection of PGE₂.

Each data column shows mean values ± s.e.m (n = 4).

Column labelled p shows the statistical probability of the change in respective variables following paired Student's t-test of absolute values before and after PBG administration.

Column labelled p(ll) shows the statistical probability of the change in respective variables compared before and after bilateral cervical vagotomy using unpaired Student's t-test.
### (a) Δ heart rate (b.p.m.)

<table>
<thead>
<tr>
<th>PGE2 (ng)</th>
<th>Baseline</th>
<th>Change</th>
<th>P</th>
<th>After Vagotomy</th>
<th>Baseline</th>
<th>Change</th>
<th>P</th>
<th>P (II)</th>
<th>Baseline</th>
<th>Systemic</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>342.00 ± 14.07</td>
<td>-2.67 ± 2.19</td>
<td>0.35</td>
<td>-0.75 ± 0.48</td>
<td>360.50 ± 26.05</td>
<td>0.22</td>
<td>0.47</td>
<td>374.67 ± 2.67</td>
<td>1.75 ± 1.75</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>351.75 ± 17.25</td>
<td>-2.00 ± 2.00</td>
<td>0.39</td>
<td>-4.00 ± 1.68</td>
<td>356.25 ± 2.14</td>
<td>0.10</td>
<td>0.47</td>
<td>363.67 ± 1.20</td>
<td>-0.50 ± 0.50</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>346.75 ± 12.79</td>
<td>-2.25 ± 1.65</td>
<td>0.27</td>
<td>-3.00 ± 1.91</td>
<td>379.00 ± 21.98</td>
<td>0.22</td>
<td>0.78</td>
<td>326.50 ± 3.48</td>
<td>5.00 ± 4.06</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>2500</td>
<td>352.67 ± 17.48</td>
<td>-7.00 ± 4.73</td>
<td>0.28</td>
<td>-4.33 ± 4.33</td>
<td>349.67 ± 4.18</td>
<td>0.42</td>
<td>0.70</td>
<td>366.00 ± 1.78</td>
<td>0.00 ± 0.00</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

### (b) Δ MABP (mmHg)

<table>
<thead>
<tr>
<th>PGE2 (ng)</th>
<th>Baseline</th>
<th>Change</th>
<th>P</th>
<th>After Vagotomy</th>
<th>Baseline</th>
<th>Change</th>
<th>P</th>
<th>P (II)</th>
<th>Baseline</th>
<th>Systemic</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>94.00 ± 10.21</td>
<td>-25.33 ± 5.81</td>
<td>0.05</td>
<td>-30.00 ± 5.58</td>
<td>120.50 ± 9.06</td>
<td>0.01</td>
<td>0.59</td>
<td>88.78 ± 1.85</td>
<td>0.00 ± 0.00</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>92.75 ± 6.09</td>
<td>-29.75 ± 4.59</td>
<td>0.01</td>
<td>-29.25 ± 5.01</td>
<td>114.50 ± 7.93</td>
<td>0.01</td>
<td>0.94</td>
<td>98.11 ± 1.75</td>
<td>-7.58 ± 3.09</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>91.25 ± 4.06</td>
<td>-31.50 ± 1.19</td>
<td>0.0001</td>
<td>-35.25 ± 5.17</td>
<td>111.50 ± 4.29</td>
<td>0.01</td>
<td>0.53</td>
<td>97.25 ± 1.74</td>
<td>-12.5 ± 1.71</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>2500</td>
<td>98.67 ± 3.71</td>
<td>-38.67 ± 0.33</td>
<td>0.0001</td>
<td>-38.87 ± 8.57</td>
<td>108.67 ± 8.35</td>
<td>0.03</td>
<td>1</td>
<td>94.75 ± 2.20</td>
<td>-25.17 ± 1.08</td>
<td>0.04</td>
<td></td>
</tr>
</tbody>
</table>

### (c) Δ breath-breath interval (s)

<table>
<thead>
<tr>
<th>PGE2 (ng)</th>
<th>Baseline</th>
<th>Change</th>
<th>P</th>
<th>After Vagotomy</th>
<th>Baseline</th>
<th>Change</th>
<th>P</th>
<th>P (II)</th>
<th>Baseline</th>
<th>Systemic</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>0.78 ± 0.09</td>
<td>-0.15 ± 0.08</td>
<td>0.24</td>
<td>-0.16 ± 0.06</td>
<td>1.22 ± 0.11</td>
<td>0.06</td>
<td>0.95</td>
<td>0.77 ± 0.06</td>
<td>0.06 ± 0.05</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>0.85 ± 0.09</td>
<td>-0.14 ± 0.04</td>
<td>0.04</td>
<td>-0.10 ± 0.02</td>
<td>1.23 ± 0.13</td>
<td>0.01</td>
<td>0.36</td>
<td>0.77 ± 0.10</td>
<td>-0.06 ± 0.02</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>0.79 ± 0.11</td>
<td>-0.12 ± 0.07</td>
<td>0.17</td>
<td>-0.16 ± 0.05</td>
<td>1.30 ± 0.13</td>
<td>0.04</td>
<td>0.63</td>
<td>0.79 ± 0.04</td>
<td>-0.01 ± 0.05</td>
<td>0.82</td>
<td></td>
</tr>
<tr>
<td>2500</td>
<td>0.87 ± 0.01</td>
<td>-0.12 ± 0.05</td>
<td>0.17</td>
<td>-0.22 ± 0.04</td>
<td>1.47 ± 0.21</td>
<td>0.04</td>
<td>0.28</td>
<td>0.88 ± 0.10</td>
<td>0.23 ± 0.41</td>
<td>0.68</td>
<td></td>
</tr>
</tbody>
</table>
Table v  Onset latencies of changes (Δ) in (a) heart rate, (b) MABP and (c) breath-breath interval, following intraventricular injection of PGE₂, before vagotomy, after bilateral cervical vagotomy and after intra arterial injection of PGE₂.

Each data column shows mean values ± s.e.m (n = 4).
Latency (s)

(a) Δ heart rate

<table>
<thead>
<tr>
<th>PGE2 (ng)</th>
<th>Before Vagotomy</th>
<th>After Vagotomy</th>
<th>Systemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>0.0 ± 0.0</td>
<td>0.1 ± 0.1</td>
<td>1.6 ± 1.6</td>
</tr>
<tr>
<td>500</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>1000</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>1.9 ± 1.3</td>
</tr>
<tr>
<td>2500</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

(b) Δ MABP

<table>
<thead>
<tr>
<th>PGE2 (ng)</th>
<th>Before Vagotomy</th>
<th>After Vagotomy</th>
<th>Systemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>0.6 ± 0.4</td>
<td>0.6 ± 0.4</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>500</td>
<td>0.2 ± 0.2</td>
<td>1.3 ± 0.4</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>1000</td>
<td>0.5 ± 0.5</td>
<td>0.7 ± 0.4</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>2500</td>
<td>0.5 ± 0.5</td>
<td>1.0 ± 0.3</td>
<td>1.5 ± 0.6</td>
</tr>
</tbody>
</table>

(c) Δ breath-breath interval

<table>
<thead>
<tr>
<th>PGE2 (ng)</th>
<th>Before Vagotomy</th>
<th>After Vagotomy</th>
<th>Systemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>3.9 ± 1.6</td>
<td>4.5 ± 1.5</td>
<td>4.8 ± 0.3</td>
</tr>
<tr>
<td>500</td>
<td>6.8 ± 1.1</td>
<td>4.7 ± 0.8</td>
<td>2.4 ± 0.7</td>
</tr>
<tr>
<td>1000</td>
<td>3.5 ± 1.4</td>
<td>6.4 ± 1.8</td>
<td>2.1 ± 1.1</td>
</tr>
<tr>
<td>2500</td>
<td>7.0 ± 2.8</td>
<td>7.2 ± 1.9</td>
<td>1.8 ± 1.1</td>
</tr>
</tbody>
</table>
Table vi Changes (Δ) and onset latencies of changes in (a) heart rate, (b) MABP and (c) breath-breath interval, following intraventricular injection of veratridine before and after bilateral cervical vagotomy in group I animals.

Each data column shows mean values ± s.e.m (n = 4).

Column labelled p shows the statistical probability of the change in respective variables following paired Student's t-test of absolute values before and after veratridine administration.
### (a) Δ heart rate (b.p.m.)

<table>
<thead>
<tr>
<th>Veratridine (µg)</th>
<th>Baseline</th>
<th>Change</th>
<th>P</th>
<th>Latency (s)</th>
<th>Baseline</th>
<th>Change</th>
<th>After Vagotomy</th>
<th>P</th>
<th>P (II)</th>
<th>Latency (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>349.00 ± 5.70</td>
<td>-2.25 ± 2.25</td>
<td>0.4</td>
<td>0.00 ± 0.00</td>
<td>360.25 ± 8.02</td>
<td>0.00 ± 0.00</td>
<td>0.4</td>
<td>0.00 ± 0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>346.00 ± 4.53</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>355.50 ± 8.77</td>
<td>0.00 ± 0.00</td>
<td>0.4</td>
<td>0.00 ± 0.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>345.50 ± 4.41</td>
<td>-4.00 ± 4.00</td>
<td>0.4</td>
<td>0.43 ± 0.43</td>
<td>361.25 ± 8.46</td>
<td>0.00 ± 0.00</td>
<td>0.4</td>
<td>0.00 ± 0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>327.67 ± 7.32</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>352.25 ± 14.91</td>
<td>0.00 ± 0.00</td>
<td>0.4</td>
<td>0.00 ± 0.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>347.00 ± 5.77</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>351.25 ± 15.11</td>
<td>-3.5 ± 3.5</td>
<td>0.4</td>
<td>0.53 ± 0.36</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### (b) Δ MABP (mmHg)

<table>
<thead>
<tr>
<th>Veratridine (µg)</th>
<th>Baseline</th>
<th>Change</th>
<th>P</th>
<th>Latency (s)</th>
<th>Baseline</th>
<th>Change</th>
<th>After Vagotomy</th>
<th>P</th>
<th>P (II)</th>
<th>Latency (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>89.25 ± 3.17</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>91.50 ± 6.12</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>88.75 ± 4.96</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>91.25 ± 4.84</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>86.00 ± 5.87</td>
<td>-2.75 ± 2.75</td>
<td>0.4</td>
<td>0.37 ± 0.37</td>
<td>88.75 ± 5.66</td>
<td>1.00 ± 1.00</td>
<td>0.4</td>
<td>0.27 ± 0.37</td>
<td></td>
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</tr>
<tr>
<td>10</td>
<td>81.00 ± 6.06</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>90.75 ± 6.61</td>
<td>3.00 ± 2.12</td>
<td>0.25</td>
<td>0.25 ± 0.49</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>89.33 ± 7.75</td>
<td>0.33 ± 1.26</td>
<td>0.84</td>
<td>0.35 ± 0.35</td>
<td>89.75 ± 5.44</td>
<td>-6.25 ± 9.11</td>
<td>0.54</td>
<td>0.65 ± 0.47</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### (c) Δ breath-breath interval (s)

<table>
<thead>
<tr>
<th>Veratridine (µg)</th>
<th>Baseline</th>
<th>Change</th>
<th>P</th>
<th>Latency (s)</th>
<th>Baseline</th>
<th>Change</th>
<th>After Vagotomy</th>
<th>P</th>
<th>P (II)</th>
<th>Latency (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.86 ± 0.08</td>
<td>1.18 ± 0.87</td>
<td>0.27</td>
<td>6.51 ± 1.77</td>
<td>1.24 ± 0.12</td>
<td>-0.29 ± 0.04</td>
<td>0.005</td>
<td>0.2</td>
<td>3.44 ± 1.55</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.82 ± 0.05</td>
<td>3.03 ± 0.87</td>
<td>0.04</td>
<td>3.45 ± 1.08</td>
<td>1.29 ± 0.11</td>
<td>-0.27 ± 0.08</td>
<td>0.05</td>
<td>0.03</td>
<td>3.14 ± 1.21</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.92 ± 0.04</td>
<td>11.81 ± 2.97</td>
<td>0.03</td>
<td>3.08 ± 1.03</td>
<td>1.33 ± 0.13</td>
<td>-0.32 ± 0.08</td>
<td>0.03</td>
<td>0.03</td>
<td>2.09 ± 1.38</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.89 ± 0.19</td>
<td>10.01 ± 2.90</td>
<td>0.08</td>
<td>3.32 ± 1.39</td>
<td>1.24 ± 0.13</td>
<td>-0.29 ± 0.04</td>
<td>0.01</td>
<td>0.07</td>
<td>1.78 ± 0.82</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.81 ± 0.10</td>
<td>31.04 ± 3.95</td>
<td>0.02</td>
<td>2.38 ± 0.91</td>
<td>1.48 ± 0.11</td>
<td>0.24 ± 0.61</td>
<td>0.72</td>
<td>0.01</td>
<td>0.54 ± 0.11</td>
<td></td>
</tr>
</tbody>
</table>
Table VII Changes (Δ) and onset latencies of changes in (a) heart rate, (b) MABP and (c) breath-breath interval, following intraventricular injection of veratridine before and after bilateral cervical vagotomy in group II animals.

Each data column shows mean values ± s.e.m (n = 4).
Column labelled p shows the statistical probability of the change in respective variables following paired Student's t-test of absolute values before and after veratridine administration.
### (a) Δ heart rate (b.p.m.)

<table>
<thead>
<tr>
<th>Veratridine (µg)</th>
<th>Before Vagotomy</th>
<th>After Vagotomy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Change</td>
</tr>
<tr>
<td>4</td>
<td>389.75 ± 19.29</td>
<td>-6.5 ± 4.27</td>
</tr>
<tr>
<td>6</td>
<td>389.25 ± 17.33</td>
<td>-2.25 ± 2.25</td>
</tr>
<tr>
<td>8</td>
<td>388.50 ± 16.78</td>
<td>-6.25 ± 3.75</td>
</tr>
<tr>
<td>10</td>
<td>390.75 ± 19.35</td>
<td>-14.5 ± 2.33</td>
</tr>
<tr>
<td>20</td>
<td>394.25 ± 14.04</td>
<td>-18.5 ± 4.7</td>
</tr>
</tbody>
</table>

### (b) Δ MABP (mmHg)

<table>
<thead>
<tr>
<th>Veratridine (µg)</th>
<th>Before Vagotomy</th>
<th>After Vagotomy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Change</td>
</tr>
<tr>
<td>4</td>
<td>93.00 ± 10.58</td>
<td>-10.5 ± 4.23</td>
</tr>
<tr>
<td>6</td>
<td>94.67 ± 9.72</td>
<td>-5.92 ± 3.76</td>
</tr>
<tr>
<td>8</td>
<td>92.25 ± 8.26</td>
<td>-12.92 ± 4.99</td>
</tr>
<tr>
<td>10</td>
<td>94.58 ± 7.70</td>
<td>-8.92 ± 3.85</td>
</tr>
<tr>
<td>20</td>
<td>93.67 ± 8.29</td>
<td>-7.83 ± 3.7</td>
</tr>
</tbody>
</table>

### (c) Δ breath-breath interval (s)

<table>
<thead>
<tr>
<th>Veratridine (µg)</th>
<th>Before Vagotomy</th>
<th>After Vagotomy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Change</td>
</tr>
<tr>
<td>4</td>
<td>0.99 ± 0.14</td>
<td>1.44 ± 0.57</td>
</tr>
<tr>
<td>6</td>
<td>1.02 ± 0.13</td>
<td>1.30 ± 0.65</td>
</tr>
<tr>
<td>8</td>
<td>0.95 ± 0.09</td>
<td>1.70 ± 0.61</td>
</tr>
<tr>
<td>10</td>
<td>0.97 ± 0.12</td>
<td>2.30 ± 0.45</td>
</tr>
<tr>
<td>20</td>
<td>0.90 ± 0.08</td>
<td>4.66 ± 2.30</td>
</tr>
</tbody>
</table>
Table viii  Changes (Δ) and onset latencies of changes in (a) heart rate, (b) MABP and (c) breath-breath interval, following intraventricular injection of veratridine before and after bilateral cervical vagotomy in group III animals.

Each data column shows mean values ± s.e.m.

Column labelled p shows the statistical probability of the change in respective variables following paired Student’s t-test of absolute values before and after veratridine administration.
### a) 
**Δ heart rate (b.p.m.)**

<table>
<thead>
<tr>
<th>Dose interval (min)</th>
<th>Baseline</th>
<th>Change</th>
<th>P</th>
<th>Latency (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>349.33 ± 14.67</td>
<td>-8.00 ± 5.51</td>
<td>0.28</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>15</td>
<td>342.67 ± 9.40</td>
<td>-8.00 ± 5.69</td>
<td>0.29</td>
<td>0.4 ± 0.4</td>
</tr>
<tr>
<td>15</td>
<td>339.00 ± 14.80</td>
<td>-5.67 ± 3.48</td>
<td>0.25</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td>15</td>
<td>371.33 ± 15.72</td>
<td>-1.33 ± 1.33</td>
<td>0.42</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dose interval (min)</th>
<th>Baseline</th>
<th>Change</th>
<th>P</th>
<th>Latency (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>-9.67 ± 7.75</td>
<td>0.34</td>
<td>0.8 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>-2 ± 1.15</td>
<td>0.23</td>
<td>0.5 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-3.67 ± 3.67</td>
<td>0.42</td>
<td>0.6 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-0.67 ± 0.67</td>
<td>0.42</td>
<td>0.4 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>

### b) 
**Δ MABP (mmHg)**

<table>
<thead>
<tr>
<th>Dose interval (min)</th>
<th>Baseline</th>
<th>Change</th>
<th>P</th>
<th>Latency (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>100.78 ± 12.03</td>
<td>-12.89 ± 6.78</td>
<td>0.2</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>15</td>
<td>99.11 ± 10.18</td>
<td>-14.56 ± 7.94</td>
<td>0.21</td>
<td>0.7 ± 0.5</td>
</tr>
<tr>
<td>15</td>
<td>102.11 ± 14.88</td>
<td>-8.22 ± 4.11</td>
<td>0.18</td>
<td>0.7 ± 0.4</td>
</tr>
<tr>
<td>15</td>
<td>101.78 ± 7.48</td>
<td>-8.67 ± 3.60</td>
<td>0.2</td>
<td>1.1 ± 0.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dose interval (min)</th>
<th>Baseline</th>
<th>Change</th>
<th>P</th>
<th>Latency (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>96.89 ± 11.78</td>
<td>-11.44 ± 5.76</td>
<td>0.19</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>5</td>
<td>97.78 ± 11.67</td>
<td>-8.67 ± 4.40</td>
<td>0.19</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>2</td>
<td>92.89 ± 12.65</td>
<td>-7.22 ± 4.45</td>
<td>0.25</td>
<td>0.5 ± 0.5</td>
</tr>
<tr>
<td>1</td>
<td>85.44 ± 13.12</td>
<td>0.00 ± 0.00</td>
<td>0.0 ± 0.0</td>
<td></td>
</tr>
</tbody>
</table>

### c) 
**Δ breath-breath interval (s)**

<table>
<thead>
<tr>
<th>Dose interval (min)</th>
<th>Baseline</th>
<th>Change</th>
<th>P</th>
<th>Latency (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>1.45 ± 0.32</td>
<td>2.02 ± 0.97</td>
<td>0.17</td>
<td>7.7 ± 0.8</td>
</tr>
<tr>
<td>15</td>
<td>1.41 ± 0.22</td>
<td>3.39 ± 0.63</td>
<td>0.03</td>
<td>7.7 ± 0.4</td>
</tr>
<tr>
<td>15</td>
<td>1.36 ± 0.23</td>
<td>3.27 ± 1.92</td>
<td>0.23</td>
<td>6.6 ± 0.2</td>
</tr>
<tr>
<td>15</td>
<td>1.52 ± 0.43</td>
<td>8.66 ± 1.67</td>
<td>0.04</td>
<td>3.8 ± 1.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dose interval (min)</th>
<th>Baseline</th>
<th>Change</th>
<th>P</th>
<th>Latency (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1.54 ± 0.35</td>
<td>11.10 ± 1.24</td>
<td>0.01</td>
<td>3.2 ± 1.5</td>
</tr>
<tr>
<td>5</td>
<td>1.35 ± 0.25</td>
<td>10.40 ± 2.52</td>
<td>0.05</td>
<td>3.5 ± 1.2</td>
</tr>
<tr>
<td>2</td>
<td>1.42 ± 0.23</td>
<td>13.38 ± 2.82</td>
<td>0.04</td>
<td>4.3 ± 0.5</td>
</tr>
<tr>
<td>1</td>
<td>1.49 ± 0.25</td>
<td>9.33 ± 3.09</td>
<td>0.09</td>
<td>2.3 ± 0.4</td>
</tr>
</tbody>
</table>
Figure i  Digitised photomicrograph illustrating Fos positive labelling within the NTS of representative sections (a) at the level of obex and (b) approximately 0.5mm rostral to obex from a stimulated group animal.

Arrows indicate examples of Fos positive labelling.

Abbreviations

AP  area postrema
DVN  dorsal vagal motor nucleus
NTS  nucleus tractus solitarii
Ts  solitary tract
Figure ii  Digitised photomicrograph illustrating Fos positive labelling within (a) the intermediate ventrolateral medulla and (b) the rostral ventrolateral medulla of representative sections from a stimulated group animal.

Arrows indicate examples of Fos positive labelling.

Abbreviations

IVLM  intermediate ventrolateral medulla
LPGi  lateral paragigantocellular nucleus
LRT  lateral reticular nucleus
NA   nucleus ambiguus
RVLMrostral ventrolateral medulla
Figure iii  Digitised photomicrograph illustrating Fos positive labelling within (a) the nucleus raphe magnus, (b) the locus coeruleus and (c) A5 noradrenergic cell area of representative sections from a stimulated group animal.

Arrows indicate examples of Fos positive labelling.

Abbreviations

7n    facial nerve or its root
A5    A5 noradrenaline cells
BC    brachium conjunctiva
LC    locus coeruleus
Me5   mesencephalic trigeminal nucleus
MPB   medial parabrachial nucleus
NRM   nucleus raphe magnus
py    pyramidal tract
Figure iv  Digitised photomicrograph illustrating Fos positive labelling within (a) the pontine parabrachial nucleus, (b) the mesencephalic parabrachial nucleus and (c) the Kölliker Fuse nucleus of representative sections from a stimulated group animal.

Arrows indicate examples of Fos positive labelling.

Abbreviations

BC  brachium conjunctiva
KF  kölliker-fuse nucleus
LPBC central lateral parabrachial nucleus
LPBE external lateral parabrachial nucleus
Me5  mesencephalic trigeminal nucleus
MPB  medial parabrachial nucleus
MPBE external medial parabrachial nucleus
Figure v  Digitised photomicrograph illustrating Fos positive labelling within (a) the periaqueductal grey approximately 8.30mm caudal to bregma, (b) the paramedian raphe nucleus and (c) the dorsal raphe nucleus of representative sections from a stimulated group animal.

Arrows indicate examples of Fos positive labelling.

Abbreviations
Aq  aqueduct
DRN  dorsal raphe nucleus
Me5  mesencephalic trigeminal nucleus
Mlf  medial longitudinal fasciculus
MRN  median raphe nucleus
PAG  peraqueductal grey
PMR  paramedian raphe nucleus
RtTg  reticulotegmental pontine nucleus
Figure vi  Digitised photomicrograph illustrating Fos positive labelling within (a) the central amygdaloid nucleus, (b) the paraventricular hypothalamic nucleus, and (c) the medial hypothalamus of representative sections from a stimulated group animal.

Arrows indicate examples of Fos positive labelling.

Abbreviations

CNA  central amygdaloid nucleus
DMH  dorsomedial hypothalamic nucleus
PVN  paraventricular hypothalamic nucleus
VMH  ventromedial hypothalamic nucleus