Chapter 1

Literature Review

Chapter 1 Literature review

1.1 Introduction

In the normal adult mammal, arterial blood pressure is held within a tightly controlled range, with little variation during the day. Although hormonal and renal mechanisms are vital for chronic management of arterial blood pressure, short term minute to minute regulation is largely due to central neural mechanisms generating normal sympathetic vasomotor tone leading to regional increases in vascular resistance and altering heart rate (Sun, 1995; Pilowsky and Goodchild, 2002; Dampney *et al.*, 2003). Several regions within the central nervous system are essential for the normal regulation of the sympathetic nervous system, with the medulla oblongata playing a particularly important role. Many other vital autonomic functions such as generation of normal respiratory rhythm and cardiorespiratory reflexes, such as the baroreflex, sympathetic chemoreflex and somatosympathetic reflex are also integrated within the medulla. Tachykinins (such as substance P) are short peptides that are found throughout the mammalian central nervous system and are implicated in many of these vital brainstem autonomic functions.

In this chapter I review the sympathetic nervous system, brainstem autonomic reflexes such as the baroreflex, sympathetic chemoreflex, somatosympathetic reflex and the central control of respiration. In the second section (beginning section 1.11) the role of tachykinins, in particular substance P and its receptor (the neurokinin-1 (NK1) receptor) in these vital brainstem functions is discussed.

1.2 Sympathetic nerve activity and the RVLM

Under resting conditions there is a basal level of sympathetic activity (sympathetic tone) that is dependent on supraspinal innervation of spinal sympathetic preganglionic neurons (SPNs) (Dembowsky *et al.*, 1985; Dampney, 1994). Acute spinal transection almost completely abolishes sympathetic tone and leads to a fall in blood pressure to about 50mm Hg, the so-called 'spinal level' (Alexander, 1946). A limited number of discrete regions within the supraspinal central nervous system project to spinal sympathetic preganglionic neurons, as demonstrated by transneuronal pseudorabies viral labelling experiments (Strack *et al.*, 1989). The largest of these are the rostral ventrolateral and ventromedial medulla (RVLM and RVMM respectively), the midline medulla, the paraventricular nucleus (PVN) of the hypothalamus, and the A5 cell group of the pons (Strack *et al.*, 1989). Bilateral inactivation or ablation of neurons in the A5 region or midline medulla has little effect on baseline sympathetic nerve activity (SNA) or blood pressure (McCall and Harris, 1987; Koshiya and Guyenet, 1994a). Only a modest decrease in blood pressure is seen following bilateral lesions in the RVMM (Varner *et al.*, 1994). The large decrease in SNA and blood pressure seen following inhibition of the PVN with the $GABA_A$ receptor agonist muscimol is attenuated by blockade of excitatory and inhibitory amino acid receptors in the RVLM, suggesting the PVN affects SNA and blood pressure through an RVLM dependent mechanism (Allen, 2002). The RVLM is thus the most important supraspinal region for the maintenance of normal resting sympathetic tone and blood pressure, and for the final integration of brainstem autonomic reflexes that affect the sympathetic nervous system such as the baroreflex (see section 1.5), sympathetic chemoreflex (see section

1.6), and somato-sympathetic reflex (see section 1.7), and the maintenance of normal cerebral vascular tone (see section 1.8).

1.3 Properties of RVLM pre-sympathetic neurons

The RVLM is essential for the maintenance of sympathetic tone, as bilateral but not unilateral ablation or inactivation of RVLM neurons in baroreceptor intact animals leads to profound falls in arterial blood pressure and sympathetic nerve activity to 'spinal' levels (Feldberg and Guertzenstein, 1972; Dampney and Moon, 1980; Willette *et al.*, 1983; Sun and Reis, 1996). In baroreceptor denervated animals, unilateral RVLM inactivation leads to significant falls in blood pressure and sympathetic nerve activity, suggesting that the baroreflex plays a role in maintaining sympathetic tone (see sections 1.4 and 1.5) (Horiuchi and Dampney, 1998).

In the RVLM, there is a cell column which lies ventral to the ventral respiratory group (VRG) and caudal to the inferior pole of the facial nucleus, where neurons that are spontaneously active and powerfully inhibited by elevations in arterial blood pressure (and thus termed 'barosensitive') are found (Brown and Guyenet, 1984; Brown and Guyenet, 1985; Lipski *et al.*, 1995b; Lipski 1998; Dembowsky and McAllen, 1990; Zagon and Spyer, 1996; Lipski *et al.*, 1998; Verberne *et al.*, 1999). Barosensitive neurons at the rostral pole of this cell column (0-300μm caudal to facial nucleus inferior pole) project to the spinal cord (see below) whereas barosensitive neurons found more caudally (i.e. 600-800μm caudal to the facial nucleus) project rostrally to the hypothalamus and basal forebrain (Sawchenko and Swanson, 1982; Tucker *et al.*,

1987; Petrov *et al.*, 1993; Otake *et al.*, 1995; Verberne *et al.*, 1999). The rostrally projecting barosensitive neurons will not be discussed further in this review.

RVLM presympathetic neurons project to the spinal cord, as demonstrated by antidromic spinal cord stimulation in the region of the intermediolateral cell column of the spinal cord (IML) while recording single barosensitive RVLM neurons intracellularly (Lipski *et al.*, 1995a; Lipski *et al.*, 1995b) and extracellularly (Sun and Spyer, 1991a; Sun and Spyer, 1991b; Schreihofer and Guyenet, 1997; Verberne *et al.*, 1999). Retrograde tracing experiments have demonstrated that presympathetic RVLM neurons project to the IML (Amendt *et al.*, 1978; Amendt *et al.*, 1979; Goodchild *et al.*, 1984; Makeham *et al.*, 2001). It is thought that the RVLM presympathetic neurons projecting to the IML innervate vasoconstrictor sympathetic preganglionic neurons (SPNs) (Ross *et al.*, 1981; Polson *et al.*, 1992). Definitive evidence for a monosynaptic connection between bulbospinal RVLM presympathetic neurons and IML vasoconstrictor SPNs has not been provided, however indirect evidence suggests this is the case. In cats, there is a close correlation (i.e. sharp narrow peak with 2msec bin width on cross-correlograms) between firing of some pairs of medullary barosensitive neurons and filaments of the cervical sympathetic trunk (McAllen *et al.*, 1994). Further, electrical stimulation of the RVLM results in excitatory post-synaptic potentials in identified thoracic SPNs, an effect abolished by AMPA/kainate antagonists (Deuchars *et al.*, 1995). Curiously, inhibitory post- synaptic potentials have also been identified in thoracic SPNs following RVLM electrical stimulation, the source of which is unknown (Deuchars *et al.*, 1997).

1.3.1 Morphology and discharge properties

Intracellular labelling has demonstrated that barosensitive bulbospinal neurons of the RVLM have cell bodies with a mean diameter of approx. 28μm, each with 3 to 8 primary dendrites that branch 2 to 4 times and extend 300-650μm (Lipski *et al.*, 1995b). A large number of these dendrites project to the ventral medullary surface and terminate immediately beneath the pia mater, the significance of which is uncertain (Lipski *et al.*, 1995b). The axons of these neurons project dorsomedially to the dorsomedial medulla where they turn caudally and project to the spinal cord (Lipski *et al.*, 1995b). There is no significant difference in the morphological characteristics of the cell body or dendrites between catecholaminergic (e.g. C1) and non-catecholaminergic (non-C1) neurons (Lipski *et al.*, 1995b). Differences in axonal fibre types (unmyelinated or myelinated) have been demonstrated (see below) (Morrison *et al.*, 1988; Schreihofer and Guyenet, 1997).

RVLM presympathetic neurons are spontaneously active and barosensitive (Brown and Guyenet, 1984; Brown and Guyenet, 1985; Lipski *et al.*, 1995b; Lipski *et al.*, 1998; Verberne *et al.*, 1999). Falls in blood pressure lead to an increase in firing rates up to a point where no further increase in firing rate occurs (between 10 and 30 spikes/ sec.), at a mean arterial pressure (MAP) of approximately 60-85 mm Hg (Brown and Guyenet, 1985). Increases in arterial blood pressure from this level decrease neuronal firing rate in an almost linear fashion up to a MAP of 145-155 mm Hg, where neuronal firing is completely inhibited (see section 1.5) (Brown and Guyenet, 1984; Brown and Guyenet, 1985). Pulse triggered neuronal activity histograms demonstrate pulse modulation of unit activity only when a moderate degree of baroreceptor mediated inhibition is present (i.e. MAP > 100mm Hg) (Verberne *et al.*, 1999), with maximal

inhibition occurring 75-100msec after the R-wave (Brown and Guyenet, 1985; Miyawaki *et al.*, 1997).

Bulbospinal barosensitive neurons of the RVLM are a heterogeneous cell population when firing rates and axonal conduction velocities are considered. Two groups of bulbospinal barosensitive neurons are described, those with axonal conduction velocities less than 1m/sec and therefore with predominantly unmyelinated axons, and those with faster conduction velocities (e.g. 2-8m/sec) which are myelinated neurons (Brown and Guyenet, 1984; Brown and Guyenet, 1985; Morrison *et al.*, 1988; Verberne *et al.*, 1999). Schreihofer and Guyenet demonstrated in 1997 that all RVLM bulbospinal barosensitive neurons with conduction velocities in the C-fibre range (i.e. conduction velocities less than 1m/sec) express tyrosine hydroxylase (TH) and are C1 neurons, whereas those with faster conduction velocities (1-7m/sec) were only lightly TH- immunoreactive (50%) or do not express TH (non-C1 neurons) (Schreihofer and Guyenet, 1997). Further, those neurons with axonal conduction velocities in the C-fibre range (less than 1m/sec) have a significantly lower neuronal firing rate than neurons with higher conduction velocities (Schreihofer and Guyenet, 1997), agreeing with earlier work by Brown and Guyenet in 1985 (Brown and Guyenet, 1985).

There is anatomical evidence that bulbospinal barosensitive C1 neurons contain two subpopulations- those with unmyelinated and those with lightly myelinated axons (Morrison *et al.*, 1988). Consistent with this, bulbospinal barosensitive C1 neurons contain two distinct subpopulations with fast and slow axonal conduction velocities, which each have significantly different neuronal firing rates (Schreihofer and Guyenet, 1997). It must be noted that in contrast to these earlier findings, Verberne et al in 1999 did not demonstrate any significant difference in conduction velocities or firing rates

between C1 and non-C1 barosensitive bulbospinal neurons of the RVLM, although the number of neurons examined was limited (9 and 5 respectively) (Verberne *et al.*, 1999). The exact nature of these different subpopulations remains to be determined.

1.3.2 Neurotransmitter and receptor content

The bulbospinal RVLM neurons involved in the baroreflex and generation of sympathetic tone are excitatory and release glutamate in the IML as their primary fast neurotransmitter (Mills *et al.*, 1988; Minson *et al.*, 1991; Llewellyn-Smith IJ *et al.*, 1998; Stornetta *et al.*, 2002a; Stornetta *et al.*, 2002b; Morrison, 2003). Although glutamate is thought to be the primary fast neurotransmitter in these neurons, they are in fact a heterogeneous cell population with multiple other neurotransmitters expressed within subgroups (Figure 1.1). For instance, approximately 70% of electrophysiologically identified barosensitive bulbospinal RVLM neurons are catecholaminergic (i.e. express phenylethanolamine-*N*-methyltransferase (PNMT)) and are termed 'C1' neurons (Schreihofer and Guyenet, 1997; Verberne *et al.*, 1999). Interestingly, bulbospinal C1 neurons are not essential for the maintenance of normal resting sympathetic tone and blood pressure, as destruction of up to 80% of these neurons with selective neurotoxins has no significant effect on these parameters (Guyenet *et al.*, 2001; Madden and Sved, 2003). The precise role of C1 neurons in the normal control of sympathetic activity remains to be determined.

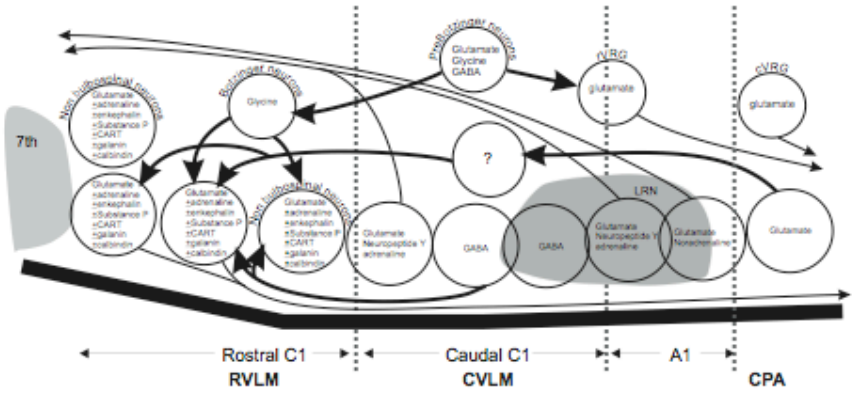

Figure 1.1. Schematic parasagittal section through the ventral medulla indicating the complex neuronal cell groups, their connections and neurotransmitters. The relative rostrocaudal position (not to scale) is given by the vertical dotted lines- RVLM, rostral ventrolatral medulla; CVLM, caudal ventrolateral medulla; A1 region and ; CPA, caudal pressor area. The thick solid lines indicate intramedullary connections and the thin solid lines distant projections. Adapted from Pilowsky and Goodchild 2002.

Many other putative neurotransmitters are found within subpopulations of bulbospinal, barosensitive RVLM neurons (Fig. 1.1). These include substance P (see section 1.11.8.3) (Pilowsky *et al.*, 1986; Li *et al.*, 2005), neuropeptide Y (Polson *et al.*, 1992; Tseng *et al.*, 1993; Minson *et al.*, 1994; Stornetta *et al.*, 1999), calbindin (Miura *et al.*, 1996; Goodchild *et al.*, 2000), preprogalanin (Sweerts *et al.*, 1999), cocaine and amphetamine related transcript (CART) (Dun *et al.*, 2002), and opiates as demonstrated by enkephalin / preproenkephalin content (Polson *et al.*, 1992; Boone and Corry, 1996; Stornetta *et al.*, 2001). The exact role of these substances is uncertain, but given that glutamate is the most likely primary neurotransmitter (Mills *et al.*, 1988; Minson *et al.*, 1991; Llewellyn-Smith IJ *et al.*, 1998; Stornetta *et al.*, 2002a; Stornetta *et al.*, 2002b; Morrison, 2003), they are most likely modulatory neurotransmitters.

Along with multiple neurotransmitters, the bulbospinal presympathetic neurons of the RVLM are made up of heterogeneous subpopulations that express different receptors. Receptors such as $GABA_A$ and $GABA_B$ (Avanzino *et al.*, 1994; Li and Guyenet, 1996), alpha 2A-adrenergic receptors (Hayar and Guyenet, 2000), mu opioid (Aicher *et al.*, 2001), and angiotensin 1A receptors (Li and Guyenet, 1996; Tagawa *et al.*, 1999; Allen, 2001). Chapter 4 investigates whether the receptor for substance P, the neurokinin-1 receptor, is expressed by bulbospinal RVLM neurons. The function of receptors is the source of much current investigation. The best understood are the GABA receptors, being essential for the inhibition of RVLM presympathetic neurons by both baroreceptor dependent and independent mechanisms (see section 1.5.3) (Li and Guyenet, 1995; Sved *et al.*, 2000). Other receptors may be responsible for differential reflex responses integrated within bulbospinal presympathetic RVLM

neurons. For instance, activation of 5-HT_{1A} or delta opioid receptors in the RVLM via local microinjection of specific agonists reduces sympathetic nerve activity and significantly attenuates the somato-sympathetic reflex (see section 1.7), but does not affect the sympathetic baroreflex (Miyawaki *et al.*, 2001; Miyawaki *et al.*, 2002a). In contrast, activation of RVLM mu-opioid receptors in the same experimental paradigm attenuates the sympathetic baroreflex without affecting the somato-sympathetic reflex (Miyawaki *et al.*, 2002a). This suggests subpopulations of bulbospinal presympathetic RVLM neurons are being activated differentially by different reflex stimuli. This apparent functional specificity is investigated further in chapter 5, with sympathetic nerve activity, sympathetic baroreflex, somato-sympathetic reflex and sympathetic chemoreflex responses to activation and inactivation of RVLM substance P receptors (the neurokinin-1 receptor) being determined.

1.4 Origin of the sympathetic tone

The exact origin of basal sympathetic tone is not yet fully understood. The two major competing theories are the 'pacemaker' and 'network' theories.

1.4.1 The Pacemaker Theory

The pacemaker theory suggests that RVLM presympathetic neurons maintain their level of tonic activity by generating intrinsic pacemaker action potentials. This was initially suggested by an *in vitro* intracellular recording study in rat brainstem slices (Sun *et al.*, 1988b). In an *in vitro* intracellular recording study in 1995 Kangrga and

Loewy suggested that bulbospinal C1 neurons could be divided into pacemaker and non-pacemaker neurons, and that the pacemaker potentials derived from a persistent sodium current (Kangrga and Loewy, 1995). A similar result was obtained by Li et al, in the same year (Li *et al.*, 1995). These *in vitro* data from neonatal brain slices were later challenged by *in vivo* evidence that RVLM barosensitive bulbospinal neurons have irregular firing patterns and display synaptic activity (excitatory (EPSP) and inhibitory (IPSP) post synaptic potentials) (Lipski *et al.*, 1996). Further, individual action potentials in these neurons are usually preceded by identifiable fast EPSPs, and no intrinsic pacemaker properties were identified in this study (Lipski *et al.*, 1996). This suggests that the primary mechanism for the maintenance of tonic activity in RVLM presympathetic neurons *in vivo* is via synaptic inputs. The issue has been clouded by recent evidence that basal sympathetic tone may result, in part, from nickel-chloride sensitive ion channels (perhaps low voltage activated T-type Ca^{2+} channels) on RVLM presympathetic neurons (Miyawaki *et al.*, 2003).

1.4.2 The Network Theory

The network theory is currently thought to be the most likely mechanism for the generation of basal sympathetic tone. This theory suggests that the tonic activity of RVLM presympathetic neurons derives from the sum of excitatory and inhibitory synaptic inputs from neurons in other regions.

RVLM neurons are essential for the maintenance of sympathetic tone. Bilateral ablation or inactivation of RVLM neurons leads to a fall in blood pressure and sympathetic nerve activity to ‘spinal’ levels (i.e. the level attained when all supraspinal

inputs have been removed by C1 level spinal transection) (Feldberg and Guertzenstein, 1972; Dampney and Moon, 1980; Sun and Reis, 1996; Sakima *et al.*, 2000).

The RVLM is thought to receive tonic excitatory inputs, as bilateral RVLM microinjections of ionotropic glutamate receptor antagonists result in falls in sympathetic nerve activity and blood pressure in cats (Abrahams *et al.*, 1994; Barman *et al.*, 2000) and blood pressure in rabbits (Blessing and Namath, 2000). The functional significance of this glutamatergic input is uncertain, as bilateral RVLM microinjection of the ionotropic glutamate receptor antagonist kynurenate result in very little, if any, change in sympathetic nerve activity or blood pressure in the anaesthetized rat (Kiely and Gordon, 1994; Ito and Sved, 1997; Tagawa *et al.*, 1999).

Tonic inhibitory inputs to the RVLM are also present, the most important of which is thought to be GABAergic input, although RVLM microinjection of glycine also results in sympatho-inhibition and a decrease in blood pressure (Sakima *et al.*, 2000). RVLM presympathetic neurons are inhibited by a GABA dependent mechanism (Willette *et al.*, 1983; Sun and Guyenet, 1985; Sun and Guyenet, 1987; Blessing, 1988). Microinjection of the $GABA_A$ receptor antagonist bicuculline in the RVLM results in large increases in blood pressure and sympathetic nerve activity (Willette *et al.*, 1983; Willette *et al.*, 1984a; Sun and Guyenet, 1987; Dampney *et al.*, 1988; Smith and Barron, 1990; Miyawaki *et al.*, 2002b).

In 1997, Ito and Sved inhibited the caudal ventrolateral medulla (CVLM) by microinjecting muscimol (a long lasting $GABA_A$ receptor agonist), resulting in disinhibition of the RVLM and a large increase in blood pressure (Ito and Sved, 1997). Subsequent microinjection of kynurenate into the RVLM caused blood pressure to fall to spinal levels, suggesting that net sympathetic tone is maintained by a balance of

excitatory (probably glutamatergic) and inhibitory inputs to the RVLM (Ito and Sved, 1997; Sved *et al.*, 2001). Disinhibiton by bilateral RVLM bicuculline microinjection results in a large increase in sympathetic nerve activity and blood pressure (Miyawaki *et al.*, 2002b). Miyawaki et al demonstrated that subsequent RVLM kynurenate microinjection results in a decrease in sympathetic nerve activity and blood pressure, but not to spinal levels, in contrast to the findings from Ito and Sved (Ito and Sved, 1997; Miyawaki *et al.*, 2002b). One possibility to explain this discrepancy is that muscimol in the CVLM may also have inhibited excitatory inputs from the CVLM to the RVLM, and that the tonic excitatory input is not wholly due to glutamate (Ito and Sved, 1997). In support of this, Horiuchi et al found that after CVLM inhibition with muscimol, removal of tonic excitatory amino acid drive to the RVLM with kynurenate results in only modest falls in blood pressure and no effect on renal sympathetic nerve activity (Horiuchi *et al.*, 2004). This suggests that the excitatory component of the tonic activity of RVLM presympathetic neurons is either due to excitatory inputs that use non-EAA (excitatory amino acid) receptors or possibly intrinsic autoactivity that is unmasked by blockade of all EAA receptor-mediated inputs, as hypothesized by Lipski et al (Lipski *et al.*, 1996).

Sympathetic tone under resting conditions is most likely mediated by the balance between inhibitory (predominantly GABAergic) and excitatory (predominantly non-EAA with an uncertain contribution from glutamate) synaptic inputs.

1.5 Sympathetic Baroreflex

1.5.1 Baroreceptor inputs to Nucleus Tractus Solitarius (NTS)

The baroreceptor reflex arc begins with the baroreceptor afferent neurons, located in the carotid sinus, aortic arch, atria, and ventricles. These baroreceptors are stimulated by increases in arterial pressure over a wide range (e.g. 40-120mmHg), allowing the baroreceptor signal to describe a wide variation in pressures (Fidone and Sato, 1969; Angell-James, 1971; Brown *et al.*, 1976; Andresen, 1984; Seagard *et al.*, 1990; Seagard *et al.*, 1993). The baroreceptor neurons are 'pseudo-unipolar' neurons that have their cell bodies in the petrosal and nodose ganglia, and a single axon which splits to form an efferent projection and a projection with afferent terminals in the vessel wall (Kumada *et al.*, 1990). The efferent projections from the baroreceptor neurons travel centrally in the IXth and Xth cranial nerves. In the rat and rabbit, the baroreceptor information is transmitted by the aortic depressor nerve, which terminates in the NTS (Lipski *et al.*, 1975; Panneton and Loewy, 1980; Wallach and Loewy, 1980; Donoghue *et al.*, 1981; Ciriello *et al.*, 1981b; Donoghue *et al.*, 1982; Ciriello, 1983; Donoghue *et al.*, 1984). Multiple subnuclei within the NTS receive these baroreceptor afferent fibres, including the commissural subnucleus; the medial, lateral and dorsolateral subnuclei at the level of the obex; and the medial, lateral and ventrolateral subnuclei at the level rostral to the obex (Ciriello *et al.*, 1981a). The aortic nerve in the rat and rabbit contains only baroafferent fibres (Sapru and Krieger, 1977; Sapru *et al.*, 1981; Numao *et al.*, 1985; Dworkin *et al.*, 2000; Petiot *et al.*, 2001), however in the cat chemoreceptor fibres are present (Baccelli *et al.*, 1964).

The baroreceptor information is carried in the rat aortic depressor nerve in A-fibre (myelinated) and C- fibre (unmyelinated) components (Thoren and Jones, 1977; Brown *et al.*, 1978; Fan and Andresen, 1998; Fan *et al.*, 1999). Under experimental conditions, low voltage electrical aortic depressor nerve stimulation (i.e. approx. 5V at 10 Hz) activates the A-fibres, with higher voltages (up to about 20V) required to recruit C-fibres in the rat (Fan and Andresen, 1998; Fan *et al.*, 1999).

The NTS is essential for the transmission of baroreceptor information, since lesions of the NTS completely abolish all baroreflexes in rats (Akemi *et al.*, 2001), cats (Nathan and Reis, 1977), and humans (Biaggioni *et al.*, 1994). Increases in arterial blood pressure result in an increase in firing rate of extracellularly recorded NTS neurons that receive monosynaptic input from aortic depressor nerve fibres in the rat (Zhang and Mifflin, 2000).

Which neurotransmitters convey the baroreceptor information to the NTS? Initially it was thought that the major neurotransmitter was substance P, however, this is now considered more likely to exert a modulatory neurotransmitter role in the NTS (for discussion of NTS substance P in the baroreflex, see section 1.11.8.1). The most likely candidate to be the major neurotransmitter of baroreceptor information in the NTS is the excitatory amino acid, glutamate (Talman *et al.*, 1980; Perrone, 1981; Granata *et al.*, 1984; Guyenet *et al.*, 1987; Kubo and Kihara, 1988a; Kubo and Kihara, 1988c; Lawrence and Jarrott, 1994; Machado *et al.*, 2000). Microinjection of L-glutamate into the NTS simulates baroreceptor activation, with a fall in arterial blood pressure and heart rate (Talman, 1997). Bilateral blockade of excitatory amino acid receptors in the NTS by microinjection of kynurenate abolishes the baroreflex (Talman *et al.*, 1980; Reis *et al.*, 1981; Kubo and Kihara, 1988b; Leone and Gordon, 1989; Talman, 1989;

Sved and Curtis, 1993). The response to excitatory amino acids in the NTS is thought to be heterogeneous, as baroactivated NTS neurons respond to NMDA and non-NMDA receptor antagonists differently (Zhang and Mifflin, 1998a; Yen *et al.*, 1999; Machado, 2001). Further, some neurons in the NTS retain their barosensitivity after application of ionotropic EAA antagonists, a result suggesting another receptor or transmission system is active in these neurons (Zhang and Mifflin, 1998a). Although ionotropic EAA receptors are thought to play the major role, there is evidence that metabotropic glutamate receptors can also modulate NTS neurotransmission (Pawloski-Dahm and Gordon, 1992; Liu *et al.*, 1998; Pamidimukkala and Hay, 2001).

Other substances, although not the primary neurotransmitters for baroreceptor information in the NTS, are involved in the modulation of the signal. These are substances such as substance P (see section 1.11.8.1) angiotensin II (Campagnole-Santos *et al.*, 1988; Paton and Kasparov, 1999), serotonin (N'Diaye *et al.*, 2001), catecholamines (Sved *et al.*, 1992), GABA (Sved and Tsukamoto, 1992; Suzuki *et al.*, 1993; Zhang and Mifflin, 1998b), neuropeptide Y (Kubo and Kihara, 1990), nitric oxide (Machado and Bonagamba, 1992; Zanzinger *et al.*, 1995a; Paton *et al.*, 2001), purines (Mosqueda-Garcia *et al.*, 1989; Phillis *et al.*, 1997), and opioids (Li *et al.*, 1996). These will not be discussed in depth in this review, except for substance P, which is described in detail in section 1.11.8.1.

In summary, baroreceptor information arrives in the NTS from cranial nerves IX and X and excites NTS neurons, predominantly via an EAA (presumably glutamate), which acts differentially on NMDA and non-NMDA receptors. These effects are modulated by a myriad of neurotransmitters, as mentioned above.

A schematic diagram indicating the major pathways involved in the sympathetic baroreflex is shown in Figure 1.2

1.5.2 NTS to CVLM

The CVLM is an essential link in the baroreceptor pathway. Lesions of the CVLM abolish depressor responses (Murugaian *et al.*, 1989), and the sympathoinhibition seen in the splanchnic nerve (Cravo *et al.*, 1991) following aortic depressor nerve stimulation in the rat. Chemical inhibition of neurotransmission in the CVLM blocks baroreflex sympathoinhibition and vasodepressor responses to NTS stimulation (Agarwal *et al.*, 1990; Kubo *et al.*, 1991). The baroreceptor pathway from the NTS to the CVLM is excitatory, as blocking excitatory neurotransmission in the CVLM with kynurenate or NMDA and AMPA/kainate receptor antagonists abolishes the sympathetic baroreflex (Somogyi *et al.*, 1989; Kubo *et al.*, 1991; Miyawaki *et al.*, 1997). Neurons within the NTS project to the CVLM, and those projections form close appositions with CVLM neurons that project to the RVLM (Yu and Gordon, 1996).

The CVLM is thought to be a sympathoinhibitory region as lesions of the CVLM (Murugaian *et al.*, 1989; Cravo *et al.*, 1991; Cravo and Morrison, 1993), inhibition with muscimol (Blessing and Reis, 1983; Ito and Sved, 1997; Horiuchi *et al.*, 2004), and kynurenate (Guyenet *et al.*, 1987) all produce long lasting pressor and sympathoexcitatory responses. Further, stimulation of the CVLM results in depressor and sympathoinhibitory responses (Feldberg and Guertzenstein, 1976; Willette *et al.*, 1987; Blessing, 1988; Agarwal *et al.*, 1989).

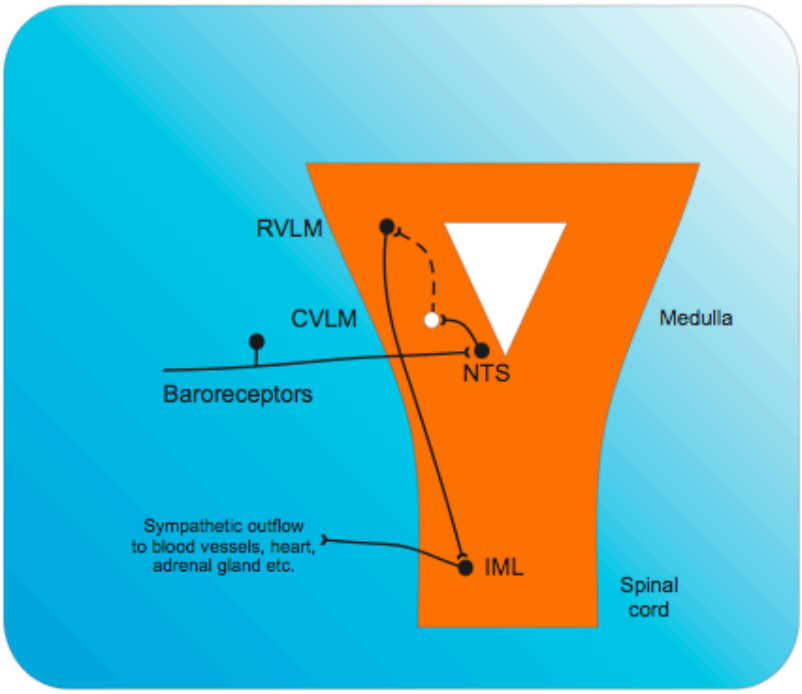

Figure 1.2. Schematic diagram of the baroreflex pathway. Filled circles indicate excitatory neurons and open circles inhibitory neurons. Continuous lines indicate excitatory pathways and dashed lines indicate inhibitory pathways. RVLM, rostral ventrolateral medulla; CVLM, caudal ventrolateral medulla; NTS, nucleus tractus solitarius; IML, intermediolateral cell column. Adapted from Pilowsky and Goodchild, 2002.

1.5.3 CVLM to RVLM

The CVLM contains barosensitive neurons that are sympathoinhibitory and essential components of the baroreceptor reflex arc (see above section 1.5.2). The primary neurotransmitter within these neurons is thought to be GABA (Blessing, 1988; Dampney *et al.*, 1988; Li *et al.*, 1991; Minson *et al.*, 1997). Barosensitive inhibitory neurons of the CVLM project monosynaptically to bulbospinal sympathoexcitatory neurons in the RVLM bilaterally (Agarwal and Calaresu, 1991; Jeske *et al.*, 1995). Activation of the CVLM with L-glutamate inhibits, and CVLM inhibition with GABA agonists excites RVLM bulbospinal sympathoexcitatory neurons recorded extracellularly (Li *et al.*, 1991). Bicuculline, a $GABA_A$ receptor antagonist, in the RVLM blocks the baroreceptor mediated inhibition of RVLM sympathoexcitatory neurons (Sun and Guyenet, 1985).

Although barosensitive CVLM inhibitory neurons are a vital component of the arterial baroreflex, there are also baro-*insensitive* neurons in the CVLM that are tonically active and inhibit RVLM presympathetic neurons (Cravo and Morrison, 1993; Drolet *et al.*, 1993; Sved *et al.*, 2000). Dampney et al showed that following baroreceptor denervation or disruption of the baroreceptor reflex arc with NTS lesions, bicuculline microinjected into the RVLM still resulted in a large increase in arterial blood pressure, suggesting GABA as the neurotransmitter within these baroinsensitive inhibitory neurons (Dampney *et al.*, 1988).

In addition to the GABAergic inhibitory (both barosensitive and baroinsensitive) projections from the CVLM to the RVLM, there is some evidence for an excitatory projection from the CVLM to the RVLM (Campos and McAllen, 1999; Natarajan and Morrison, 2000). Activation of a region of the brainstem at the level of the pyramidal

decussation known as the caudal pressor area (CPA) results in a robust pressor and sympathoexcitatory response (Natarajan and Morrison, 2000). Blockade of EAAs in the CVLM abolishes the pressor and sympathoexcitatory effects of CPA disinhibition with bicuculline, suggesting the CVLM is the site of an essential interneuron (Natarajan and Morrison, 2000). The idea of an excitatory projection from the CVLM to the RVLM mediating CPA induced sympatho-excitation is contradicted by a study by Horiuchi and Dampney in 2002 where the fall in arterial blood pressure and sympathetic nerve activity seen following inhibition of the CPA was blocked by RVLM GABA receptor blockade (Horiuchii and Dampney, 2002). This suggests the sympathoexcitation following CPA activation is due to RVLM disinhibition rather than RVLM glutamatergic excitation. Blockade of glutamate receptors within the RVLM does not alter the depressor and sympathoinhibitory effects of CPA inhibition, again suggesting an excitatory CVLM to RVLM interneuron is not essential for the CPA induced alterations in blood pressure or sympathetic nerve activity (Campos *et al.*, 1994; Horiuchii and Dampney, 2002).

1.5.4 RVLM projections

Barosensitive neurons in the CVLM project monosynaptically to RVLM bulbospinal neurons which are sympathoexcitatory (Agarwal and Calaresu, 1991; Jeske *et al.*, 1995). Bulbospinal RVLM presympathetic neurons are inhibited by baroreceptor activation such as aortic depressor nerve stimulation or elevations in blood pressure *in vivo* when recorded intracellularly (Lipski *et al.*, 1995a) or extracellularly (Schreihofer and Guyenet, 1997; Verberne *et al.*, 1999). The baroreceptor mediated inhibition of bulbospinal RVLM presympathetic neurons is mediated by GABA, as blockade of

RVLM $GABA_A$ receptors abolishes the sympathoinhibition seen following CVLM activation (Sun and Guyenet, 1985).

As previously mentioned, approximately 70% of electrophysiologically identified bulbospinal barosensitive neurons of the RVLM are catecholaminergic (Schreihofer and Guyenet, 1997; Verberne *et al.*, 1999). These barosensitive C1 neurons, although not essential for maintenance of resting sympathetic tone, are involved in the baroreflex as evidenced by the approximately 40% reduction in baroreflex gain following chemical depletion of these bulbospinal catecholaminergic neurons (Schreihofer and Guyenet, 2000; Guyenet *et al.*, 2001). It must be noted that Madden et al have shown that in awake rats with >80% C1 neuronal depletion, there is a slight decrease in resting blood pressure (approx 10mmHg.), and significant attenuation of the sympathoexcitatory responses to baroreceptor unloading, chemoreceptor activation and electrical stimulation of the sciatic nerve (Madden and Sved, 2003).

1.6 Chemoreflex

1.6.1 Chemoreceptor projections to the brainstem

The arterial chemoreceptors provide short term information about the chemical composition of the arterial blood and are thought to be type 1 glomus cells in the aortic and carotid bodies (De Kock, 1951; De Kock, 1954; Biscoe, 1971; Pequignot *et al.*, 1984; Hansen, 1985; Fidone *et al.*, 1988; Gonzalez *et al.*, 1994). These type 1 glomus cells respond to hypoxic and hypercapnoeic stimulation by releasing neurotransmitters that excite nearby chemoafferent terminals e.g. carotid sinus nerves (Verna, 1979;

McDonald, 1980; McDonald and Mitchell, 1981; Morgan *et al.*, 1981; Gonzalez *et al.*, 1994; Marshall, 1994; Prabhakar, 2000; Lahiri *et al.*, 2001). The type 1 glomus cells contain and release multiple neurotransmitters into the synaptic cleft activating pre-synaptic (i.e. on the type 1 glomus cells) and post-synaptic (i.e. chemoafferent terminals) such as catecholamines (especially dopamine), acetylcholine, met and leu-enkaphalins, substance P, neuropeptide Y, galanin, calcitonin-gene-related peptide (CGRP), serotonin, and endothelins (Gonzalez *et al.*, 1994; Verna, 1997; Kusakabe *et al.*, 2003). Of these, the most important in the adult are thought to be dopamine and acetylcholine (Gonzalez *et al.*, 1994; Verna, 1997; Fitzgerald, 2000).

The carotid body is innervated by chemoafferent fibres of the carotid sinus with cell bodies in the petrosal ganglion (Kondo, 1976; McDonald and Mitchell, 1981; Martin and Longhurst, 1986; Gonzalez *et al.*, 1994).

The principle sites of termination of chemoafferents arising from the carotid body are the commissural and medial subnuclei of the NTS at the level of the obex (Davies and Edwards, 1973; Lipski *et al.*, 1977; Ciriello *et al.*, 1981b; Housley *et al.*, 1987; Finley and Katz, 1992).

The major neurotransmitter of the chemoreceptor afferents projecting to the NTS is thought to be an excitatory amino acid, probably glutamate (Vardhan *et al.*, 1993a; Mizusawa *et al.*, 1994). Glutamate microinjection into the commissural NTS produces respiratory responses identical to chemoreceptor activation, with an increase in phrenic amplitude, frequency and minute ventilation (Vardhan *et al.*, 1993a; Vardhan *et al.*, 1993b). Glutamate is released in the NTS during hypoxia when measured by *in vivo* microdialysis, an effect abolished by carotid body denervation (Mizusawa *et al.*, 1994). Respiratory and sympathetic components of the chemoreflex are abolished by

excitatory amino acid receptor antagonists (simultaneous blockade of NMDA and non-NMDA receptors) microinjected into the commissural NTS in anaesthetised rats (Vardhan *et al.*, 1993a). The respiratory response to hypoxia is significantly attenuated by commissural NTS pre-treatment with the NMDA-receptor antagonist MK-801 or the ionotropic non-specific glutamate receptor antagonist, kynurenate (Mizusawa *et al.*, 1994). In anaesthetised rats, the pressor response to carotid body stimulation with CO_2 or electrical stimulation of the carotid sinus nerve is significantly attenuated by NTS microinjections of kynurenate (Zhang and Mifflin, 1993).

A recent paper by Machado et al has clouded the issue by suggesting that glutamate may not be the primary neurotransmitter in the NTS, at least in the pressor response to chemoreceptor activation (Machado and Bonagamba, 2005). Microinjections of kynurenate (non-specific EAA antagonist) into the NTS significantly increases mean arterial blood pressure due to disinhibition of RVLM bulbospinal sympathoexcitatory neurons (see section 1.5.1). This may result in reduction in the pressor response to chemoreceptor stimulation. In awake rats, when blood pressure is restored to normal levels with a sodium nitroprusside infusion following commissural NTS kynurenate microinjection, the pressor response to carotid body stimulation is not significantly different from controls (Machado and Bonagamba, 2005). This suggests that a neurotransmitter other than glutamate is involved in the transmission of the pressor component of the chemoreflex in the NTS. The fact that these experiments were conducted in awake, anaesthetic free rats may also explain the discrepancy between this study and the findings of others that the pressor response is attenuated by kynurenate in anaesthetized rats (Zhang and Mifflin, 1993; Vardhan *et al.*, 1993a; Machado and Bonagamba, 2005). It is possible that the respiratory chemoreflex and

pressor chemoreflex are mediated by different neurotransmitters and receptors within the NTS.

1.6.2 Projections from NTS to RVLM

The RVLM is essential for the sympathoexcitatory and pressor responses seen following peripheral chemoreceptor activation. Activation of peripheral chemoreceptors excites RVLM bulbospinal sympathoexcitatory neurons (Sun and Spyer, 1991a; McAllen, 1992; Koshiya *et al.*, 1993; Sun and Reis, 1993a; Sun and Reis, 1993b; Miyawaki *et al.*, 1996a). Bilateral microinjections of kynurenate into the RVLM abolish the sympathoexcitatory and pressor responses following peripheral chemoreceptor activation, suggesting glutamate mediated neurotransmission (Koshiya *et al.*, 1993; Amano *et al.*, 1994; Sun and Reis, 1994a). The EAA receptors responsible were initially thought to be NMDA receptors only (Kubo *et al.*, 1993; Sun and Reis, 1995b). However, following intravenous MK-801 (a non-competitive NMDA receptor antagonist), brief hypoxia still results in pressor and sympathoexcitatory responses, with an increase in firing rate of RVLM bulbospinal presympathetic neurons (Miyawaki *et al.*, 1996a). These effects are abolished by RVLM microinjection of 6-cyano-7-nitroquinoxaline-2, 3-dione (CQNX -a selective AMPA/kainite receptor antagonist)(Miyawaki *et al.*, 1996a). These results suggest both NMDA and AMPA/ kainite receptors are involved in sympathetic chemoreflex signal transduction in the RVLM (Kubo *et al.*, 1993; Sun and Reis, 1995b; Miyawaki *et al.*, 1996a).

Definitive evidence for a monosynaptic connection between electrophysiologically identified chemoreceptor activated NTS neurons and bulbospinal RVLM presympathetic neurons is lacking. There is evidence that electrophysiologically

identified neurons in the commissural nucleus of the NTS that are excited by carotid chemoreceptor activation project to the RVLM (Koshiya and Guyenet, 1996a). Further, some NTS neurons make monosynaptic connections with a subgroup of RVLM bulbospinal sympathoexcitatory neurons, the C1 cell group, when examined ultrastructurally. Whether these neurons are chemo-activated is unknown (Aicher *et al.*, 1996). The possibility exists for a chemoreflex pathway from the NTS to the RVLM that involves local excitatory interneurons.

The large increase in sympathetic nerve activity seen following peripheral chemoreceptor activation is strongly coupled to central respiratory drive, as shown by significant respiratory entrainment with measured phrenic nerve output (Koshiya *et al.*, 1993; Koshiya and Guyenet, 1996b). This suggests that some of the chemoreflex information reaches the RVLM presympathetic neurons via the respiratory network. The respiratory entrainment occurs distal to the NTS, as chemoreceptor activated NTS neurons have no discernable respiratory modulation (Koshiya and Guyenet, 1996a). This respiratory input is not essential as muscimol injection in the region of the pre-Bötzinger complex (the putative rhythm generating region- see section 1.10.6.1) abolishes central respiratory output and the respiratory entrainment, but not the magnitude of peripheral chemoreceptor induced sympathoexcitation (Koshiya and Guyenet, 1996b). The exact source of respiratory related inputs to the RVLM presympathetic neurons is poorly understood and discussed further in section 1.9.

1.6.3 The Pons and the A5 cell group

The A5 cell group are noradrenergic neurons located in the ventrolateral pons and are involved in the sympathetic chemoreflex (Byrum and Guyenet, 1987; Huangfu *et al.*,

1992; Guyenet *et al.*, 1993; Koshiya and Guyenet, 1994b). Hypoxia activates the A5 cell when examined by cFos expression (Hirooka *et al.*, 1997). Further, extracellular recording of putative A5 neurons demonstrates an increase in firing rate and respiratory rhythmicity following hypoxic stimulation of peripheral chemoreceptors (Guyenet *et al.*, 1993). Bilateral microinjections of muscimol ($GABA_A$ receptor agonist) in the A5 region attenuates the sympathetic chemoreflex by 50%, but does not abolish it (Koshiya and Guyenet, 1994c). As previously mentioned, bilateral RVLM microinjections of kynurenate abolish the sympathetic chemoreflex completely (see section 1.6.2), whereas A5 inhibition only attenuates the reflex by approximately 50% (Koshiya and Guyenet, 1994c). Given that A5 noradrenergic neurons project directly to the IML (Loewy *et al.*, 1979; Byrum and Guyenet, 1987), it may be that A5 neurons excite IML SPNs in parallel with RVLM presympathetic neurons, excited by a common pool of RVLM interneurons, although A5 neurons acting by exciting RVLM presympathetic neurons is also consistent with available anatomical evidence (Andrezik *et al.*, 1981; Sun and Guyenet, 1986; Byrum and Guyenet, 1987; Lipski *et al.*, 1995b). Microinjection of anti-DBH-saporin, a catecholaminergic neuron neurotoxin, into the rat thoracic spinal cord results in the destruction of bulbospinal A5 neurons (approx. 98%) and almost complete abolition of the sympathetic chemoreflex (Schreihofer and Guyenet, 2000). This is not specific for A5 neurons as close to 75% of C1 neurons and 84% of C3 neurons were also destroyed in this study, and the sympathetic chemoreflex inhibition may be due to neuronal destruction in these regions (Schreihofer and Guyenet, 2000).

1.6.4 Are RVLM neurons central chemoreceptors?

In peripherally chemodenervated rats, brief episodes of hypoxia result in robust sympathoexcitation and an increase in firing rate of RVLM presympathetic neurons (Sun and Reis, 1994c; Sun and Reis, 1995a). A similar result is seen with cyanide microinjection into the RVLM (simulating hypoxia), an effect abolished by cobalt (Co^{2+}) but not local kynurenate, suggesting this excitation was due to a Ca^{2+}-dependent mechanism rather than glutamate neurotransmission (Sun *et al.*, 1992). Exposure of RVLM brainstem slices to hypoxia or cyanide *in vitro* increases the firing rate of putative RVLM presympathetic neurons, or depolarisation in the presence of TTX, suggesting the response is not due to synaptic inputs (Sun and Reis, 1994b; Sun and Reis, 1994c). Once again Co^{2+} abolished this response, suggesting a Ca^{2+} dependent mechanism (Sun and Reis, 1994b).

Although the above evidence suggests that RVLM bulbospinal presympathetic neurons may be intrinsically chemosensitive, they should be interpreted with caution, as neuronal responses to brainstem hypoxia after peripheral chemodenervation may not be specific to these neurons. Cyanide microinjected into the RVLM excites 75% of RVLM respiratory neurons, and amongst unidentified RVLM neurons, cyanide *inhibits* approximately 50% (Sun and Reis, 1994c). Kawai et al demonstrated in 1999 that acutely dissociated RVLM bulbospinal neurons recorded by whole cell patch-clamp *in vitro* are depolarised by hypoxia and cyanide in the presence of TTX, consistent with the idea that RVLM presympathetic neurons are intrinsically chemosensitive (Kawai *et al.*, 1999). However, this response was not specific for RVLM presympathetic neurons, and sensitivity to hypoxia was in fact seen in a wide variety of neurons within the RVLM (Kawai *et al.*, 1999).

The sympathetic chemoreflex, baroreflex, somato-sympathetic reflex and phrenic nerve output following ventilation with 5, 10 and 15% CO_2 in rats is investigated in chapter 3.

1.7 Somato-sympathetic reflex

Stimulation of somatic afferent nerves from skeletal muscles and skin results in alterations in sympathetic nerve activity and blood pressure (Katz and Perryman, 1965; Koizumi *et al.*, 1970; Sato *et al.*, 1981; McAllen, 1985). Low frequency and low intensity electrical stimulation of A-δ fibres results in a fall in blood pressure and stimulation of those same fibres with high frequency and intensity results in a pressor response (Katz and Perryman, 1965; Koizumi *et al.*, 1970; Sato *et al.*, 1981). Electrical stimulation of slowly conducting C-fibres from both muscle and skin causes pressor responses (Katz and Perryman, 1965; Koizumi *et al.*, 1970; Sato *et al.*, 1981).

Within the spinal cord, the dorsal horn is thought to be the major site for the termination of primary afferent axons, ending predominantly in laminae I and II (Light and Perl, 1979; Sugiura *et al.*, 1987). Substance P plays a major role in the transmission of nociceptive somatic afferent input to the spinal cord and is discussed further in section 1.11.8.6. Anterograde tracing experiments have demonstrated projections from lamina I to the entire rostrocaudal extent of the ventrolateral medulla, overlapping with the C1 (rostrally) and A1 (caudally) catecholaminergic cell groups, with terminals from lamina I neurons demonstrated on ventrolateral tyrosine hydroxylase immunoreactive neurons when examined ultrastructurally (Craig, 1995; Westlund and Craig, 1996).

Electrical stimulation of somatic nerve afferent fibres (e.g. sciatic nerve stimulation) results in excitation of efferent sympathetic nerves with a characteristic pattern (see Fig. 1.3) (McAllen, 1985; Morrison and Reis, 1989; Zanzinger *et al.*, 1994; Nagata *et al.*, 1995; Miyawaki *et al.*, 1996a; Miyawaki *et al.*, 2001). There is an early excitatory component thought to be due to stimulation of A-δ fibres and termed the somato-sympathetic 'A' reflex, and a late excitatory component most likely due to C-fibre activation, termed the somato-sympathetic 'C' reflex (Morrison and Reis, 1989; Zanzinger *et al.*, 1994; Nagata *et al.*, 1995; Miyawaki *et al.*, 1996a; Miyawaki *et al.*, 2001).

Morrison and Reis in 1989 demonstrated that electrophysiologically identified bulbospinal RVLM neurons are excited by single sciatic nerve electrical stimulation, preceding the biphasic increases in sympathetic nerve activity (Morrison and Reis, 1989). The two peaks seen in sympathetic nerve activity during the early somato-sympathetic 'A' reflex (see Fig. 1.3) (Morrison and Reis, 1989; Miyawaki *et al.*, 1996a; Miyawaki *et al.*, 2001; Miyawaki *et al.*, 2002a) are most likely due to the different conduction velocities seen within subpopulations of bulbospinal RVLM presympathetic neurons (Brown and Guyenet, 1985; Verberne *et al.*, 1999) rather than different conduction velocities in efferent neurons to the RVLM (Miyawaki *et al.*, 2001). This is shown by evidence that the interval between the two early excitatory peaks seen in sympathetic nerve activity following electrical stimulation of the sciatic nerve or the RVLM is identical (Miyawaki *et al.*, 2001).

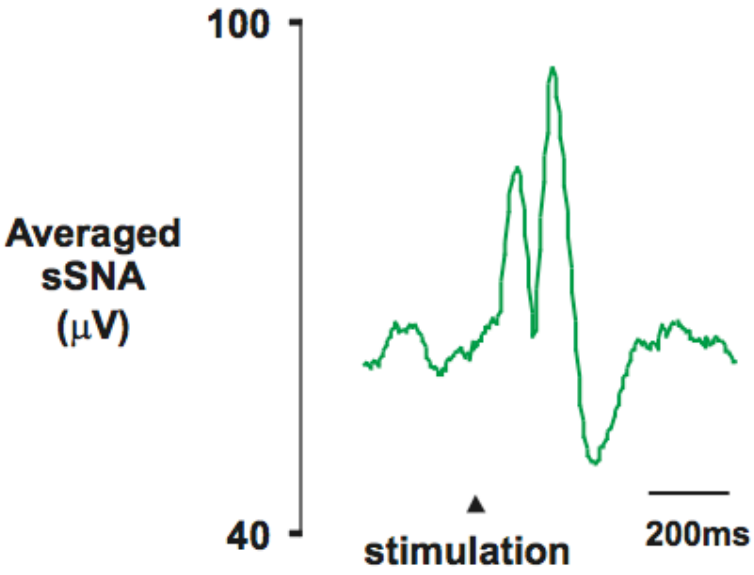

Figure 1.3. The somato-sympathetic 'A' reflex. Electrical stimulation of the tibial nerve (arrow) evokes an early sympatho-excitatory response which demonstrates a characteristic biphasic appearance on peri-stimulus sSNA waveform averaging. For discussion , see section 1.7.

The RVLM is crucial for the characteristic sympathoexcitatory response to somatic afferent electrical stimulation (Morrison and Reis, 1989; Kiely and Gordon, 1993; Zanzinger *et al.*, 1994). Cooling of the RVLM abolishes the somatosympathetic reflex (Zanzinger *et al.*, 1994). Excitatory amino acid receptors in the RVLM are vital for transmission of the somato-sympathetic reflex. Intracisternal administration of kynurenate attenuates the sympathoexcitatory and pressor responses of sciatic nerve electrical stimulation (Sun *et al.*, 1988a). More specifically, bilateral microinjection of kynurenate in the RVLM abolishes the somato-sympathetic reflex (Kiely and Gordon, 1994). This effect is thought to be due to non-NMDA receptors as microinjections of the selective non-NMDA receptor antagonist DNQX (Kiely and Gordon, 1993) or AMPA/kainate receptor antagonist CNQX (Miyawaki *et al.*, 1996a) bilaterally in the RVLM abolishes the somato-sympathetic reflex, whereas the selective NMDA receptor antagonists D-AP7 (Kiely and Gordon, 1993) or APV (Miyawaki *et al.*, 1996a) do not.

Although EAAs are thought to be the major excitatory neurotransmitters for the somato-sympathetic reflex in the RVLM, the reflex can be attenuated and modulated by many other substances. For instance, selective activation of delta-opioid receptors in the RVLM with [D-Pen^{2,5}]-enkaphalin (DPDPE) abolishes the somato-sympathetic reflex whereas selective activation of mu-opioid receptors with [D-Ala^2, *N*-Me-Phe^4, Gly-ol^5]-enkaphalin (DAMGO) has no effect (Miyawaki *et al.*, 2002a). Similarly, bilateral RVLM microinjection of the selective 5-HT$_{1A}$-receptor agonist 8-hydroxy-di-*n*-propylamino tetraline (8-OH-DPAT) abolishes the somato-sympathetic reflex without affecting other brainstem reflexes such as the sympathetic baroreflex or chemoreflex (Miyawaki *et al.*, 2001). The most likely mechanism for the effects of

both delta-opioid and 5-HT_{1A} receptor agonists is via pre-synaptic modulation of excitatory inputs to the RVLM presympathetic neurons (Miyawaki *et al.*, 2001; Miyawaki *et al.*, 2002a). Endogenous angiotensin may also play a role, as microinjection of the non-specific angiotensin receptor antagonist [Sar^1, Thr^8]-angiotensin II in the RVLM significantly attenuates the somato-sympathetic response induced by sciatic nerve stimulation in the cat (Hirooka and Dampney, 1995). It should be noted that there is evidence that [Sar^1, Thr^8]-angiotensin II is non-specific in its action in the RVLM, as the depressor effect of RVLM microinjection of [Sar^1, Thr^8]-angiotensin II may not be due to activation of angiotensin II receptors (Ito and Sved, 2000). Within the RVLM, a neuromodulatory role for nitric oxide has also been demonstrated, with an increase in the somato-sympathetic reflex following inhibition of nitric oxide synthase in the RVLM (Zanzinger *et al.*, 1995b).

The integration of the somato-sympathetic reflex within the RVLM remains poorly understood. The role of substance P and the neurokinin-1 receptor in the somato-sympathetic reflex is discussed further in section 1.11.8.6. The effects of hypercapnoeia and activation / blockade of RVLM neurokinin-1 receptors on the somato-sympathetic reflex are investigated in chapters 3 and 5 respectively.

1.8 RVLM and cerebral blood flow

Stimulation of the sympathoexcitatory neurons of the RVLM electrically (Saeki *et al.*, 1989; Golanov *et al.*, 2000a; Golanov *et al.*, 2001), chemically (Saeki *et al.*, 1989; Chida *et al.*, 1995; Chida *et al.*, 1998), or with hypoxia (Underwood *et al.*, 1992; Underwood *et al.*, 1994; Golanov and Reis, 1999; Golanov *et al.*, 2000b; Golanov *et*

al., 2001), results in widespread increase in regional cerebral blood flow (rCBF) without a change in glucose utilization (Underwood *et al.*, 1992; Underwood *et al.*, 1994), and a decrease in cerebral vascular resistance. This is thought to be due to a polysynaptic pathway as the RVLM does not directly innervate the cerebral cortex (Ruggiero *et al.*, 1989). The pathway for RVLM induced increases in rCBF is poorly understood, however there is evidence that a region dorso-caudal to the RVLM C1 region, adjacent to the nucleus ambiguus may be the region of the first synapse (Golanov *et al.*, 2000a; Golanov *et al.*, 2000b; Golanov *et al.*, 2001). This region has been named the medullary cerebral vasodilator area (MCVA)(Golanov *et al.*, 2000b). Chemical or electrical stimulation of the MCVA increases rCBF and decreases cerebral vascular resistance without a corresponding increase in glucose metabolism, a finding similar to chemical or electrical RVLM stimulation (Underwood *et al.*, 1992; Golanov and Reis, 1994; Golanov *et al.*, 2000b). Lesions of the MCVA abolish the increase in rCBF seen in RVLM activation, however RVLM lesions have no effect on the rCBF increases following MCVA activation (Golanov *et al.*, 2000b). Further down the chain, lesion of a limited subthalamic area, encompassing the medial pole of the zona incerta, the prerubral zone and Forel's field, abolish the increases in rCBF seen after MCVA activation (Golanov *et al.*, 2001). This region has been named the subthalamic cerebrovasodilator area (SVA) (Golanov *et al.*, 2001). An early, poorly understood, pathway is beginning to emerge, where activation of the RVLM relays cerebral vasodilator information from the RVLM to MCVA, and from the MCVA relayed to the SVA (Golanov *et al.*, 2000a; Golanov *et al.*, 2000b; Golanov *et al.*, 2001). Downstream projections from the SVA are unknown, as are the neurotransmitters responsible for conveying cerebral vasodilator information in the

MCVA and SVA regions, as the majority of the work conducted by Golanov and co-workers has been conducted using electrical stimulation and electrolytic lesions (Golanov *et al.*, 2000a; Golanov *et al.*, 2000b; Golanov *et al.*, 2001). However, the MCVA does appear to excited by local microinjection of glutamate or nicotine (Golanov *et al.*, 2000b), while the SVA is excited by kainic acid (Golanov *et al.*, 2001).

The effect of microinjection of neurokinin-1 receptor agonists and antagonists in various regions within the medulla on rCBF is demonstrated in chapter 6.

1.9 Respiratory modulation of SNA

Whole nerve recordings such as from the splanchnic, lumbar or renal sympathetic nerves record the summed action potentials of large numbers of individual fibres within the nerve. When these summed action potentials form bursts in recorded activity, the discharges are said to be 'synchronized' (Malpas, 1998).

Sympathetic nerve activity displays a rhythmic bursting pattern that closely correlates with recorded phrenic nerve output (Miyawaki *et al.*, 1995; Koshiya and Guyenet, 1996b; Miyawaki *et al.*, 2002b). Extracellular recording from electrophysiologically identified RVLM presympathetic neurons also demonstrate respiratory related modulation of neuronal firing rates (Miyawaki *et al.*, 1995; Miyawaki *et al.*, 1996a).

Phrenic nerve activity triggered averaging of sympathetic nerve activity demonstrates typical patterns in different sympathetic nerves. For instance, splanchnic sympathetic nerve activity is characterized by an inspiratory peak (I-peak) during the phrenic burst

with a further peak immediately after phrenic cessation (post-inspiratory, PI-peak) (Miyawaki *et al.*, 1996a; Miyawaki *et al.*, 2002b). In contrast, lumbar sympathetic nerves demonstrate the PI-peak only (Miyawaki *et al.*, 2002b). This difference in respiratory modulation may be due to functional specificity for sympathetic nerves supplying different tissue beds, the significance of which is uncertain.

What is the source of the respiratory related modulation of sympathetic nerve activity? The respiratory-related activity recorded in sympathetic nerves persists following bilateral vagotomy and paralysis, indicating that respiratory entrainment is not due to activation of pulmonary stretch receptors or other vagal afferent neurons (Numao *et al.*, 1987; Habler *et al.*, 1994; Pilowsky, 1995). Further, as discussed in section 1.6.2, chemoafferent nerve fibres and respiratory-related NTS neurons do not demonstrate significant respiratory modulation, indicating that the modulation is occurring centrally (Koshiya and Guyenet, 1996a).

The most likely source of the respiratory input to sympathetic preganglionic neurons is either the putative respiratory rhythm-generating centre within the brainstem, the pre-Bötzinger complex (see section 1.10.6.1), or closely associated respiratory-related interneurons. This is demonstrated by two microinjection experiments. Microinjection of muscimol in the region of the pre-Bötzinger complex abolishes all central respiratory activity, including the respiratory related modulation of sympathetic nerve activity, but not chemoreceptor activation induced sympatho-excitation (Koshiya and Guyenet, 1996b). However, inhibition of the CVLM region with muscimol abolishes phrenic nerve output (probably by inhibiting bulbospinal phrenic premotor neurons) without affecting the phasic, respiratory-like modulation of SNA or the sympathetic chemoreflex (Koshiya *et al.*, 1993).

Blockade of ionotropic excitatory amino acid receptors in the RVLM with either kynurenate, AMPA-kainate receptor antagonists or NMDA-receptor antagonists abolishes the post-inspiratory peak in lumbar (Guyenet *et al.*, 1990; Miyawaki *et al.*, 1996a; Miyawaki *et al.*, 2002b)and splanchnic (Miyawaki *et al.*, 1996a; Miyawaki *et al.*, 2002b) sympathetic nerves. These drugs have no effect on the inspiratory peak, indicating that ionotropic excitatory amino acid neurotransmission is not responsible for this peak.

There is some evidence for a tonic inhibition of the respiratory-related excitatory inputs to RVLM presympathetic neurons. Bilateral RVLM microinjections of bicuculline (GABA-A receptor antagonist) induce a robust increase in the post-inspiratory peak in SNA in both lumbar and splanchnic sympathetic nerves, an effect abolished by subsequent microinjection of kynurenate (Miyawaki *et al.*, 2002b). The source of this tonic inhibition is unknown, however disfacilitation of the CVLM with AMPA-kainate receptor antagonists results in a similar increase in post-inspiratory discharge, suggesting CVLM GABA-ergic neurons projecting to the RVLM may be responsible (Miyawaki *et al.*, 1996a).

Little is known about the source of the inspiratory peak in splanchnic SNA, however it should be noted that the inspiratory peak became undetectable following bicuculline microinjection in the study conducted by Miyawaki *et al* (Miyawaki *et al.*, 2002b). As mentioned in section 1.5.3, the CVLM has monosynaptic GABAergic projections to RVLM presympathetic neurons (Agarwal and Calaresu, 1991; Jeske *et al.*, 1995). Further evidence for the involvement of the CVLM in the respiratory modulation of sympathetic nerve activity is seen with recent evidence that some barosensitive GABAergic neurons in the CVLM demonstrate respiratory modulated neuronal firing

patterns (Mandel and Schreihofer, 2006). These neurons may be those neurons that project to RVLM presympathetic neurons, however direct anatomical evidence of this is lacking.

Direct anatomical evidence for respiratory neurons projecting to RVLM presympathetic neurons is sparse (Pilowsky *et al.*, 1994; Sun *et al.*, 1997). Bötzinger neurons project to presumed RVLM presympathetic neurons, however these neurons are inhibitory during expiration and thus unlikely to contribute to the EAA-mediated post-inspiratory peak (Sun *et al.*, 1997).

The exact pathways of the respiratory modulation of RVLM presympathetic neurons and SNA remain to be determined.

1.10 VLM and respiratory function

Brainstem respiratory neurons involved in afferent signal processing, rhythm generation and premotor output shaping are found in several bilaterally symmetric, longitudinally oriented nuclei within the pons, and within the dorsal (DRG) and ventral (VRG) respiratory groups of the medulla.

1.10.1 The Dorsal Respiratory Group (DRG)

The DRG consists primarily of neurons within the ventrolateral subnucleus of the NTS that process afferent respiratory information and relay the signal to other medullary areas (Hilaire *et al.*, 1990). This region has been discussed in detail in sections 1.6.1 and 1.6.2.

1.10.2 The Ventral Respiratory Group (VRG)

The VRG (also known as the ventral respiratory column) consists of longitudinally oriented nuclei found within the ventrolateral medulla immediately ventral and lateral, although overlapping slightly, the cranial motoneurons of the nucleus ambiguus (see Fig. 1.1) (Núñez-Abades *et al.*, 1992).Based on the rostro-caudal level of the obex the VRG has been subdivided into a rostral part (rVRG) where a predominance of premotor inspiratory neurons are found (Stornetta *et al.*, 2003b), and a caudal part (cVRG) where many premotor expiratory neurons are located (Shen and Duffin, 2002). At the level of the obex an intermediate level is found where there is a mixture of inspiratory and expiratory cell types (Shen and Duffin, 2002; Stornetta *et al.*, 2003b). Inspiratory neurons of the VRG project to, and synapse with, phrenic motor neurons (Lipski *et al.*, 1994).

1.10.3 The Bötzinger cell group and pre-Bötzinger Complex (preBötC)

Rostral to the rVRG but still in line with the longitudinal extent of the VRG are the Bötzinger cell group and the preBötC. The Bötzinger cell group contains inhibitory expiratory neurons, which are predominantly glycinergic (Sun *et al.*, 1997; Schreihofer *et al.*, 1999; Ezure *et al.*, 2003). Bötzinger neurons lie immediately dorsal to the RVLM bulbospinal presympathetic neurons, and have been shown to project to presumed RVLM presympathetic neurons (Sun *et al.*, 1997). As these expiratory Bötzinger neurons are predominantly inhibitory and glycinergic (Schreihofer *et al.*,

1999), they may be involved in the respiratory modulation of SNA discussed in section 1.9.

Immediately caudal to the Bötzinger cell group but rostral to the rVRG lies a small group of neurons called the preBötzinger Complex (preBötC). This region is essential for the generation of normal respiratory rhythm (Smith *et al.*, 1991; Gray *et al.*, 1999) and will be discussed further in section 1.10.6.1.

1.10.4 Other respiratory related neurons

The respiratory related hypoglossal motoneurons innervating the tongue are dorsal to the VRG and closer to the midline (Fregosi and Fuller, 1997). The upper cervical inspiratory neurons (UCINs) described by Lipski *et al* in 1993 are caudal to the VRG (Lipski *et al.*, 1993). These cell groups will not be discussed further in this review.

1.10.5 Patterns of respiratory related neuronal firing

Respiratory related neurons within the VLM demonstrate different firing patterns throughout the respiratory cycle (Feldman *et al.*, 2003; Duffin, 2004). Throughout inspiration, inspiratory neurons (I) can vary their firing rate upwards (augmenting, I-AUG), downwards (decrementing, I-DEC), or span inspiration with a constant firing rate (constant, I-CON). Similarly, expiratory neurons (E) can be augmenting (E-AUG) or decrementing (E-DEC) (Feldman *et al.*, 2003; Duffin, 2004). Further, expiratory neurons can be subdivided into those neurons that are active early / post inspiratory (E1) or late / pre-inspiratory (E2) (Feldman *et al.*, 2003; Duffin, 2004). The physiological reason for the subdivision of expiratory neurons into E1 and E2 phases is uncertain. It may be that E1 activity increases airway resistance during expiration,

prolonging lung inflation and allowing better gas exchange (Paton and Dutschmann, 2002). The E2 phase may serve to prevent premature inspiration (Duffin, 2004).

1.10.6 Generation of respiratory rhythm

1.10.6.1 The pre-Bötzinger complex (preBötC)

In a landmark paper in 1991, Smith *et al* described a small group of propriobulbar neurons immediately rostral to the rVRG and caudal to the Bötzinger neurons that are essential for the generation of respiratory-related motor nerve output in rat *in vitro* medullary slice preparations (Smith *et al.*, 1991). The region was termed the preBötzinger complex (preBötC) and was postulated to represent the major respiratory rhythm generator (Smith *et al.*, 1991). Lesions in this area of the brainstem severely disrupt normal breathing in adult mammals (Fung *et al.*, 1994; Hsieh *et al.*, 1998; Ramirez *et al.*, 1998; Mutolo *et al.*, 2002). As mentioned in section 1.6.2 inhibition of the preBötC with bilateral muscimol microinjections abolishes central respiratory output in rats(Koshiya and Guyenet, 1996b). In neonatal mice, markedly abnormal breathing patterns occur following genetic deletion of the transcription factor *MafB* (v-maf musculoaponeurotic fibrosarcoma oncogene homologue B) (Blanchi *et al.*, 2003). The principle abnormality seen in these mice is a marked reduction in preBötC neurons (Blanchi *et al.*, 2003).

In 1999 Gray *et al* proposed that the preBötC could be neuroanatomically defined by the presence of immunoreactivity to the neurokinin-1 receptor (Gray *et al.*, 1999). While there is significant overlap of neurokinin-1 immunoreactive neurons and the preBötC, the presence of the neurokinin-1 receptor within cells in this region is not

sufficient to anatomically define the putative respiratory rhythm generating neurons (Wang *et al.*, 2001; Makeham *et al.*, 2001) (see results chapter 4). Many other substances have been described in putative rhythm generating neurons of the preBötC, including preproenkaphalin (Guyenet *et al.*, 2002), the glutamate transporter VGLUT2 (Guyenet *et al.*, 2002), μ-opiate receptors (Gray *et al.*, 1999), and somatostatin (Stornetta *et al.*, 2003a). For further discussion of substance P and neurokinin-1 receptors in the preBötC, see section 1.11.8.4.

The mechanism by which the preBötC generates respiratory related rhythm is the source of fierce debate. The two major contenders are the pacemaker and the group-pacemaker theories (Feldman *et al.*, 2003; Feldman and Del Negro, 2006).

The pacemaker theory suggests that neurons with intrinsic pacemaker properties are essential for normal respiratory rhythm generation. Indeed, when Cl^--mediated synaptic inhibition is blocked *in vitro*, respiratory-related rhythm is unaffected, suggesting intrinsic pacemaker properties (Feldman and Smith, 1989; Onimaru *et al.*, 1989). Approximately 5-25% of preBötC inspiratory neurons demonstrate voltage-dependent pacemaker properties (Del Negro *et al.*, 2002a; Pena and Ramirez, 2004; Del Negro *et al.*, 2005; Pagliardini *et al.*, 2005), derived primarily from persistent Na^+ currents (I_{NaP}) (Smith *et al.*, 1991; Johnson *et al.*, 1994). This I_{NaP} current is Cd^{2+}-insensitive but blocked by riluzole (Del Negro *et al.*, 2002a; Ptak *et al.*, 2005). A further voltage-insensitive cation inward current (I_{CAN}) has been demonstrated in a small subset (less than 10%) of preBötC neurons (Pena and Ramirez, 2004; Del Negro *et al.*, 2005). Blockade of the I_{NaP} current with bath application of riluzole *in vitro* fails to block respiratory rhythm generation (Del Negro *et al.*, 2002b; Del Negro *et al.*,

2005). This was an unexpected finding and suggests that these pacemaker neurons may not be essential for respiratory rhythm generation.

The group-pacemaker hypothesis suggests that respiratory rhythm is an emergent feature, derived from recurrent excitatory synaptic connections initiating positive feedback within preBötC neurons (Rekling *et al.*, 1996; Del Negro *et al.*, 2005; Feldman and Del Negro, 2006). In this model, the I_{NaP} and I_{CAN} currents contribute by amplifying the synaptic depolarization and thus generating the respiratory burst, however are not essential for network activity (Feldman and Del Negro, 2006). The generation of inspiratory rhythm remains controversial, especially in light of recent evidence for other possible respiratory rhythm generating regions within the brainstem such as the parafacial respiratory group (pFRG) (see section 1.10.6.2).

1.10.6.2 The parafacial respiratory group / retrotrapezoid nucleus

Recent evidence suggests that there is a second major region within the brainstem, located ventral to the facial nucleus, that contributes to respiratory rhythm generation (Smith *et al.*, 1989; Feldman *et al.*, 1990; Onimaru and Homma, 2003). This lies in the region of the retrotapezoid nucleus (RTN), and has also been termed the parafacial respiratory group (pFRG). Bilateral lesions in this RTN/pFRG region significantly decrease respiratory frequency, suggesting a role in respiratory rhythm generation (Onimaru and Homma, 2003). Brainstem transactions rostral to the facial nucleus that leave both RTN/pFRG and preBötC regions intact do not alter inspiratory or expiratory respiratory bursting activity (Janczewski and Feldman, 2006). A lower brainstem transection through the rostral RTN/pFRG that leaves the preBötC intact abolishes

expiratory motor bursting activity but leaves inspiratory motor bursting activity unaffected (Janczewski and Feldman, 2006). Using direct visualization with Ca^{2+} sensitive dyes, Omimaru *et al* demonstrated a cluster of rhythmically active neurons in the RTN/pFRG with a predominantly pre-inspiratory discharge pattern (Onimaru and Homma, 2003). It has been proposed that these pre-inspiratory rhythmically active RTN/pFRG neurons interact with the preBötC to generate respiratory rhythm in a coupled oscillator fashion, with the RTN/pFRG generating expiratory patterning and the preBötC generating inspiratory patterning (Janczewski *et al.*, 2002; Mellen *et al.*, 2003; Janczewski and Feldman, 2006).

In contrast, others have proposed that neurons in the RTN/pFRG region are central chemoreceptors which project to the preBötC region of the VRG and provide tonic respiratory drive through the release of glutamate (Weston *et al.*, 2004; Mulkey *et al.*, 2004; Guyenet *et al.*, 2005). These cells are tonically active when disconnected from other brainstem respiratory input, but most likely receive respiratory synchronous inhibitory inputs to develop their characteristic respiratory bursting pattern (Guyenet *et al.*, 2005). Further, the RTN/pFRG region receives peripheral chemoreceptor information via a direct projection from chemoactivated NTS neurons, the majority of which (>90%) are glutamatergic (Takakura *et al.*, 2006).

The generation of respiratory rhythm remains a controversial subject and the subject of much current research.

1.11 Tachykinins and their receptors

1.11.1 Historical introduction

In 1931, Ulf von Euler and John Gaddum in London discovered that a substance derived from horse intestine and brain through alcoholic extraction resulted in hypotension when injected intravenously and contraction of isolated rabbit intestine in a bath preparation. These effects were atropine resistant. This substance was termed "preparation P" (von Euler and Gaddum, 1931). The dried powder derived from the alcohol extraction was later termed "substance P" (Gaddum and Schild, 1935).

In 1953, Bengt Pernow, working from von Euler's laboratory in Sweden, published his PhD thesis. In it he described the purification of substance P, and studies on the distribution demonstrated higher substance P levels in the grey compared to white matter in the brain, with some areas demonstrating particularly high levels, such as the substantia nigra and hypothalamus. He also demonstrated that the dorsal half of the spinal cord contained higher levels of substance P than the ventral half and that substance P was present in autonomic nerves, sympathetic trunk and spinal ganglia (Pernow, 1953)

It was not until 1971 that substance P was identified as a peptide, in bovine hypothalamus, and the amino acid sequence determined as the undecapeptide H-Arg^{1}-Pro^{2}-Lys^{3}-Pro^{4}-Gln^{5}-Gln^{6}-Phe^{8}-Gly^{9}-Leu^{10}-Met^{11}-NH_2 (Chang *et al.*, 1971). This group was also the first to synthesize substance P (Tregear *et al.*, 1971) and develop a radioimmunoassay (Powell *et al.*, 1973).

1.11.2 Substance P as a neurotransmitter

Pioneering work in 1953 by Lembeck, demonstrated a substance within the spinal cord that had very similar actions to substance P and found in higher concentrations in dorsal rather than ventral roots of spinal nerves. He postulated that this substance was substance P and that it was involved in neurotransmission (Lembeck, 1953).

After the discovery of the structure of substance P, the field progressed very rapidly. Substance P immunoreactivity was demonstrated in cell bodies of the dorsal root ganglia and axons in the dorsal horn (Hökfelt *et al.*, 1975a; Hökfelt *et al.*, 1975b). Substance P immunoreactivity was also demonstrated in 50% of electrophysiologically identified C-fibres of the rat lumbar dorsal root ganglia, and 20% of the Aδ-fibre neurons (McCarthy and Lawson, 1989).

Release of substance P has been demonstrated in the spinal cord following electrical stimulation of dorsal nerve roots (Otsuka and Konishi, 1976) and peripheral nerve C-fibres (Yaksh *et al.*, 1980), chemical stimulation with capsaicin (Akagi *et al.*, 1980), and mechanical and inflammatory stimuli (Kuraishi *et al.*, 1989).

Dorsal horn neurons, when exposed to substance P by iontophoresis *in vivo* undergo prolonged depolarisation (Henry, 1976), as do cultured spinal neurons after bath application of substance P (Nowak and Macdonald, 1982). Both bath application of substance P and electrical stimulation of a dorsal root result in depolarisation of intracellularly recorded dorsal root neurons in rat spinal cord slices (Urban and Randic, 1984; Randic *et al.*, 1986). Tachykinin antagonists or substance P antibodies block both the electrically induced and substance P induced depolarisations (Urban and Randic, 1984; Randic *et al.*, 1986).

Given the anatomical, physiological and pharmacological evidence it is now well accepted that tachykinins, especially substance P, can act as neurotransmitters within the CNS.

1.11.3 Biosynthesis of substance P / tachykinins

All members of the tachykinin family, such as substance P, neurokinin A and neurokinin B, possess a common C-terminal sequence (**Phe-X-Gly-Leu-Met-NH_2**) (see Table 1.1).

Substance P	Arg-Pro-Lys-Pro-Gln-Glln-**Phe-Gly-Leu-Met-NH_2**
Neurokinin A	His-Lys-Thr-Asp-Ser-**Phe-Val-Gly-Leu-Met-NH_2**
Neurokinin B	Asp-Met-His-Asp-Phe-**Phe-Val- Gly-Leu-Met-NH_2**
Neuropeptide K	Asp-Ala-Asp-Ser-Ser-Ile-Glu-Lys-Gln-Val-Ala-Leu-Leu-Lys-Ala-Leu-Tyr-Gly-His-Gly-Gln-Ile-Ser-His-Lys-Arg-His-Lys-Thr-Asp-Ser-**Phe-Val-Gly-Leu-Met-NH_2**
Neuropeptide γ	Asp-Ala-Gly-His-Gly-Glln-Ile-Ser-His-Lys-Thr-Asp-Ser-**Phe-Val-Gly-Leu-Met-NH_2**
Hemokinin-1 (rat/mouse)	Arg-Ser-Arg-Thr-Arg-Gln-**Phe-Tyr-Gly-Leu-Met-NH_2**
Hemokinin-1 (human)	Thr-Gly-Lys-Ala-Ser-Gln-**Phe-Phe-Gly-Leu-Met-NH_2**
Endokinin C (human)	Lys-Lys-Ala-Tyr-Gln-Leu-Glu-His-Thr-**Phe-Gln-Gly-Gly-Leu-Leu-NH_2**
Endokinin D (human)	Val-Gly-Ala-Tyr-Gln-Leu-Glu-His-Thr-**Phe-Gln-Gly-Leu-Leu-NH_2**

Table 1.1. Amino acid sequences of the mammalian tachykinins (Khawaja and Rogers, 1996; Zhang *et al.*, 2000b; Kurtz *et al.*, 2002; Page *et al.*, 2003)

Mammalian tachykinins are derived from three genes. Substance P is derived from the pre-protachykinin-A (PPT-A) gene, which also encodes for neuropeptide K, neuropeptide γ, and neurokinin A. A second gene, the pre-protachykinin-B (PPT-B) gene encodes for neurokinin B (Nawa *et al.*, 1983; Kotani *et al.*, 1986; Bonner *et al.*, 1987). A recently discovered tachykinin, named haemokinin-1 (HK-1), is encoded by a third preprotachykinin gene, cloned in the human and rat (pre-protachykinin-C, PPT-C) (Zhang *et al.*, 2000b; Kurtz *et al.*, 2002; Page *et al.*, 2003).

Through alternative splicing, the PPT-A gene can express 3 different forms of mRNA, named α-PPT, β-PPT and γ-PPT (Nawa *et al.*, 1984; Krause *et al.*, 1987). These differ only in the exons used for the protein coding region. For instance, α-PPT lacks exon 6, γ-PPT lacks exon 4, and β-PPT uses all 7 exons of the PPT-A gene (Carter and Krause, 1990). Each of these mRNAs produce different peptides- α-PPT produces substance P; β-PPT produces substance P, neurokinin A and neuropeptide K; and γ-PPT produces substance P, neurokinin A and neuropeptide γ (Nawa *et al.*, 1984; Krause *et al.*, 1987; MacDonald *et al.*, 1989).

The second gene, PPT-B, encodes for only neurokinin B (Bonner *et al.*, 1987).

The third gene, PPT-C, consists of 5 exons in the human (Kurtz *et al.*, 2002; Page *et al.*, 2003). As in PPT-A, alternative splicing of the PPT-C primary transcript can result in four distinct mRNAs (α, β, γ, δ). The α PPT-C mRNA encodes for two forms of HK-1 named EKA and EKC. The β PPT-C encodes for a form of HK-1 named EKB and a tachykinin named EKD. The γ PPT-C and δ PPT-C mRNAs encode for EKB only (Kurtz *et al.*, 2002; Page *et al.*, 2003). A schematic diagram illustrating the biosynthesis of mammalian tachykinins is shown in Fig. 1.4.

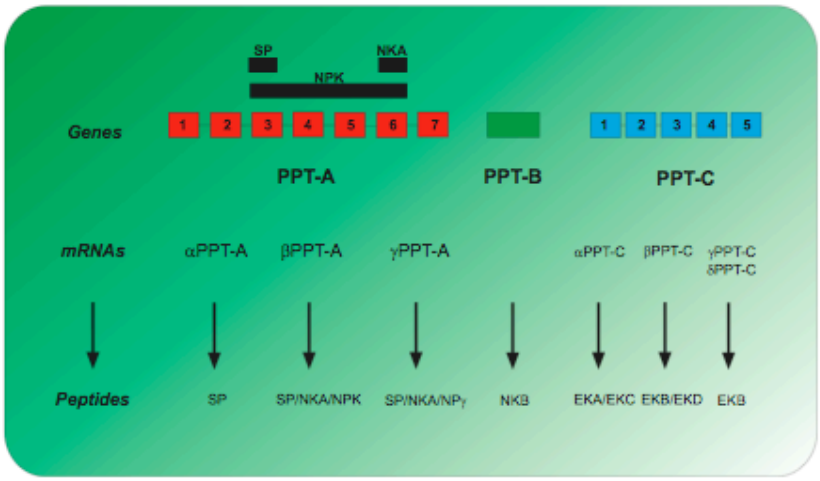

Figure 1.4. Schematic representation of the biosynthesis of mammalian tachykinins. For discussion, see section 1.11.3

The distribution and expression of the different mRNAs is species dependent. In the bovine brain, α-PPT is expressed to a greater degree than β-PPT, a situation that is reversed in peripheral tissues (Nawa *et al.*, 1984). In the human basal ganglia β-PPT is the predominant form expressed (Bannon *et al.*, 1992). In rat, however, there is only small expression of α-PPT, although β-PPT and γ-PPT are found widely, wherever the expression of neurokinins can be found (Nakanishi, 1987; Carter and Krause, 1990).

Preprotachykinins are synthesized in membrane bound ribosomes of the peptidergic cell (Harmar *et al.*, 1980; Harmar and Keen, 1982). These preprotachykinins contain the amino acid sequence of substance P. After a trypsin like cleavage, an inactive propeptide (substance P-Gly^{12}-Lys^{13}) is produced and packaged into vesicles in the golgi apparatus (Cuello *et al.*, 1977; Floor *et al.*, 1982). Once packaged into vesicles, substance P is transported via axonal transport to the terminal endings at a mean velocity of approximately 5-6mm/hr (Brimijoin *et al.*, 1980). It is interesting to note that the axonal transport of the substance P containing vesicles is sometimes specific to different parts of the cell. For instance, in primary afferent neurons where the single axon arising from the cell body splits into central and peripheral branches within the dorsal root ganglion, up to 4 times as much substance P is transported to the peripheral versus the central branches (Harmar and Keen, 1982). At the terminals substance P is liberated from its precursor within these vesicles via specific proteases, from six groups of proteolytic enzymes called convertases (Steiner *et al.*, 1992).

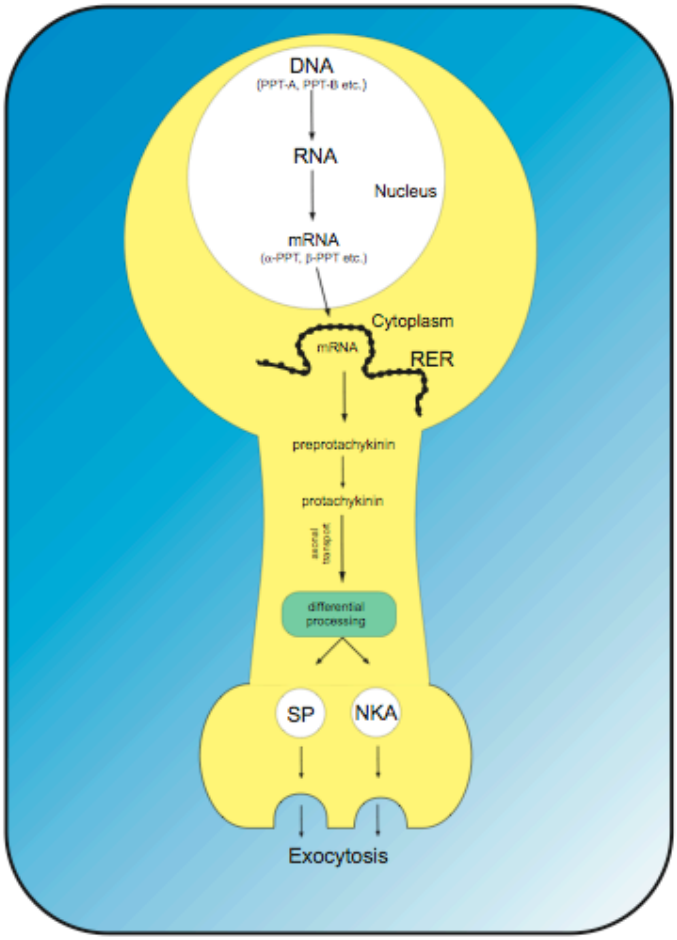

Figure 1.5. Model of the biosynthesis, axonal transport and release of substance P. RER, rough endoplasmic reticulum. SP, substance P. NKA, neurokinin A. Adapted from Snijdelaar *et al*, 2000.

Tachykinins are stored primarily within nerve terminals in large granular vesicles about 100nm in diameter (Floor *et al.*, 1982; Difiglia *et al.*, 1982a; Difiglia *et al.*, 1982b). A schematic diagram illustrating the biosynthesis, axonal transport and release of substance P is shown in Figure 1.5.

1.11.3.1 Release of substance P

Most research conducted on the release of tachykinins and substance P from nerve endings has been conducted on either sensory nerve endings or enteric neurons. The majority of these studies lead to the conclusion that tachykinins, including substance P, are released via a calcium dependent mechanism. Using specific Ca^{2+} channel blockers, such as ω-conotoxin GVIA, it has been demonstrated that the release of tachykinins in sensory neurons is predominantly due to N-type voltage gated calcium channels (Maggi *et al.*, 1990; Maggi *et al.*, 1992; Evans *et al.*, 1996; Kageyama *et al.*, 1997). There is some evidence that there may be a small contribution from L-type voltage gated calcium channels, however this study was conducted using potassium depolarisation rather than electrical field stimulation (Kageyama *et al.*, 1997). Within enteric neurons, both N- and non-N-type but not L-type voltage gated calcium channels are responsible for release of tachykinins, including substance P (De Luca *et al.*, 1990; Katsoulis *et al.*, 1992).

1.11.3.2 Inactivation of substance P

Neuropeptides are inactivated by peptidases. The peptidase hydrolyses the neuropeptide, converting it to a different biologically active agent, or alternatively,

renders it inactive. Some peptidases are bound to the cell membrane with active regions facing the extracellular space, such as neutral endopeptidase (also known as enkephalinase) (Matsas *et al.*, 1983; Hooper *et al.*, 1985). Neutral endopeptidase cleaves substance P between Gln^6 and Phe^7 and between Gly^9 and Leu^{10} (Matsas *et al.*, 1986). Within the CNS, neutral endopeptidase immunoreactivity corresponds with substance P immunoreactivity (Matsas *et al.*, 1986). Further, neutral endopeptidase inhibitors such as phosphoramidon potentiate the action of substance P in slices of rat substantia nigra (Mauborgne *et al.*, 1987). Other peptidases are specific for substance P, such as one discovered by Nyberg and co-workers which hydrolyses substance P and forms substance P (1-7), also an active substance (Nyberg *et al.*, 1984). Angiotensin-converting enzyme (ACE), although not specific for substance P, has been shown to play a part in its metabolism, cleaving it at the Phe^8 to Gly^9 and Gly^9 to Leu^{10} bonds to yield fragments substance P(1-7) and substance P(1-8) (Yokosawa *et al.*, 1983).

The question then arises- in the CNS, which of these many possibilities for substance P metabolism is the most important? Within the striatum of the rat, which is the most commonly studied CNS region for substance P metabolism, it would appear that neutral endopeptidase is the most important primary degradation enzyme, assisted by aminopeptidases such as dipeptidyl aminopeptidase IV or prolyl endopeptidase in primary substance P cleavage (Heymann and Mentlein, 1978; Blumberg *et al.*, 1980; Michael-Titus *et al.*, 2002). At least within the rat striatum, ACE is thought to predominantly play a role in the secondary hydrolysis of substance P degradation products, even though it has been shown to cleave substance P into substance P(1-7) and substance P(1-8) (Yokosawa *et al.*, 1983). This is demonstrated by evidence that

within the striatum of the rat, captopril, an ACE inhibitor, does not prevent the degradation of substance P, but does prevent the hydrolysis of substance P(1-7), a daughter fragment (Michael-Titus *et al.*, 2002).

1.11.4 Tachykinin receptors NK1, NK2, NK3

After the discovery of Neurokinin A and B in the early 1980's (Kangawa *et al.*, 1983; Minamino *et al.*, 1984), the hunt began for receptors for these new tachykinins. The possibility of multiple neurokinin receptors was suggested by Teichberg and co-workers (Teichberg *et al.*, 1981). A receptor, termed the SP-P receptor, was described that was activated by substance P and physalaemin at low concentrations (nanomolar) and a further receptor, the SP-E receptor, was described that was activated by nanomolar concentrations of kassinin, eledoisin and NKA (Iverson *et al.*, 1982; Lee *et al.*, 1982a). Neurokinin B was found to be the preferred ligand for a third type of neurokinin receptor, subsequently named the SP-N receptor (Beaujouan *et al.*, 1984; Laufer *et al.*, 1985). A flood of papers in the early 1980's led to a confusing array of names for the tachykinin receptors. This was remedied at the Montreal Tachykinin Symposium in 1986 where it was decided that the receptors would be named Neurokinin 1 receptor (NK_1- previously SP-P), Neurokinin 2 receptor (NK_2- previously SP-E, NK-A, SP-K) and Neurokinin 3 receptor (NK_3- previously SP-N and NK-B) (Henry, 1987).

In the late 1990's it was proposed that there is a fourth human neurokinin receptor which is highly homologous with the neurokinin-3 receptor and was thus termed the neurokinin-4 or neurokinin-3B receptor (Donaldson *et al.*, 1996; Krause *et al.*, 1997; Donaldson *et al.*, 2001). Other studies have cast doubt on there being a separate class

of receptors (Sarau *et al.*, 2000; Page and Bell, 2002; Pinto *et al.*, 2002). These papers point out that separate neurokinin-4 receptor expression is difficult to demonstrate in either human or rat tissues and that the proposed structure is, in fact, almost identical to the guinea-pig neurokinin-3 receptor (Sarau *et al.*, 2000; Page and Bell, 2002; Pinto *et al.*, 2002).

1.11.4.1 The general structure of neurokinin receptors

In 1987, the first tachykinin receptor to be successfully cloned was the neurokinin-2 receptor (Masu *et al.*, 1987). This paper demonstrated the amino acid sequence of the bovine gastric neurokinin-2 receptor, expressed in *Xenopus* oocytes. This was soon followed by the determination of the amino acid sequences of the neurokinin-3 receptor (Shigemoto *et al.*, 1990) and the neurokinin-1 receptor (Yokota *et al.*, 1989; Hershey and Krause, 1990).

Tachykinin receptors belong to the large family of "rhodopsin-like" G-protein-coupled receptors. The general structure of this family of receptors consists of 7 hydrophobic transmembrane domains (TM I-VII). These domains link three intracellular (IL-1, IL-2 and IL-3) and three extracellular (EL-1, E-2 and EL-3) loops. There is a cytoplasmic carboxy-terminus and an extracellular amino-terminus and the whole structure is coupled to intracellular G-proteins (see Fig. 1.6) (Masu *et al.*, 1987; Sasai and Nakanishi, 1989; Yokota *et al.*, 1989; Shigemoto *et al.*, 1990).

Although each tachykinin receptor has a tachykinin which binds with the highest affinity, there is a degree of cross reactivity between tachykinins and the different receptors (Hardwick *et al.*, 1997).

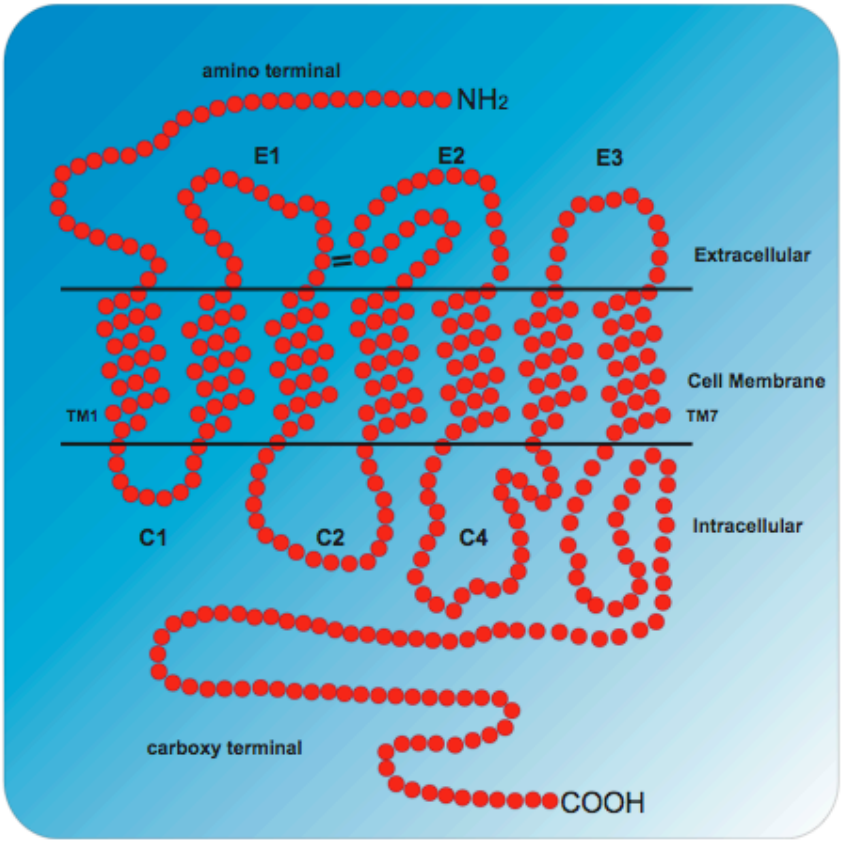

Figure 1.6. Schematic model of the neurokinin-1 receptor. E1-E3 indicate extracellular loops, while C1-C3 indicate intracellular loops. TM1-TM7 indicate transmembrane domains. The = between E1 and E2 indicates disulphide linkage. Adapted from Khawaja et al, 1996.

1.11.4.2 The NK1 receptor

The NK1 receptor has the general characteristics of rhodopsin-like G-protein coupled receptors mentioned above. There is a high degree of sequence homology between many different species, including rat, mouse, humans, and guinea-pig (Gerard *et al.*, 1993). Indeed, between man and rat the mRNA sequence is 92% identical (Gerard *et al.*, 1991). Figure 1.6 is a schematic representation of the Neurokinin 1 receptor.

1.11.4.3 Ligands for tachykinin receptors

The neurokinin-1 receptor is potently activated by substance P. Likewise, neurokinin A activates the neurokinin-2 receptor and neurokinin B, the neurokinin-3 receptor (Teichberg *et al.*, 1981; Iverson *et al.*, 1982; Lee *et al.*, 1982a; Beaujouan *et al.*, 1984; Laufer *et al.*, 1985). Following these discoveries, however, a misconception arose. It came to be assumed that the neurokinin-1 receptor was "the substance P receptor". Likewise it was assumed that neurokinin A was the physiological ligand for the neurokinin-2 receptor and neurokinin B was the physiological ligand for the neurokinin-3 receptor. Some difficulties arose with this assumption, however, especially in relation to neurokinin A. Neurokinin A is abundantly expressed in the CNS (Kanazawa *et al.*, 1984; Arai and Emson, 1986). The neurokinin-2 receptor, in contrast, is expressed in very low quantities within the CNS, and is often expressed in regions where neurokinin A expression cannot be demonstrated (Saffroy *et al.*, 2001; Saffroy *et al.*, 2003). Why is the neurokinin-2 receptor in regions where there appears to be no endogenous neurokinin A to activate it?

The idea of three tachykinins (substance P, neurokinin A and neurokinin B) with three specific receptors (neurokinin-1, neurokinin-2 and neurokinin-3 respectively) held sway until the 1990's, when homologous binding experiments were finally performed (Huang *et al.*, 1995; Krause *et al.*, 1997). It was then demonstrated that all tachykinins have agonist effects on all tachykinin receptors, but with different affinities (see Table 1.2).

Table 1.2 Binding affinities for human NK-1, NK-2, and NK-3 receptors.

	NK_1	NK_2	NK_3
Substance P	0.12	240	780
Neurokinin A	14	0.72	640
Neurokinin B	0.5	n.a.	1.1
Hemokinin-1 (human)	1.8	480	370
Hemokinin-1 (mouse)	$0.12_{\text{mouseNK-1}}$ $0.13_{\text{human NK-1}}$	74	3200

In **Table 1.2**, the indicated affinities of substance P, Neurokinin A and Hemokinin-1 (Half maximal inhibitory concentrations (IC_{50}) in nM) are from homologous radioligand-binding experiments (Kurtz *et al.*, 2002). The data for Neurokinin B is based on half maximal excitatory concentration (EC_{50}) values for the stimulation of phosphatidyl inositol turnover in transfected cells (Krause *et al.*, 1997).

Hemokinin-1 has also been demonstrated to be a full agonist at all three neurokinin receptors, with highest affinity for the neurokinin-1 receptor and a functional activation profile similar to that of substance P (Morteau *et al.*, 2001; Kurtz *et al.*, 2002; Camarda *et al.*, 2002; Bellucci *et al.*, 2002).

1.11.5 Interaction between tachykinin and receptor

1.11.5.1 Agonists

Most research has been conducted on substance P / neurokinin-1 receptor interactions. As previously mentioned, the natural ligand with the highest affinity for the neurokinin-1 receptor is substance P itself. The other major tachykinins can activate the neurokinin-1 receptor, but with lower affinity (see section 1.11.4.3). The C-terminal sequence of substance P is essential for significant agonist activity, with the shortest fragment that results in significant activation being the C-terminal hexapeptide. Initially analogues such as [pGlu^{6}, MePhe^{8}, Sar^{9}]-substance P(5-11) were developed which produced hypotension when injected intravenously in both the rabbit and rat (Sandberg *et al.*, 1981; Lee *et al.*, 1982b). This was later found to be a fairly non-specific agent, activating NK3 receptors with greater affinity than NK1 receptors (Drapeau *et al.*, 1987). Subsequently, research into more specific agonist compounds was conducted. The first major group are considered 'classical' agonists, and include substance P methyl ester (Fox *et al.*, 1996), [Sar^{9}Met(O_2)^{11}]-substance P (Figini *et al.*, 1997), and substance P sulfone (Tousignant *et al.*, 1991). The second group are the 'septide like' compounds, including septide itself (Petitet *et al.*, 1992; Pradier *et al.*, 1994), GR73,632 and [Glu(Obzl)^{11}]substance P (Meini *et al.*, 1995). These 'septide like' agonists are problematic as they are potent activators of the neurokinin-1 receptor, however septide does not demonstrate any significant affinity for the neurokinin-1 receptor in competition binding experiments using radiolabelled substance P (Maggi and Schwartz, 1997). Possible explanations include two distinct binding sites on the neurokinin-1 receptor for substance P and the 'septide like' compounds, or alternatively there may be two active conformations of the receptor,

which are differentially activated (Maggi and Schwartz, 1997). Indeed, an intriguing paper was published in 2002 by Sachon et al, suggesting that there are analogues of substance P which are agonists at one binding site on the neurokinin-1 receptor, and antagonists at a second binding site using a different second messenger system (Sachon *et al.*, 2002)

1.11.5.2 Antagonists

To further characterize the tachykinin / receptor interactions, selective antagonists to substance P were developed, being the only tachykinin known at the time. This was initially via substitution with D-amino acids for L-amino acids on the substance P backbone, creating the first substance P antagonist, [D-Pro^{2}, D-Trp^{7,9}]-substance P (Folkers *et al.*, 1981; Leander *et al.*, 1981; Holmdahl *et al.*, 1981). Unfortunately, these 'first generation' substance P antagonists suffered from several drawbacks, including neurotoxicity (Post and Paulsson, 1985), potent mast cell degranulation (Fewtrell *et al.*, 1982), and poor selectivity (Yachnis *et al.*, 1984).

The 'second generation' antagonists are peptides that have alterations in the Gly^{9} region of the C-terminal hexapeptide fragment. These peptide antagonists, such as FK888 (Hagiwara *et al.*, 1994; Wang *et al.*, 1994a), FR113680 (Hagiwara *et al.*, 1992), and S18523 (Bonnet *et al.*, 1996) were more potent, more selective and less neurotoxic than the first generation antagonists. The peptide structure of these agents makes them susceptible to enzymatic degradation, limiting their usefulness, as did poor oral bioavailability (for human use) and CNS penetration (Khawaja and Rogers, 1996; Harrison and Geppetti, 2001).

The 'third generation' antagonists are non-peptide compounds whose discovery represented a significant breakthrough. The first of these was CP 96,345 (Snider *et al.*, 1991), followed by CP 99 994 (Desai *et al.*, 1992; McLean *et al.*, 1993) and SR 140333 (Emonds-Alt *et al.*, 1993). Limitations were subsequently found in the use of CP 96,345, because of binding to L-type calcium channels (Schmidt *et al.*, 1992), non-selective effects on neurotransmission, and local anaesthetic activity (Wang *et al.*, 1994b). The number of non-peptide antagonists has grown immensely in recent years, and in a review by Quartara and Maggi in 1997, over 35 non-peptide tachykinin antagonists were described (Quartara and Maggi, 1997).

1.11.5.3 Neurokinin-1 receptor binding sites

Recent evidence suggests that substance P binds to the extracellular loops of the neurokinin-1 receptor, making contact with the second and third extracellular loops (Ulfers *et al.*, 2002). This paper also suggested that the N-terminal of the receptor is confers a degree of flexibility, allowing it to fold over the binding pocket, thus stabilising the interaction (Ulfers *et al.*, 2002). Other studies have also suggested that the third extracellular loop is involved in substance P binding (Fong *et al.*, 1992b). The first transmembrane domain and the amino terminal have also been implicated in normal substance P binding (Gether *et al.*, 1993a). Further research is required to elucidate the exact binding sites of substance P to the neurokinin-1 receptor.

The first antagonist to have binding sites on the neurokinin-1 receptor described to any degree was CP 96,345, which was shown to bind to the extracellular portions of the 5th and 6th transmembrane segments and the 4th transmembrane domain (Fong *et al.*, 1992b; Gether *et al.*, 1993a; Gether *et al.*, 1993b). Since then, multiple neurokinin-1

receptor antagonists have had their bindings sites described, mostly using site directed neurokinin-1 receptor mutagenic experiments (Jenson *et al.*, 1994; Gether *et al.*, 1994; Fong *et al.*, 1994; Huang *et al.*, 1994; Cascieri *et al.*, 1995; Greenfeder *et al.*, 1999).

1.11.5.4 Receptor internalisation

The neurokinin-1 receptor, along with the other tachykinin receptors, undergoes rapid agonist-induced endocytosis and recycling (Grady *et al.*, 1995; Mantyh *et al.*, 1995; McConalogue *et al.*, 1998; Jenkinson *et al.*, 2000; Schmidlin *et al.*, 2002; Schmidlin *et al.*, 2003). This endocytosis is dependent on β-arrestins, which rapidly translocate from the cytosol to the membrane following receptor activation and are essential for agonist-induced G-protein coupled endocytosis in many receptor systems, including neurokinin receptors (Ferguson *et al.*, 1995; Goodman *et al.*, 1996; Schmidlin *et al.*, 2002; Schmidlin *et al.*, 2003).

1.11.6 Intracellular effectors

There are multiple intracellular effector systems that are activated following ligand activation of a tachykinin receptor. Figure 1.7 is a schematic representation of the intracellular effects of neurokinin receptor activation. The most important intracellular effector is phospholipase C (PLC) (Hanley *et al.*, 1980; Watson and Downes, 1983). In G-protein coupled tachykinin receptors, the G-protein is coupled to one of the intracellular loops of the receptor. The G-protein consists of several associated subunits, termed α, β and γ, which are bound to a guanosine diphosphate molecule (GDP). After activation of the tachykinin receptor there is a change in the receptor

shape that results in the G-protein exchanging GDP for guanosine triphosphate (GTP). This activates the G–protein, causing the α subunit to dissociate and activate (after binding to) the major intracellular effector, PLC (Hanley *et al.*, 1980; Watson and Downes, 1983). Activation of PLC results in the cleavage of phosphatidyl inositol biphosphate (PIP_2) within the cell membrane, to create two second messengers-inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (Taylor *et al.*, 1986; Song *et al.*, 1988). IP_3 binds to receptors on the sarcoplasmic reticulum and causes the release of intracellular Ca^{2+} stores. DAG, acting with Ca^{2+} activates protein kinase C (PKC) and subsequently opens voltage sensitive Ca^{2+} channels further increasing the levels of intracellular Ca^{2+} (Gallacher *et al.*, 1990). The increase in intracellular Ca^{2+} is thought to result in the major tissue responses, especially smooth muscle contraction and secretion in salivary glands, to neurokinin receptor activation. The G-protein α subunit also hydrolyses the GTP back to GDP and binds to the other subunits (β and γ), ending the continued activation of the messenger cascade.

Other intracellular effector mechanisms also play a role, such as activation of adenylate cyclase and subsequent increase in intracellular cyclic AMP formation at high tachykinin concentrations (Narumi and Maki, 1978; Yamashita *et al.*, 1983; Nakajima *et al.*, 1992).

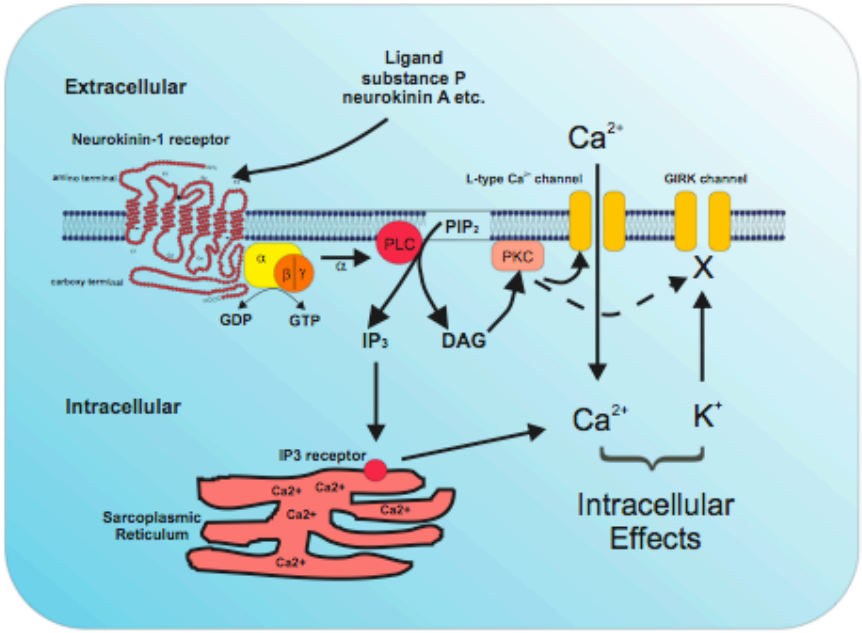

Figure 1.7. Intracellular effectors for the neurokinin-1 receptor. For a description, see section 1.11.6. GDP, guanosine diphosphate; GTP, guanosine triphosphate; PLC, phospholipase C; PKC, protein kinase C; PIP_2, phosphotidyl inositol biphosphate; IP_3, inositol triphosphate; DAG, diacylglycerol. GIRK, G-protein coupled inward rectifier K^+. The dotted line indicates inhibition of GIRK channels by activated PLC

Tachykinins evoke membrane depolarisation in both neurons and smooth muscle, primarily through a reduction in K^+ conductance (Hösli *et al.*, 1981; Minota *et al.*, 1981; Fujisawa and Ito, 1982; Li and Guyenet, 1997a). Of particular interest is evidence that activated PLC and subsequently activated PKC result in an inhibition of G-protein coupled inward rectifier K^+ (GIRK) channels (Mao *et al.*, 2003). This may be an important mechanism in the substance P evoked depolarisation of neurons in the RVLM, described by li et al (Li and Guyenet, 1997). This paper, although demonstrating that an alteration in K^+ conductance is most likely responsible for sympathoexcitatory RVLM neuron depolarisation, did suggest that reduction in a potassium leak current, rather than GIRK inhibition, was the major factor. This mechanism has also been demonstrated in rat spinal motor neurons (Fisher and Nistri, 1993). It may be that a combination of the two mechanisms is responsible.

1.11.6.1 Tachykininergic co-transmission

As described in section 1.11.3, substance P and neurokinin A both derive from the PPT-A gene. These peptides are co-localised and co-released from enteric neurons and sensory nerves (Hua *et al.*, 1985; Helke and Niederer, 1990; Maggi, 1995; Holzer and HolzerPetsche, 1997). As discussed in section 1.11.4.3, the different tachykinins, such as substance P and neurokinin A can all bind and activate the different tachykinin receptors, albeit with different binding affinity (Huang *et al.*, 1995; Krause *et al.*, 1997; Kurtz *et al.*, 2002). This leads to a system of duplication where a single neurotransmitter can activate multiple different receptors and that a single receptor is available to be activated by multiple different co-released neurotransmitters (Maggi, 2000). With the development of specific potent receptor antagonists, several of the

complex mechanisms by which co-transmission occurs have been demonstrated such as summation, cooperation and specialization (Maggi, 2000). Summation refers to the co-transmitters each activating their preferred receptor on the target cell and the effectors inducing a summed response that does not show any sign of specialization. This mechanism has been demonstrated in the rat urinary bladder (Maggi *et al.*, 1991; Meini and Maggi, 1994), rat small intestine (Maggi and Giuliani, 1995) and guinea pig common bile duct (Patacchini *et al.*, 1998). Cooperation refers to the adding of the two effector signals (as in summation) in the target cell to reach a threshold whereby a final event, such as the firing of an action potential, occurs. In this situation, blocking one of the effector signals (i.e. with a neurokinin-1 receptor antagonist) is sufficient to prevent the final event from occurring (Zagorodnyuk *et al.*, 1995). This has been demonstrated in circular muscle of guinea pig duodenum (Maggi *et al.*, 1994; Zagorodnyuk *et al.*, 1995) and in the human small intestine (Zagorodnyuk *et al.*, 1997). Specialization has been demonstrated with activation of neurokinin-1 or neurokinin-2 receptors in the guinea pig colon (Maggi *et al.*, 1997). In this paper, electrically induced colon smooth muscle contraction could be entirely blocked by a selective neurokinin-1 receptor antagonist if the stimulation was for a brief period, however only when the stimulation was for a prolonged period was any significant attenuation obtained with a selective neurokinin-2 receptor antagonist. This physiological response is separated both in temporal terms, and in the effector mechanisms which produce the final response (Maggi *et al.*, 1997).

1.11.6.2 Tachykinin receptor isoforms

Tachykinin receptors are encoded by genes with five exons, separated by introns. This is unusual in G-protein coupled receptors, most of which have genes that do not contain introns (Minneman, 2001). The introns are located in identical places in the tachykinin receptors, separating the exons (Hershey *et al.*, 1991; Gerard *et al.*, 1993). The presence of introns in the genes encoding for the tachykinin receptors may allow for alternative splicing and the expression of different receptor isoforms. In fact, different isoforms of the neurokinin-1 receptor have been demonstrated in several studies. These isoforms differ in the length of their carboxy-terminal tail (Aanonsen *et al.*, 1992; Fong *et al.*, 1992a; Mantyh *et al.*, 1996; Beaujouan *et al.*, 2000; Baker *et al.*, 2003). In the cane toad, the three identified neurokinin-1 receptor isoforms differ in substance P and neurokinin A binding affinities, raising the possibility of specific physiological roles (Liu *et al.*, 2004). Some differential expression of neurokinin-1 receptor isoforms occurs in humans, with the truncated carboxy-terminal tail isoform being more prevalent in peripheral tissues, and the long form more prevalent in the brain (Caberletto *et al.*, 2003). The neurokinin-2 receptor also has different isoforms, identified in both the rat and human (Candenas *et al.*, 2002; Bellucci *et al.*, 2004; Meini *et al.*, 2005). The functional significance, if any, of these neurokinin receptor isoforms remains to be determined.

1.11.6.3 Tachykinin receptor conformation differences

There are many papers suggesting that G-protein coupled receptors can exist in different conformations. These different conformations can be activated in a ligand specific manner, and they can have different intracellular effects (Palanche *et al.*, 2001;

Gazi *et al.*, 2003; Swaminath *et al.*, 2004; Perez and Karnik, 2005). This has been demonstrated for the neurokinin-1 receptor (Rosenkilde *et al.*, 1994; Hastrup and Schwartz, 1996), the neurokinin-2 receptor (Palanche *et al.*, 2001; Lecat *et al.*, 2002), the dopamine (D2) receptor (Gazi *et al.*, 2003), and other receptors (for review, see (Perez and Karnik, 2005)). It may be a general feature of G-protein-coupled receptors that each ligand induces a unique conformational change in the receptor, and that different ligands acting on the same receptor result in different intracellular effects (Perez and Karnik, 2005).

1.11.7 Distribution of tachykinins and neurokinin receptors

1.11.7.1 Distribution of tachykinins

The distribution of tachykinins in mammals has been investigated by radioimmunoassay (Kanazawa *et al.*, 1984; Ogawa *et al.*, 1985; Shults *et al.*, 1985; Arai and Emson, 1986; Tateishi *et al.*, 1989). Using *in situ* hybridisation, the distribution of tachykinin precursor mRNAs (i.e. PPT-A and PPT-B) in the CNS has been studied and there is broad agreement with the radioimmunoassay experiments (Warden and Young, 1988; Harlan *et al.*, 1989). These studies demonstrate that substance P and neurokinin A are generally distributed in the same regions, predominantly within the CNS, spinal cord and primary afferent sensory neurons supplying peripheral tissues (Holzer, 1988). Occasionally neurokinin A is not co-expressed with substance P (Dalsgaard *et al.*, 1985). Further, neurokinin B is expressed primarily in the CNS and spinal cord in a different distribution to that of substance P/ neurokinin A, and is expressed in very low quantities in peripheral tissues (Warden

and Young, 1988; Harlan *et al.*, 1989; Tateishi *et al.*, 1990; Moussaoui *et al.*, 1992). Although substance P, neurokinin A and neurokinin B are extensively expressed in neural tissue, their expression has also been demonstrated in multiple non-neural tissues, indicating extremely diverse physiological roles. Substance P has been demonstrated in mouse and human Leydig cells (Chiwikata *et al.*, 1991), inflammatory and immune cells (Pascual and Bost, 1990; Ho *et al.*, 1997; Lai *et al.*, 1998), human endothelial cells (Linnik and Moskowitz, 1989), epithelial cells (Chu *et al.*, 2000), fibroblasts (Bae *et al.*, 2002), enterochromaffin cells (Simon *et al.*, 1992), smooth muscle cells (Khan and Collins, 1994; Maghni *et al.*, 2003), and within the uterus (Patak *et al.*, 2003). Neurokinin B mRNA has been demonstrated in uterine and placental tissue (Page *et al.*, 2000; Pinto *et al.*, 2001; Patak *et al.*, 2003). A summary of the distribution of tachykinins and their receptors in neural and selected non-neural tissues is provided in Table 1.3

Table 1.3. Distribution of Tachykinins and Tachykinin Receptors.

	Tachykinins, pmol/g			**Receptors**			**Comment**
Region	**SP**	**NKA**	**NKB**	**NK_1**	**NK_2**	**NK_3**	
Cerebral cortex	7.0	4.0	2.9	+	-	++++	1
Striatum	312.2	26.2	1.2	++	-	++	
Globus Pallidus	114.7	68.9	8.3				
Substantia nigra	1154.2	115.0	2.8	-	-	+	2
Ventral Tegmentum	115.4	68.9	17.2	-	-	+	3
Nucleus accumbens	122.9	70.4		++	++	+/-	
Hipppocampus	4.1	3.0	1.1	++	+	++++	4
Hypothalamus	201.4	171.3	31.4	-	-	++++	5
Habenula	406.0	240.2		+++	-	+	6
Interpeduncular nucleus	474.6	168.3		+	+	++++	
Cerebellum	4.7	1.6	1.6	+++	-	+	
NTS	459.0	389.2	21.7	++	-	+++	
Raphe nuclei	275.0	83.0		+++	-	+	7
Medulla Oblongata	226.4	71.1	7.2				
Dorsal motor nucleus Vagus				++++	-	+++	
Hypoglossal nucleus				+++	-	-	
Nucleus Ambiguus				++	-	-	
Spinal trigeminal Nucleus				++	-	-	
Spinal cord							
Dorsal Horn	503.7	65.9	9.1	++	-	++++	8
IML	121.6	126.0	31.6	++++	-	-	8
Ventral Horn	117.4	16.1	2.0	++	-	-	8
Ileum	47.8	23.9	0.44	+++	++	+	8

Colon	20.1	16.1	2.0	++++	++++	+	8
Parotid Gland	16.2	0.4	0.4	+++	-	-	8
Submandibular Gland	12.5	5.0	0.5	++++	-	-	
Heart	0.4	0.2	0.3	-	-	-	
Bladder	1.7	3.6	0.3	++++	++++	+	
Adrenal Gland	1.2	0.8	1.0	-	++++	-	
Skin	2.5			+	-	-	

Data for tachykinin concentrations in pmol/g are derived from (Otsuka and Yoshioka, 1993). Tachykinin receptor distribution data in CNS are from Saffroy et al, except for data for ventral tegmentum, which is from Mantyh et al (Saffroy *et al.*, 2003; Mantyh *et al.*, 1989). Comments: 1=layer 5 frontal cortex area 10, 2= substantia nigra, compact part. Reticular part had no NK_3 receptor, 3=data from (Mantyh *et al.*, 1989), 4=CA1 field, ventral pyramidal cell layer, 5=paraventricular nucleus, magnocellular part, 6= medial habenular nucleus, 7 =dorsal raphe nuclei, 8 = data from relative mRNA for receptors (Tsuchida *et al.*, 1990).

1.11.7.2 Distribution of neurokinin receptors

In mammals, the distribution of neurokinin-1 receptors within the CNS has been studied by autoradiography (Dam and Quirion, 1986; Danks *et al.*, 1986; Saffroy *et al.*, 1988), by neurokinin-1 receptor mRNA expression (Sivam and Krause, 1992; Aubry *et al.*, 1994; Whitty *et al.*, 1995; Whitty *et al.*, 1997), and by immunohistochemistry (Shigemoto *et al.*, 1993).

The distribution of the neurokinin-2 receptor has also been extensively studied. Within the CNS, it has been studied by autoradiography (Quirion *et al.*, 1991; Saffroy *et al.*, 2001; Saffroy *et al.*, 2003), in situ hybridisation mRNA studies (Stumm *et al.*, 2001; Bensaid *et al.*, 2001), and immunohistochemistry (Steinberg *et al.*, 1998).

Neurokinin-3 receptor distribution has been studied by autoradiography (Mantyh *et al.*, 1989; Saffroy *et al.*, 2003), and mRNA distribution (Sasai and Nakanishi, 1989;

Shigemoto *et al.*, 1990). As for the distribution of tachykinins, the distribution of tachykinin receptors is summarized in Table 1.3.

1.11.8 Substance P in the ventral medulla

1.11.8.1 Substance P in the baroreflex

As previously described in section 1.5, the baroreceptor reflex pathway begins with innervation of the NTS by baroreceptor afferents from the carotid sinus and aortic arch (Paintal, 1973; Kirchheim, 1976). Multiple nuclei within the NTS receive afferent fibres from the carotid sinus nerve and aortic arch, including the commissural subnucleus; the medial, lateral and dorsolateral subnuclei at the level of the obex; and the medial, lateral and ventrolateral subnuclei at the level rostral to the obex (Ciriello *et al.*, 1981a). These afferent fibres contain both baroreceptor and chemoreceptor information (Ciriello *et al.*, 1981a). The bulk of the research carried out on substance P and its role in the baroreflex has been focused on the NTS.

In the NTS significant substance P immunoreactivity is present in nerve terminals (Cuello and Kanazawa, 1978; Helke *et al.*, 1980b). Significant substance P immunoreactivity is also found within nerves of multiple modality, including the 9th cranial nerve, the nodose ganglion and within the vagus nerve (Ljungdahl *et al.*, 1978; Lundberg *et al.*, 1978).

An important paper by Helke *et al* in 1980, demonstrated that substance P immunoreactivity was present within the NTS where baroreceptor afferents are known to terminate (Helke *et al.*, 1980b). This paper also demonstrated substance P immunoreactivity within the tunica adventitia of the aortic arch and carotid sinus, and

within the vagus nerve and nodose ganglia, agreeing with several papers from two years previously (Cuello and Kanazawa, 1978; Ljungdahl *et al.*, 1978; Lundberg *et al.*, 1978; Helke *et al.*, 1980b). New in this paper, however, was a demonstration that unilateral surgical removal of the nodose ganglion ipsilaterally decreased the substance P immunoreactivity in areas within the NTS known to receive vagal afferent fibres (Helke *et al.*, 1980b).

Many other anatomical experiments provide evidence for the involvement of substance P in the baroreceptor afferent nerves from the aortic arch and carotid sinus. The substance P precursor mRNA, β-preprotachykinin, is present within the nodose ganglion, in similar proportions (26%) to the percentage of neurons immunoreactive to substance P (Hamid *et al.*, 1991). Substance P immunoreactivity is known to be present within neurons in the afferent baroreceptor pathway, such as the aortic nerve (Helke *et al.*, 1980a), vagal and glossopharyngeal afferent neurons (Helke and Hill, 1988; Kummer, 1988), and the nodose and petrosal ganglia (Helke and Niederer, 1990; Ichikawa *et al.*, 1993).

Substance P immunoreactivity has been demonstrated within nerve terminals in the NTS (Armstrong *et al.*, 1981; Maley and Elde, 1982; Kalia *et al.*, 1984; Kubota *et al.*, 1985; Maley *et al.*, 1989; Gatti *et al.*, 1995). Of particular importance is the study by Gatti *et al* in 1995, which used a combination of immunohistochemistry, transganglionic transport of horseradish peroxidase from the carotid sinus, and electron microscopy (Gatti *et al.*, 1995). This study demonstrated extensive substance P immunoreactivity within the NTS, and also demonstrated by electron microscopy that some of the substance P immunoreactive axons and nerve terminals within the NTS were also carotid sinus nerve primary afferent fibres (Gatti *et al.*, 1995). Substance P-

immunoreactive axon terminals within the NTS make synaptic contact with NTS neurons that project to the catecholaminergic cell region of the CVLM (Kawano and Masuko, 1997).

The release of substance P within the NTS was first demonstrated *in vitro*, where substance P efflux was demonstrated following exposure to depolarising levels of capsaicin and potassium (Helke *et al.*, 1981). A further *in vivo* study in the rabbit using microdialysis has also demonstrated substance P release within the NTS (Morilak *et al.*, 1988). This paper provided good evidence that this release was associated with baroreceptor stimulation, as it was secondary to stimulation of the aortic depressor nerve, which in the rat contains only baroreceptor afferent fibres (Numao *et al.*, 1985). Activation of baroreceptors via an aortic balloon catheter in cats results in substance P release in the NTS, an effect abolished by sinoaortic denervation (Potts and Fuchs, 2001).

As previously noted in section 1.11.4.3, the receptor with the highest affinity to substance P is the neurokinin-1 receptor. The neurokinin-1 receptor has been demonstrated in the NTS in both the rat and the cat (Helke *et al.*, 1984; Danks *et al.*, 1986; Maley *et al.*, 1989). These receptors are not thought to be located on vagal afferent fibres, as destruction of these fibres does not alter the neurokinin-1 receptor binding in the NTS (Helke *et al.*, 1984; Helke *et al.*, 1985). Further, in cats, up-regulation of substance P receptors occurs in the NTS when the afferent input is removed (Segu *et al.*, 1991).

In the cat, iontophoretic application of substance P to functionally identified neurons in the NTS results in neuronal excitation (Morin-Surun *et al.*, 1984). NTS slice

preparations using extracellular recording *in vitro* have also demonstrated neuronal excitation when exposed to substance P (Barnes *et al.*, 1991). Using i*n vitro* intracellular recording, substance P also results in depolarisation and an increase in input resistance of NTS neurons in slice preparations (Jacquin *et al.*, 1989). Similar NTS neuronal excitation has been demonstrated using neurokinin-1 receptor agonists such as septide, GR73632, and substance P-O-methylester (Maubach and Jones, 1997). In this paper, these effects were blocked by specific neurokinin-1 receptor antagonists (Maubach and Jones, 1997).

Functional studies investigating substance P within baroreceptor afferent nerves terminating in the NTS have provided conflicting results. Intracerebral ventricular administration of substance P has yielded increases in arterial blood pressure (Haeusler and Osterwalder, 1980; Unger *et al.*, 1988; Brattstrom and Seidenbecher, 1992; Tschope *et al.*, 1995), no effect (Appenrodt *et al.*, 1993), and decreases (Chan *et al.*, 1990). Intracerebral ventricular administration of substance P is an extremely non-specific method of administration, as substance P has been shown to play a role in arterial blood pressure regulation in other regions within the brainstem, such as the RVLM and CVLM (Urbanski *et al.*, 1989; Wang *et al.*, 2002b).

More specific *in vivo* experiments, involving microinjection of substance P into the NTS, have also demonstrated conflicting results, with some studies suggesting a decrease in blood pressure and heart rate (Hall *et al.*, 1989; Chan *et al.*, 1990b) (Haeusler and Osterwalder, 1980; Kubo and Kihara, 1987; Feldman, 1995), whilst other studies have suggested no effect on resting blood pressure and heart rate (Talman and Reis, 1981; Seagard *et al.*, 2000). Still further, a paper in 1993 demonstrated that

NTS microinjection of substance P in conscious rats results in an increase in blood pressure and tachycardia (Abdala *et al.*, 2003).

Pre-treatment of the NTS with neurokinin-1 receptor antagonists blocks previously demonstrated falls in arterial blood pressure following microinjection of substance P into the NTS (Kubo and Kihara, 1987; Feldman, 1995; Zhang *et al.*, 2000a). Carotid sinus nerve stimulation (simulating stimulation of baroreceptor afferent nerves) increases the firing rate of NTS neurons and this effect is attenuated by iontophoresis of a substance P antagonist (Miura *et al.*, 1987).

Separate from the effects on arterial blood pressure, the effects on baroreflex sensitivity of substance P microinjection into the NTS have been investigated. Most studies demonstrate an increase in the baroreceptor reflex sensitivity (Lorez *et al.*, 1983; Hall *et al.*, 1989; Chan *et al.*, 1990b; Seagard *et al.*, 2000), however a paper by Feldman *et al* in 1995 did not show any change in baroreflex sensitivity (Feldman, 1995). Baroreflex gain is also significantly attenuated when NTS neurokinin-1 receptor immunoreactive neurons are chemically ablated with substance P conjugated with the neurotoxin, saporin (Riley *et al.*, 2002).

What are we to make of the conflicting results described above? The general trend for these studies investigating the role of substance P and the neurokinin-1 receptor in the NTS on the arterial baroreflex is that they do play a significant role, however, with several caveats. It may be that the conflicting results seen in microinjection experiments are due to regional NTS specificity, as has been demonstrated in the NTS in baroreflex sensitivity experiments (Seagard *et al.*, 2000). An experiment in conscious rats, where substance P administered into the NTS resulted in an increase in

blood pressure, also raises the possibility that anaesthesia may significantly alter the effects of substance P in the NTS (Abdala *et al.*, 2003). Further, substance P and the neurokinin-1 receptor have been shown to be involved in many brainstem autonomic reflexes (see sections 1.11.8.4-1.11.8.6), and these reflexes may interact with the baroreflex in a complex manner.

It may be that substance P plays only a modulatory neurotransmitter role on the baroreflex in the NTS. The excitatory neurotransmitter glutamate, when microinjected into the NTS, produces profound falls in arterial blood pressure and heart rate, leading to the suggestion that glutamate is the primary neurotransmitter involved in the NTS transmission of baroreceptor information (Talman *et al.*, 1980). Baroreflex sensitivity experiments have also tended to show an attenuation or little effect on baseline values, rather than an abolition of the baroreflex, when NTS neurokinin-1 receptors have been ablated or antagonists used (Chan *et al.*, 1990; Feldman, 1995; Martini *et al.*, 1995; Seagard *et al.*, 2000; Riley *et al.*, 2002). Lastly, substance P immunoreactivity is present in only approximately 15% of carotid sinus nerve terminals within the dorsolateral NTS of the cat, suggesting that substance P has a modulatory neurotransmitter role rather than being the primary neurotransmitter in the NTS (Massari *et al.*, 1998).

1.11.8.2 Substance P in the baroreflex- the CVLM

Much less research has been conducted on substance P and the neurokinin-1 receptor in the rest of the baroreflex pathway within the brainstem, such as the CVLM and RVLM. As mentioned in section 1.5.2, excitatory neuronal projections from the NTS to the CVLM are thought to be the next step in baroreceptor signal transduction.

NTS neuronal cell bodies with projections to the CVLM receive synaptic contacts with substance P-immunoreactive axon terminals (Kawano and Masuko, 1997). Again, as mentioned in section 1.5.3, the excitation of CVLM GABA-ergic neurons with projections to the RVLM is thought to be essential for baroreceptor signal transduction (Willette *et al.*, 1983a; Willette *et al.*, 1983b; Willette *et al.*, 1984b). Microinjection of [pGlu^{5}, MePhe^{8}, Sar^{9}]-SP(5-11), a relatively non-specific substance P agonist, into the CVLM causes dose dependent decreases in heart rate but no effect on arterial blood pressure (Urbanski *et al.*, 1989). There are no papers describing neurokinin-1 receptor immunoreactivity within barosensitive inhibitory neurons projecting to the RVLM. This does not mean that substance P and the neurokinin-1 receptor do not have a role to play in signal transduction within the CVLM. Two studies by Wang *et al* have suggested a modulatory neurotransitter role (Wang *et al.*, 2002a; Wang *et al.*, 2003). Ablation of neurokinin-1 receptor immunoreactive neurons within the ventrolateral medulla by microinjection of a neurokinin-1 receptor agonist conjugated to the neurotoxin saporin, results in almost complete and specific elimination of these neurons within the CVLM (Wang *et al.*, 2002a; Wang *et al.*, 2003). Microinjection of the excitatory amino acid, DL-homocysteic acid (DLH), into the CVLM in these rats results in a markedly attenuated depressor effect and a decrease in the number of

baroactivated CVLM neurons as shown by cFOS activation after i.v. phenylephrine injection (Wang *et al.*, 2003). Given that the majority of the neurokinin-1 receptor immunoreactive neurons of the VLM are glutamatergic (Guyenet *et al.*, 2002), these results were attributed to the destruction of excitatory propriomedullary neurons, perhaps from adjacent respiratory centres, that express the neurokinin-1 receptor and might facilitate the activation of GABA-ergic CVLM neurons (Wang *et al.*, 2003). It remains possible, however, that some of the neurokinin-1 receptor expressing neurons of the CVLM that were destroyed, were in fact GABA-ergic barosensitive neurons that project to the RVLM- a possibility still consistent with the data presented in this study (Wang *et al.*, 2003). A final possibility suggested in this paper was that the neurokinin-1 receptor immunoreactive GABA-ergic neurons destroyed within the CVLM might represent a subset of CVLM neurons that are depressor neurons but not involved in the baroreflex (and therefore not cFos positive following intravenous phenylephrine). Some GABA-ergic neurons that expressed the neurokinin-1 receptor were indeed demonstrated in a slightly more ventral location than the CVLM barodepressor cell column (Wang *et al.*, 2003). The existence of a group of depressor neurons that are not barosensitive has previously been suggested, however the location was thought to be caudal rather than ventral to the classic barosensitive depressor CVLM neurons (Cravo *et al.*, 1991).

1.11.8.3 Substance P in the baroreflex- the RVLM

As mentioned in section 1.5, the final common pathway for the brainstem transmission of brainstem baroreceptor information is thought to be the RVLM.

The RVLM contains a generally high level of the neurokinin-1 receptor (Helke *et al.*, 1984; Nakaya *et al.*, 1994). Ultrastructurally, the putative sympathoexcitatory C1 neurons of the RVLM receive synapses from substance P containing terminals (Milner *et al.*, 1988). These substance P containing terminals are thought to arise predominantly from neurons projecting from the raphe pallidus, with much smaller contributions from the NTS, paraventricular nucleus of the hypothalamus, the lateral hypothalamus and the raphe obscurus (Milner and Giuliano, 1996). In spite of these findings, and although neurokinin-1 receptor immunoreactivity has been demonstrated on spinally projecting RVLM neurons (Wang *et al.*, 2001), at least two papers have failed to demonstrate neurokinin-1 receptor immunoreactivity on C1 neurons (Chen *et al.*, 2000; Wang *et al.*, 2001). Chapter 4 describes a study conducted to explore the distribution of neurokinin-1 receptor expression in relation to the catecholamine containing and bulbospinal neurons of the RVLM.

Microinjection of the stable substance P agonist, [pGlu^5, MePhe^8, Sar^9]-SP(5-11), into the RVLM *in vivo* results in large dose dependent pressor responses (Urbanski *et al.*, 1989). Substance P excites unidentified extracellularly recorded RVLM neurons *in vitro* (Sun and Guyenet, 1989), and bulbospinal C1 neurons recorded with patch electrodes *in vitro* (Li and Guyenet, 1997b). Prior to the paper published from the study described in chapter 5, no studies describing the sympathetic nerve response or baroreceptor reflex function following activation or antagonism of neurokinin-1 receptors in the RVLM had been published.

As previously described, many RVLM C1 neurons are bulbospinal, barosensitive neurons that provide tonic excitatory drive to spinal sympathetic preganglionic neurons

(Guyenet *et al.*, 1989; Lipski *et al.*, 1996). The principle fast neurotransmitter within these neurons is thought to be glutamate (Stornetta *et al.*, 2002a; Stornetta *et al.*, 2002b). Many other neurotransmitters are found within subpopulations of RVLM neurons, such as substance P (Lorenz *et al.*, 1985; Pilowsky *et al.*, 1986; Milner *et al.*, 1988). Approximately 40% of spinal SPNs that are hypotension activated are neurokinin-1 receptor immunoreactive (Burman *et al.*, 2001), and substance P depolarises spinal SPNs via neurokinin-1 receptor activation (Dun and Mo, 1988; Solomon S *et al.*, 1999). The RVLM is known to be one of the main supraspinal sources of spinal substance P (along with the hypothalamus, caudal raphe nuclei and ventromedial medulla) (Menetrey and Basbaum, 1987; Sasek *et al.*, 1990). An early study suggested that the majority of RVLM adrenergic neurons contained substance P (Lorenz 1985). This was contradicted by a later study that suggested this high figure was incorrect due to antibody cross-reactivity, and that a more accurate figure was in the order of 1-2% (Pilowsky *et al.*, 1986). A recent study from our laboratory using *in situ* hybridisation and immunohistochemistry for preprotachykinin A mRNA suggest that 18-20% of bulbospinal C1 neurons are likely to express substance P or alternatively, neurokinin A (Li *et al.*, 2005).

1.11.8.4 Substance P / NK1 receptor in respiratory neurons

Microiontophoretic application of substance P onto single identified NTS respiratory neurons results in neuronal excitation (Henry and Sessle, 1985). Intracerebroventricular injections of substance P also results in increases in respiratory tidal volume and minute ventilation in anaesthetized rats (Hedner *et al.*, 1984). Similarly, dorsal medulla oblongata application of substance P in rabbit pups increases

tidal volume and respiratory frequency, an effect antagonized by substance P antagonists (Yamamoto and Lagercrantz, 1985; Prabhakar *et al.*, 1987).

As previously mentioned in section 1.10.6.1, the generation of respiratory rhythm occurs within the ventral respiratory group (VRG). The pre-Bötzinger complex (preBötC) is a small region within the VRG containing propriomedullary neurons (Ellenberger and Feldman, 1990; Connelly *et al.*, 1992; Schwarzacher *et al.*, 1995). The preBötC is thought to be the main respiratory rhythm generating area of the VRG (Smith *et al.*, 1991; Connelly *et al.*, 1992), deriving from a small group of glutamatergic neurons with intrinsic bursting properties (Johnson *et al.*, 1994; Koshiya and Smith, 1999; Rekling *et al.*, 2000).

Microinjection or bath application of substance P into the preBötC region increases respiratory frequency (Monteau *et al.*, 1996; Johnson *et al.*, 1996; Gray *et al.*, 1999).

Characterization of the phenotype and development of reliable anatomic markers of the putative rhythm generating preBötC neurons has proven difficult. In 1999, Gray *et al* suggested that the neurokinin-1 receptor could be used as a marker for the type 1 respiratory rhythm generating propriomedullary neurons of the preBötC that are very responsive to substance P (Gray *et al.*, 1999). Further studies demonstrated that the majority of VRG neurokinin-1 receptor immunoreactive neurons are excitatory (Wang *et al.*, 2001), and that a subtype of electrophysiologically identified preBötC inspiratory neurons express the neurokinin-1 receptor (Guyenet and Wang, 2001). Neurokinin-1 receptor knockout mice lack the increase in respiratory motor output that occurs in wild type mice following exogenous substance P exposure in *in vitro* brainstem-spinal cord preparations (Ptak *et al.*, 2000). Major respiratory abnormalities such as an ataxic breathing pattern and pathological respiratory responses to hypoxia,

hyperoxia and anaesthesia occur with bilateral destruction of the preBötC neurokinin-1 receptor expressing neurons with substance P-saporin in adult rats (Gray *et al.*, 2001). Similarly, selective ablation of the preBötC neurokinin-1 receptor expressing neurons abolishes the tachypnoeic response to excitatory amino microinjection into the preBötC (Wang *et al.*, 2001). These studies suggest that neurons that express the neurokinin-1 receptor play an important role in normal brainstem respiratory function, particularly within the preBötC region. Although receptor knockout and chemical neuronal ablation experiments do not address the role that substance P and the neurokinin-1 receptor play in normal respiratory function, microinjection experiments suggest an important role (Monteau *et al.*, 1996; Johnson *et al.*, 1996; Gray *et al.*, 1999). However, the original proposal that the neurokinin-1 receptor could be used as a sole specific phenotypic marker for the propriomedullary respiratory rhythm generating neurons of the preBötC (Gray *et al.*, 1999), is probably not correct. Some preBötC neurokinin-1 receptor immunoreactive neurons are bulbospinal rather than propriomedullary, and a variety of different ventral medullary neurons express the neurokinin receptor (see results chapter 4) (Wang *et al.*, 2001; Makeham *et al.*, 2001). Further, less than 50% of electrophysiologically identified putative respiratory generating propriomedullary neurons of the preBötC express the neurokinin-1 receptor (Guyenet and Wang, 2001). The respiratory rhythm generating neurons of the preBötC most likely represent a subset of the neurokinin-1 receptor containing neurons of the VRG.

1.11.8.5 Brainstem substance P in the sympathetic chemoreflex

As described in section 1.6, activation of the peripheral chemoreceptors results in robust sympathoexcitation. The role of brainstem substance P and the neurokinin-1 receptor is not well understood.

Conflicting results have been described regarding the involvement of substance P in NTS sympathetic chemoreflex neurotransmission. Intracisternal injection of substance P produces similar respiratory effects to hypoxia in rabbits (Gallagher *et al.*, 1985). Substance P release in the NTS is increased following hypoxia in rabbits, an effect abolished by carotid sinus nerve denervation (Lindefors *et al.*, 1986; Srinivasan *et al.*, 1991). Neurokinin-1 receptor binding decreases after a single acute hypoxic episode (Laferriere *et al.*, 2003), and neurokinin-1 receptor density increases in the NTS after repeated bouts of hypoxia for 6 days (Rodier *et al.*, 2001). These results are consistent with receptor activation and desensitisation following acute hypoxia and subsequent up-regulation with chronic hypoxic episodes. Hypoxia also desensitises the respiratory responses to NTS microinjection of substance P, presumably due to hypoxic neurokinin-1 receptor activation and desensitisation (Mazzone *et al.*, 1998).

Given that microinjection of substance P into the NTS can result in pressor responses in awake rats (Zhang *et al.*, 2000a; Abdala *et al.*, 2003) (see section 1.11.8.1), and that substance P is released in the NTS in response to hypoxia (Lindefors *et al.*, 1986; Srinivasan *et al.*, 1991), substance P in the NTS may be involved in the generation of hypoxia induced sympathoexcitation. In contrast to this is the finding that microinjection of the highly selective neurokinin-1 receptor antagonist, WIN 51708, into the NTS fails to block the pressor and respiratory responses to peripheral chemoreceptor activation with potassium cyanide (Zhang *et al.*, 2000a). It must also be

noted that in the majority of experiments where substance P has been microinjected into the NTS the result has been a depressor response, although usually in anaesthetized animals (see section 1.11.8.1).

As discussed in section 1.6.2, the sympathoexcitatory component of the peripheral chemoreflex is thought to result from projections from the NTS to the RVLM, either monosynaptically or via local excitatory interneurons. The studies described in chapter 5 are the first to investigate the role of substance P and the neurokinin-1 receptor in the RVLM component of the sympathetic chemoreflex (Makeham *et al.*, 2005).

1.11.8.6 Substance P in the somato-sympathetic reflex

Within the brainstem, little is known about the role of substance P and the neurokinin-1 receptor in the somato-sympathetic reflex. The somato-sympathetic reflex pathway is described in section 1.7.

Intrathecal injection of the substance P antagonist spantide ([D-Arg^{1}, D-Trp^{7,9}, Leu^{11}]-substance P in the rat results in attenuation of both the A- and C- components of the somato-sympathetic reflex (Fujino *et al.*, 1987). A similar result occurs with intrathecal administration of the substance P antagonist CP-96, 345 (Adachi *et al.*, 1993). These effects are thought to be due to substance P related neurotransmission within the spinal cord (Fujino *et al.*, 1987; Adachi *et al.*, 1993). Within the spinal cord, the dorsal horn is the major site for the termination of primary afferent axons, ending predominantly in laminae I and II (Light and Perl, 1979; Sugiura *et al.*, 1987). Substance P is found within many nociceptive afferent neurons (Lawson 1997), the majority of which terminate in laminae I and II, although some terminate in deeper levels (Alvarez and Priestley, 1990; Lawson *et al.*, 1997; Sakamoto *et al.*, 1999).

The neurokinin-1 receptor is present within a well defined area of the dorsal horn, especially laminae I, III and IV (Brown *et al.*, 1995; Littlewood *et al.*, 1995; Todd *et al.*, 1998). Many lamina I neurokinin-1 receptor immunoreactive dendrites receive synapses from substance P containing axons (McLeod *et al.*, 1998). All dorsal horn neurons that are activated by substance P are also activated by nociception (Henry, 1976). Receptor internalisation (Mantyh *et al.*, 1995) or cFos expression (Doyle and Hunt, 1999) is seen in the majority of lamina I neurokinin-1 receptor immunoreactive neurons after noxious somatic afferent stimulation.

Less well known is the role of substance P and the neurokinin-1 receptor in the brainstem integration of the somato-sympathetic reflex. Mechanical stimulation of peripheral somatic afferents results in an attenuation of the cardiac vagal baroreflex via activation of neurokinin-1 receptors in the NTS (Boscan *et al.*, 2002; Pickering *et al.*, 2003). The first of these papers from Pickering *et al* investigated the effect on the sympathetic baroreflex of blocking neurokinin-1 receptors in the NTS, but found no effect (Pickering *et al.*, 2003). No studies have been conducted assessing the effects, if any, of brainstem tachykinin receptor activation or blockade on the A- and C-components of the somato-sympathetic reflex. The study described in chapter 5 is the first to examine the somato-sympathetic reflex after activation and blocking of neurokinin-1 receptors in the RVLM (Makeham *et al.*, 2005).

1.11.9 Tachykinins in nervous system disease

1.11.9.1 Subarachnoid haemorrhage (SAH)

Subarachnoid haemorrhage results in biphasic cerebral vasospasm and a subsequent decrease in cerebral blood flow (Shiokawa and Svendgaard, 1994; Svendgaard *et al.*, 1996; Svendgaard *et al.*, 1998). There is an increase in substance P-immunoreactivity in centrifuged CSF samples following experimental SAH in rabbits (Tran Dinh *et al.*, 1994). Following experimental SAH there is a significant decrease in substance P-immunoreactivity within the dura (Keller *et al.*, 1993; Arand *et al.*, 1994), perivascular nerves within supratentorial cerebral vessel walls (Hara *et al.*, 1986; Uemura *et al.*, 1987; Edvinsson *et al.*, 1990; Arand *et al.*, 1994), and within the basilar artery (Uemura *et al.*, 1987; Linnik *et al.*, 1989). Abnormal peptidergic perivascular innervation has also been demonstrated within human aneurysm sacs, the major cause of subarachnoid haemorrhage (Buki *et al.*, 1999).

Several studies have suggested that experimental SAH results in a decrease in the normal cerebral blood vessel endothelium dependent relaxation seen in response to substance P (Pasqualin *et al.*, 1992; Onoue *et al.*, 1995; Onoue *et al.*, 1998). Other studies have shown no change in substance P induced vessel relaxation following experimental SAH (Edvinsson *et al.*, 1990; Saito *et al.*, 1991).

As previously mentioned, SAH results in a biphasic vasospasm within the cerebral vasculature. Intrathecal pre-treatment with substance P antagonist spantide (Delgado-Zygmunt *et al.*, 1990; Svendgaard *et al.*, 1996; Svendgaard *et al.*, 1998) or anti-substance P immunoglobulin (Shiokawa *et al.*, 1993; Shiokawa and Svendgaard, 1994;

Svendgaard *et al.*, 1996; Svendgaard *et al.*, 1998) abolishes or significantly attenuates both phases of vasospasm. Curiously, one study has demonstrated an *increase* in SAH induced cerebral vasospasm following intrathecal pre-treatment with substance P antiserum (Locatelli, 2000). A further study demonstrated an abolition of both phases of SAH induced vasospasm with the peptide substance P antagonist spantide but not with a non-peptide neurokinin-1 receptor antagonist (Svendgaard *et al.*, 1998). The exact significance of this is uncertain.

1.11.9.2 Depression and anxiety disorders

The aetiology of depression and anxiety disorders is poorly understood, however they are thought to involve endogenous predisposing factors and an altered response to stress (McEwen, 2000).

Maternal separation in guinea-pigs results in substance P release within the amygdala, a key region involved in the stress response (Smith *et al.*, 1999). Substance P is also intimately related to monoamine containing neurons, through which most clinically useful antidepressants are thought to mediate their effects (Duman *et al.*, 1997). Approximately 50% of human dorsal raphe neuron (DRN) 5-HT-containing neurons also express substance P (Sergeyev *et al.*, 1999). Neurokinin-1 receptor antagonists in the DRN and mice with neurokinin-1 receptor deletion result in an increase in DRN neuron firing rate without an increase in 5-HT efflux in the cerebral cortex (Froger *et al.*, 2001; Conley *et al.*, 2002). Within the locus coeruleus, blockade of neurokinin-1 receptors results in increase in burst firing of noradrenergic neurons, as do conventional antidepressants (e.g. selective 5-HT-reuptake inhibitors) (Maubach *et al.*, 2002).

Neurokinin-1 receptor null mice demonstrate reduced anxiety like behaviour such as reduced aggression (De Felipe *et al.*, 1998), increased time on the open arms of an elevated plus maze (Santarelli *et al.*, 2001), and reduced stress-induced vocalizations (Rupniak *et al.*, 2001; Santarelli *et al.*, 2001).

Central administration of substance P potentiates the acoustic startle response (Krase *et al.*, 1994) and cardiovascular defence reaction (Unger *et al.*, 1988) in rats, and cause anxiety-like responses in mice on the elevated plus maze (Teixeira *et al.*, 1996). Neurokinin-1 receptor antagonists, such as MK 0869 and L760735, cause antidepressant and anxiolytic effects such as reduced aggression and distress vocalisations (Kramer *et al.*, 1998; Rupniak *et al.*, 2001), increased social interactions (Cheeta *et al.*, 2001), and increased time in the aversive arms of the elevated plus maze (Varty *et al.*, 2002).

These promising animal studies have driven human therapeutic trials. The first neurokinin-1 receptor antagonist to be tested clinically, MK 0869, was shown to have similar efficacy in depression to paroxetine (selective serotonin reuptake inhibitor) in a double blind, randomised trial (Kramer *et al.*, 1998). A similar result has also been demonstrated for another neurokinin-1 receptor antagonist, L759274 (Kramer *et al.*, 2004). In contrast to these studies, a large Phase III trial for MK 0869 in depression has recently demonstrated no significant difference from placebo (Kramer *et al.*, 2004).

1.11.9.3 Sudden Infant Death Syndrome (SIDS)

SIDS is a major cause of postnatal infant deaths. The exact aetiology remains uncertain, however abnormal responses to chronic hypoxia and the possibility of

abnormal neuronal circuitry within the brainstem have been postulated (Steinschneider, 1972; Steinschneider *et al.*, 1982; Van der Hal *et al.*, 1985; Hunt, 1989). As mentioned in section 1.11.8.5, hypoxia results in the release of substance P within the NTS (Lindefors *et al.*, 1986; Srinivasan *et al.*, 1991). Some studies have demonstrated an increase in the concentration of substance P within the medulla oblongata and pons of human SIDS victims, most notably within the NTS and spinal trigeminal nucleus (Bergstrom *et al.*, 1984; Yamanouchi *et al.*, 1993; Ozawa and Takashima, 2002; Biondo *et al.*, 2004). Another study demonstrated no link between SIDS, sleep apnoea and substance P within brainstem trigeminal nuclei and the nucleus parabranchialis, although the NTS and preBötC / VRG region was not assessed (Sawaguchi *et al.*, 2003). As previously mentioned in section 1.11.8.4, major respiratory abnormalities such as an ataxic breathing pattern and pathological respiratory responses to hypoxia, hyperoxia and anaesthesia occur with bilateral destruction of the preBötC neurokinin-1 receptor expressing neurons (Gray *et al.*, 2001). Further, markedly sleep-disordered breathing patterns occur in rats with bilateral destruction of the preBötC neurokinin-1 receptor expressing neurons (McKay *et al.*, 2005). The role of substance P in SIDS and altered respiratory function remains poorly understood and the target of much current investigation.