Chapter 4

Neurokinin-1 Receptor Anatomy

And the Ventral Medulla:

Bulbospinal and Catecholaminergic

Neurons

Chapter 4 Neurokinin-1 receptor anatomy and the ventral medulla: bulbospinal and catecholaminergic neurons

4.1 Abstract

Bulbospinal C1 neurons are sympathoexcitatory and excited by substance P. However the substance P receptor (neurokinin-1 receptor) has been reported to be absent from the somata of C1 neurons. This study, using double and triple labelling immunofluorescence and retrograde tracing, provides evidence that the neurokinin-1 receptor is present on 5.3% of C1 neurons, and that 4.7% of C1 neurons receive close appositions from neurokinin-1 receptor immunoreactive terminals, indicating a pre-synaptic and post-synaptic site for the action of substance P. These results provide support for the sympathoexcitatory actions of substance P on C1 neurons. These results also demonstrate the neurokinin-1 receptor on bulbospinal neurons of the ventral respiratory group, in a region overlapping the preBötzinger Complex. The neurokinin-1 receptor is therefore not an exclusive anatomical marker for the preBötzinger Complex, as these putative respiratory rhythm generating neurons are thought to be propriobulbar.

The results presented in this chapter were published in 2001 in *Neuroreport* (cover illustration, see appendix)-

Makeham JM, Goodchild AK, and Pilowsky PM. 2001. NK1 receptor and the ventral medulla of the rat: bulbospinal and catecholaminergic neurons. Neuroreport 12:3663-3667.

4.2 Introduction

The rostral ventrolateral medulla (RVLM) is a sympathoexcitatory region of the brainstem that is vital for the maintenance of arterial blood pressure (Lipski *et al.*, 1995b; Verberne *et al.*, 1999). Many neurons in this region are spinally projecting, inhibited by increases in arterial blood pressure, tonically active and have pulse modulated firing patterns at higher blood pressures and are thus designated sympathoexcitatory (see section 1.3). Sixty percent of these neurons are adrenergic and thus of the C1 cell group (Lipski *et al.*, 1995b; Verberne *et al.*, 1999).

The undecapeptide substance P, a member of the mammalian tachykinin family, has sympathoregulatory effects when injected into multiple brainstem nuclei (see section 1.11.8) (Len *et al.*, 1994; Seagard *et al.*, 2000). This is thought to be a result of activation of the neurokinin-1 receptor, for which substance P is an agonist. Microinjection of the stable substance P analogue ($pGlu^5$, $MePhe^8$, Sar^9)-SP(5-11) into the RVLM *in vivo* produces large pressor responses (Urbanski *et al.*, 1989). Exposure of RVLM slices to substance P and specific neurokinin-1 receptor agonists *in vitro* causes an increase in the spontaneous firing rate of all intracellularly recorded bulbospinal putative sympathoexcitatory neurons (Li and Guyenet, 1997). Immunoreactivity to the neurokinin-1 receptor is present in the RVLM (Nakaya *et al.*, 1994), and C1 neurons receive synapses from substance P containing terminals when examined ultrastructurally (Milner *et al.*, 1988).

Curiously, however, several studies have noted an absence of neurokinin-1 receptor on C1 neurons of the RVLM (Chen *et al.*, 2000; Wang *et al.*, 2001). The neurokinin-1 receptor has recently been demonstrated on spinally projecting neurons of the ventral

medulla, but it was noted that the neurokinin-1 receptor is not present on C1 neurons (Wang *et al.*, 2001).

Immediately dorsal and caudal to the RVLM lies the preBötzinger Complex (preBötC) of the ventral respiratory group (VRG). It has been suggested that the preBötC can be anatomically distinguished from the rest of the VRG by the presence of the neurokinin-1 receptor (Gray *et al.*, 1999). The purpose of the present study was to investigate the distribution of the neurokinin-1 receptor in relation to C1 neurons and bulbospinal neurons of the ventral medulla and the VRG. This was done using a combination of double and triple label immunofluorescence.

4.3 Materials and methods

The methods have been described in detail in section 2.4. They will be briefly discussed here.

4.3.1 Retrograde labelling

Six male Sprague-Dawley rats underwent spinal cord injections in order to retrogradely label neurons projecting to the intermediolateral spinal cord (IML). After sodium pentobarbitone (60mg/kg i.p.) anaesthesia and fixation in stereotaxic apparatus, a laminectomy at T2 was performed and 100nl of cholera toxin B subunit (1% CTB; Sapphire Bioscience) was microinjected bilaterally into the IML (co-ordinates 0.4mm lateral from midline and 0.7 ventral to dorsal surface). The animals were allowed to recover and perfused 48hrs later.

4.3.2 Perfusion and sectioning

12 male Sprague-Dawley rats (300-500g) were deeply anaesthetised with sodium pentobarbitone (100mg/kg i.p.), given 5000 units heparin and 1%(w/v) sodium nitrite transcardially, and then perfused transcardially with 400ml of phosphate buffered saline (PBS, 100mM sodium phosphate buffer plus 0.9% sodium chloride: pH 7.4) followed by 700ml 4% formaldehyde in 0.1M PB (sodium phosphate buffer). The brainstem was then removed and post-fixed with gentle agitation at room temperature for 4 hours in 4% formaldehyde in 0.1M PB. Brains were cut into 50μm coronal sections on a vibrating microtome and were washed for 30min in 50% ethanol solution, followed by 3x30min washes in Tris-phosphate buffered saline (TPBS; Tris-HCl 10mM, sodium phosphate buffer 10mM, 0.9% NaCl, pH 7.4).

4.3.3 Immunohistochemistry

4.3.3.1 Dual immunofluorescence labelling experiments.

In animals that had not received spinal cord injection of CTB (n=6), the sections were incubated for 48hrs in sheep anti-PNMT (supplied by Professor Peter Howe, 1:10,000 dilution), rabbit anti-NK1R (Sigma; 1:5,000 dilution), 5% normal horse serum (NHS) and TPBS with 0.05% merthiolate (TPBS-M). Following 3x30min washes in TPBS, the sections were further incubated for 24hrs in a combination of secondary antibodies, consisting of fluorescein isothiocyanate (FITC)-conjugated donkey anti-sheep IgG (Jackson ImmunoResearch Laboratories, 1:500 dilution), Texas Red™ sulfonyl chloride (Texas Red™)-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, 1:500 dilution) and 2% normal horse serum, all diluted in TPBS-M.

After 3x30min TPBS washes the sections were mounted and coverslipped using ProLong™ Antifade (Molecular Probes).

4.3.3.2 Triple immunofluorescence labelling experiments

In animals that had undergone spinal cord injection of CTB (n=6), the sections were incubated for 48hrs in a combination of mouse anti-DBH (Chemicon; 1:500 dilution), goat anti-CTB (List Biological Laboratories, Campbell, California; 1:1,000 dilution), rabbit anti-neurokinin-1 receptor (Sigma; 1:5,000) and 5% normal horse serum diluted in TPBS-M. Following 3x30min wash in TPBS, the sections were further incubated in a combination of secondary antibodies, consisting of FITC-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, 1:500 dilution), Texas Red™-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, 1:500 dilution), 7-amino-4-methylcoumarin-3-acetic acid (AMCA)-conjugated donkey anti-goat IgG (Jackson ImmunoResearch, 1:500 dilution) and normal horse serum 2%, all diluted in TPBS-M. After 3x 30 min TPBS washes the sections were mounted and coverslipped in ProLong™ Antifade (Molecular Probes).

4.3.3.3 Microscopy

Sections were viewed with a Leica DML fluorescence microscope (Leica, Wetzlar, Germany) with appropriate filter sets (see section 2.4.6) that discriminated between the fluorophores (FITC, AMCA or Texas red). No “bleedthrough” of fluorescence was observed. Images were acquired using a Spot 2 digital camera system (Diagnostic Instruments). Fluorescence images were pseudocoloured and merged using the Spot 2

proprietary software. Some sections were also examined for dual labelling using a confocal laser scanning TCS 4D system (Leica, Germany) as described in section 2.4.7.

4.3.3.4 Analysis

Sections were mounted in sequential rostro-caudal order. All sections were examined using fluorescence microscopy. For quantitative analysis every fourth section was counted. The ventral medulla was defined as the area ventral to the nucleus ambiguus, medial to the spinal trigeminal tract and lateral to the lateral edge of the inferior olive. Double and triple labelling was determined using 40X magnification. Close appositions were defined only when the terminal and labelled cell were in focus in the same focal plane and there was no discernible gap between the two structures (Pilowsky *et al.*, 1992).

4.4 Results

The distribution of catecholamine synthetic enzymes DBH and PNMT were as described previously (Armstrong *et al.*, 1982; Minson *et al.*, 1994; Phillips *et al.*, 2001). Somata were found in a longitudinal column throughout the ventral medulla. Neurokinin-1 receptor immunoreactive somata and dendrites were all found within the ventral medulla as previously described (Chen *et al.*, 2000; Wang *et al.*, 2001).

4.4.1 C1 neurons and NK-1 receptor immunoreactivity

In 4 rats, cells in the ventral medulla were first defined as PNMT or DBH immunoreactive and were then assessed for the presence of neurokinin-1 receptor immunoreactivity. Of these, 5.3% (98/1862 cells) were immunoreactive to the neurokinin-1 receptor (Figures 4.1 and 4.2). They were found from 12mm to 13.5mm caudal to Bregma. There was no significant rostro-caudal variation in the percentage of C1 neurons that also demonstrated neurokinin-1 receptor immunoreactivity (Figure 4.2).

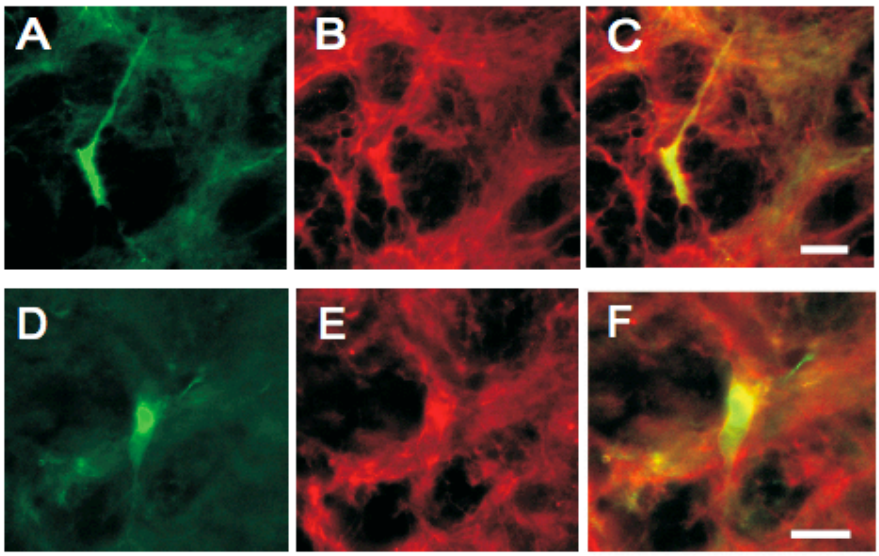

Figure 4.1. Neurokinin-1 receptor immunoreactivity in C1 neurons **(A)-(C)** and **(D)-(F)**. **(A)** C1 neuron of the RVLM labelled for PNMT (green). **(B)** The same neuron labelled for the neurokinin-1 receptor (red) **(C)** Merged image of **(A)** and **(B)** showing both labels are present in the same cell, resulting in a yellow colour. **(D)** Different RVLM C1 neuron labelled for PNMT (green). **(E)** The same neuron labelled for neurokinin-1 receptor (red). **(F)** Merged image of **(D)** and **(E)** showing both labels are present in the same cell. Bars = 10 μm.

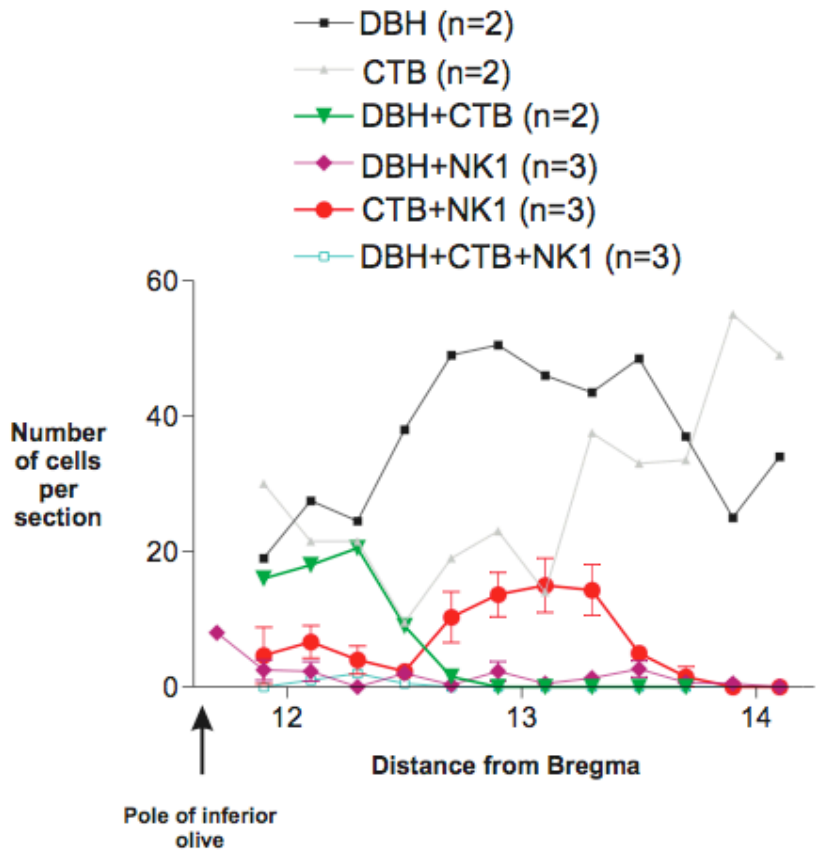

Figure 4.2. Graph indicating the number of cells per section in triple labelling experiments for various labels at different rostro-caudal levels. Note the spinally projecting, neurokinin-1 receptor immunoreactive neurons in the region of the rVRG / preBotC (see secton 4.3.3). Also note the generally low number of C1 (DBH immunoreactive) neurons that are neurokinin-1 receptor immunoreactive

4.4.2 Close appositions of NK-1 receptor immunoreactive terminals

Neurokinin-1 receptor immunoreactive terminals were also found to closely appose C1 neurons. Close appositions were quantified using light microscopy in 4 rats. A small number, 4.7% (87/1862 cells) of the PNMT or DBH immunoreactive neurons of the ventral medulla received close appositions from neurokinin-1 receptor immunoreactive terminals (Figures 4.3 and 4.4). The close appositions were only found between 12mm to 13mm caudal to Bregma, and within this region there was no significant rostro-caudal variation in the percentage of PNMT or DBH immunoreactive neurons that received close appositions. None of the C1 neurons receiving neurokinin-1 receptor immunoreactive close appositions were themselves immunoreactive to the neurokinin-1 receptor. Some sections demonstrating close appositions were taken for further analysis using confocal microscopy. Figures 4.3 and 4.4 are confocal images of close appositions between either PNMT or DBH immunoreactive neurons of the ventrolateral medulla and neurokinin-1 receptor immunoreactive terminals.

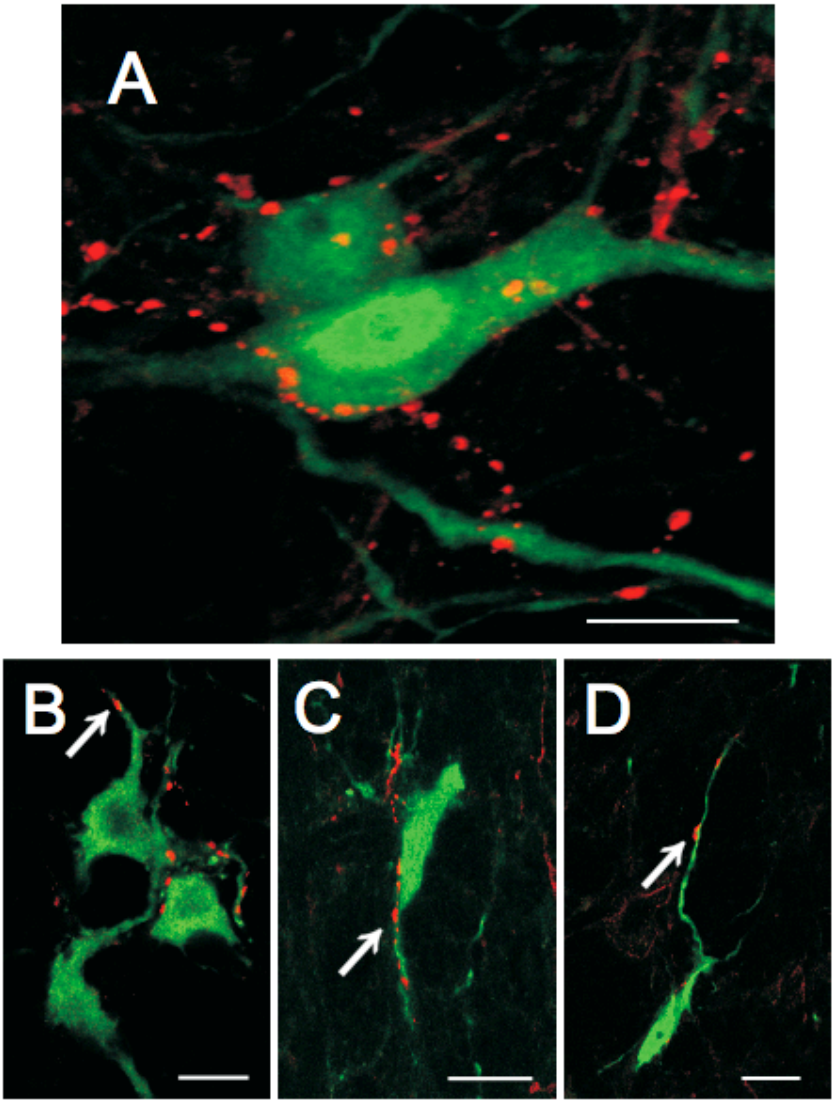

Figure 4.3. Close appositions between C1 neurons and neurokinin-1 receptor immunoreactive terminals **A.** Merged dual fluorescence 40μm thick confocal projection image demonstrating close appositions between neurokinin-1 receptor immunoreactive terminals (red) and a PNMT immunoreactive neuron (green) in the RVLM. (**B,C,D**) Single 1μm thick optical slice dual label confocal images demonstrating close appositions between neurokinin-1 receptor immunoreactive terminals (red) and DBH immunoreactive C1 neurons (green). Some examples of close appositions are demonstrated by the arrows. Bar = 10μm.

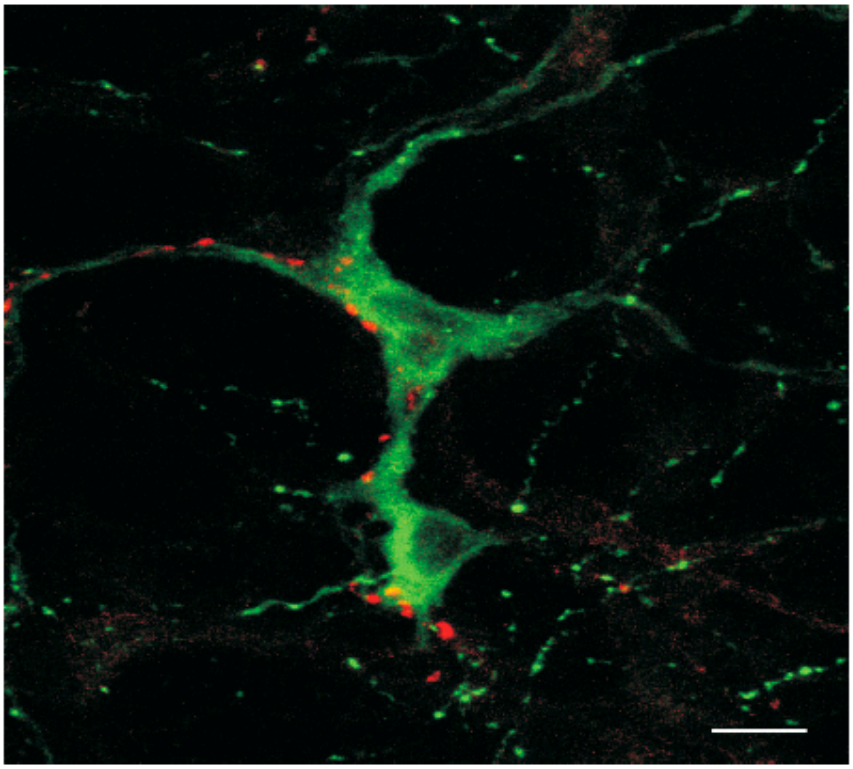

Figure 4.4. Example of neurokinin-1 receptor immunoreactive close appositions to C1 neuron in the RVLM. Single 1μm thick confocal microscope image demonstrating close appositions between neurokinin-1 receptor immunoreactive terminals (red) and PNMT immunoreactive C1 neurons (green) in the RVLM. This image was chosen as the cover illustration of the December 2001 issue of *Neuroreport* vol. 12 (17) (Makeham *et al* 2001). Bar = 10μm.

4.4.3 Bulbospinal neurons and NK-1 receptor immunoreactivity

Retrogradely labelled CTB neurons were found throughout the rostro-caudal extent of the ventral medulla. These included the bulbospinal neurons of the RVLM, many of which are immunoreactive to DBH or PNMT, as described previously (Ross *et al.*, 1984). Caudally, bulbospinal neurons were found in a region known as the rostral Ventral Respiratory Group (rVRG). This region can be easily defined since the neurons lie dorsal but adjacent to the neurons immunoreactive for DBH or PNMT (Fig. 4.5).

Neurons in the ventral medulla defined as bulbospinal were then assessed for immunoreactivity to the neurokinin-1 receptor. Many, 18.3% (201/1097; n=3), of the spinally projecting neurons were also immunoreactive to the neurokinin-1 receptor (Figures 4.5 and 4.6). However, there was a clear rostro-caudal variation in the proportion of the bulbospinal neurons that displayed neurokinin-1 receptor immunoreactivity (Fig. 4.6). At some rostro-caudal levels up to 58% of the CTB immunoreactive neurons were also labelled for the neurokinin-1 receptor. The region where the highest percentage of dual labelled neurons were found was 12.75-13.25mm caudal to Bregma, a region corresponding to the preBötC / rVRG region. Neurokinin-1 receptor immunoreactivity was also present in non bulbospinal, non catecholaminergic neurons found rostral to those of the rVRG, in an area corresponding to the preBötC. Only 7 neurons were immunoreactive to DBH, CTB and the neurokinin-1 receptor. These were found in the region corresponding to the RVLM (Figure 4.2).

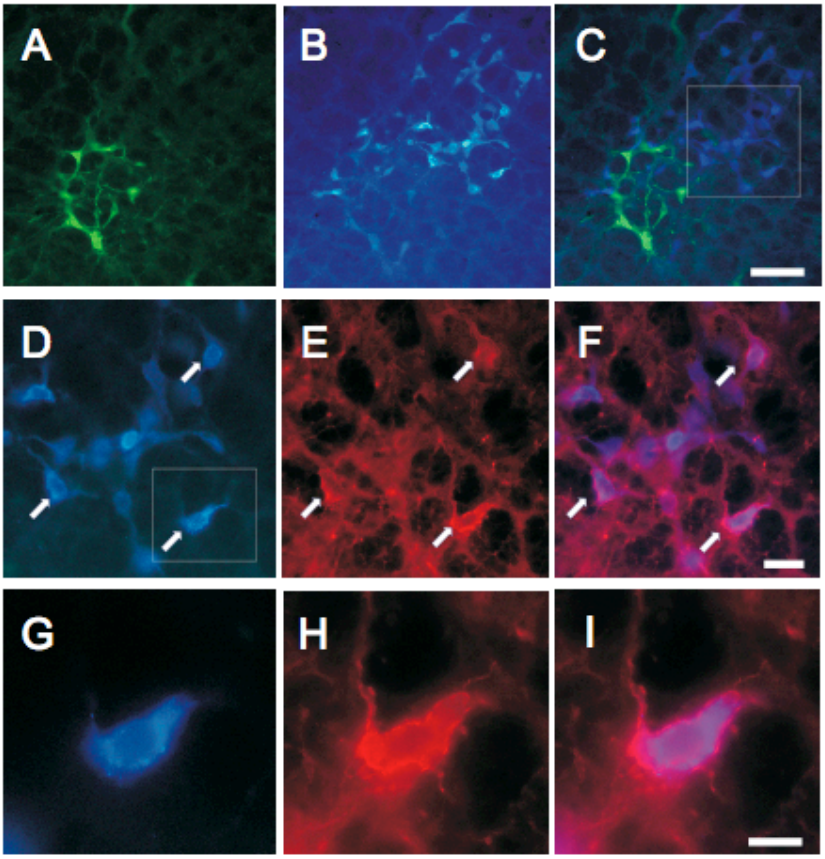

Figure 4.5. **(A-C)** Dual label immunofluorescence indicating the location of spinally projecting CTB labelled neurons of the rVRG (blue) and C1 neurons stained for DBH (green). **(D)** Enlargement of the boxed region of **(C)** demonstrating spinally projecting neurons of the rVRG labelled for CTB (blue). **(E)** The same region as **(D)** stained for neurokinin-1 receptor immunofluorescence (red). **(F)** A merged image of **(D)** and **(E)**. Note 3 of the many dually labelled neurons indicated by the arrows. **(G-I)** Enlargement of boxed region of (D). Note the cytoplasmic labelling of DBH (green) in contrast to the predominantly membrane bound neurokinin-1 receptor labelling (red). Bar=50μm (A-C), 10μm (D-F), 5μm (G-I).

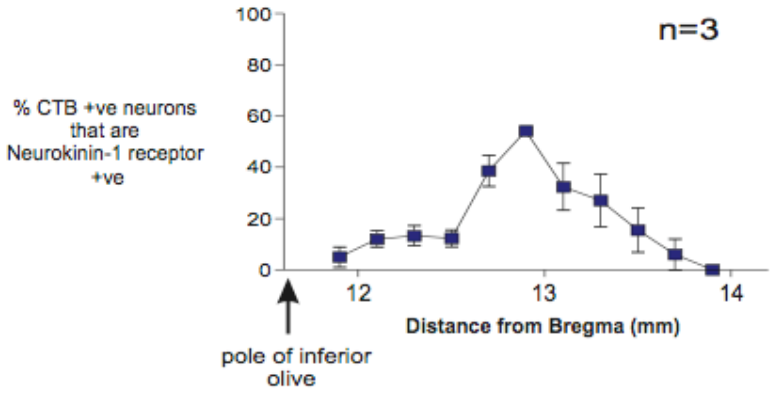

Figure 4.6. Graph indicating the percentage of CTB labelled spinally projecting cells of the ventral medulla that are also immunoreactive to the neurokinin-1 receptor relative to the caudal distance from Bregma (n=3, see section 4.3.3). Note that the highest percentage occurs at a rostro-caudal level corresponding to the rVRG.

4.5 Discussion

4.5.1 C1 neurons and the neurokinin-1 receptor

Many studies have determined the distribution of catecholamine synthesizing enzyme containing neurons in the ventral mammalian brainstem (Armstrong *et al.*, 1982; Minson *et al.*, 1994; Verberne *et al.*, 1999; Phillips *et al.*, 2001). Several studies have also combined immunohistochemistry for these enzymes in conjunction with immunoreactivity to the neurokinin-1 receptor (Chen *et al.*, 2000; Wang *et al.*, 2001). Given the reported robust arterial blood pressure elevation from the stable substance P analogue (pGlu^5, MePhe^8, Sar^9)-SP(5-11) when microinjected into the RVLM *in vivo* (Urbanski *et al.*, 1989) (and also results chapter 5), and the increase in firing frequency of bulbospinal RVLM neurons exposed to substance P *in vitro* (Li and Guyenet, 1997), it is curious that these studies failed to demonstrate the presence of neurokinin-1 receptor immunoreactivity on neurons of the C1 cell group (Chen *et al.*, 2000; Wang *et al.*, 2001). The present study demonstrates that a small proportion of the neurons of the C1 cell group are in fact immunoreactive to the neurokinin-1 receptor. Although it is not known what proportion of C1 neurons are required for a physiological response, such a small amount of post-synaptic labelling seems unlikely to be able to fully explain the reported robust pressor responses of the region to exogenous exposure to the substance P analogue (pGlu^5, MePhe^8, Sar^9)-SP(5-11) (see chapter 5) (Urbanski *et al.*, 1989).

Many bulbospinal sympathoexcitatory neurons of the RVLM are not C1 neurons (Verberne *et al.*, 1999). This study also looked at the spinally projecting neurons of this region, which are sympathoexcitatory (Brown and Guyenet, 1985; Lipski *et al.*,

1995b; Schreihofer and Guyenet, 1997; Verberne *et al.*, 1999). Very few spinally projecting (CTB positive) neurons were immunoreactive to both DBH and the neurokinin-1 receptor. This is unexpected given the high percentage of neurons that are spinally projecting in the RVLM (Verberne *et al.*, 1999). Of the 18.3% (201/1097 cells; n=3) CTB neurons that were neurokinin-1 receptor immunoreactive, all but 7 were found in the region corresponding to the rVRG, rather than the RVLM.

The small number of C1 neurons that are immunoreactive to the neurokinin-1 receptor may be an underestimate since low levels of the neurokinin-1 receptor may not be detected by the methods employed here. In this study, the quality of the immunofluorescence for the neurokinin-1 receptor was directly influenced by the duration of fixation after perfusion of the animal. Optimal immunofluorescence for the neurokinin-1 receptor was achieved after a fixation period of only 4 hours, with the quality of immunofluorescence deteriorating if left for longer periods. This may explain the differences between this and previous studies, which have used longer fixation times.

Recent studies have suggested that there are long and short isoforms of the neurokinin-1 receptor, differing in their carboxy-terminal tails (Mantyh *et al.*, 1996; Li *et al.*, 1997; Zerari *et al.*, 1998). The commercially acquired antibody used in this study, which is directed to the C-terminal of the long form, presumably does not recognize the short form. It is thus possible that some bulbospinal neurons express one or both of these isoforms, which may explain the incongruity between the physiology and the immunohistochemistry. A recent study comparing an antibody to the neurokinin-1 receptor C-terminal and an antibody raised to the receptor region of the neurokinin-1 receptor has indeed demonstrated differential expression in the spinal cord, with the

receptor region directed antibody labelling both lamina I & II, whereas the c-terminal directed antibody labelled only lamina I (Zerari *et al.*, 1998). It remains to be seen if such differential expression is also present in the ventral brainstem.

4.5.2 C1 neurons and NK-1 receptor immunoreactive close appositions

A new finding to arise from this study is that 4.7% of the C1 neurons of the RVLM receive close appositions from neurokinin-1 receptor immunoreactive terminals, indicating a possible pre-synaptic mechanism for the action of substance P on these neurons. No neurons receiving these close appositions were also immunoreactive to the neurokinin-1 receptor. However, the demonstration of close appositions must be approached with caution, as they are not conclusive evidence of a synaptic input. Nevertheless, previous work from our laboratory suggests that at least half of the cases of observed close appositions do form synapses when observed ultrastructurally (Pilowsky *et al.*, 1992). Using confocal optical slices of 1µm thickness, as in this study, demonstrates that the terminal and cell membrane are indeed closely apposed though synaptic contact needs to be fully established by electron microscopy.

An *in vitro* study on the action of substance P on C1 neurons demonstrated no change in the significant depolarization of neurons after exposure to substance P either in the presence or absence of tetrodotoxin, a synaptic transmission blocker (Li and Guyenet, 1997). This suggests that substance P has primarily post-synaptic actions on the C1 neurons of the brainstem. This study also suggested that the predominant postsynaptic receptor on RVLM vasomotor neurons is the neurokinin-1 receptor, rather than the neurokinin-2 or neurokinin-3 receptors. Pre-synaptic effects of substance P were also

noted in some cases (Li and Guyenet, 1997). This study demonstrates evidence for pre and post-synaptic neurokinin-1 receptor receptors on C1 neurons.

4.5.3 Neurokinin-1 receptor and the VRG / preBötC

A further interesting finding is the presence of the neurokinin-1 receptor on spinally projecting neurons in the VRG, overlapping with the preBötC. The preBötC region is thought to be the respiratory rhythm generating region of the brainstem (Smith *et al.*, 1991; Gray *et al.*, 1999). The preBötC region consists of propriobulbar cells, whereas the rVRG found caudal to the preBötC contains bulbospinal cells (Ellenberger and Feldman, 1990; Smith *et al.*, 1991; Dobbins and Feldman, 1994; 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 substance P and the neurokinin-1 receptor play an important role in normal brainstem respiratory function, particularly within the preBötC region. However, the publication of the results from this chapter in 2001 (Makeham *et al.*, 2001), along with work from Wang *et al* in the same year (Wang *et al.*, 2001), demonstrated that the original proposal that neurokinin-1 receptor immunoreactivity could be used as a sole specific phenotypic marker for the propriomedullary respiratory rhythm generating neurons of the preBötC (Gray *et al.*, 1999), is not correct. In the present study, at some levels up to 58% of bulbospinal neurons in the rVRG are immunoreactive to the neurokinin-1 receptor (Fig. 4.6). In addition, less than 50% of electrophysiologically identified putative respiratory generating propriomedullary neurons of the preBötC express the neurokinin-1 receptor (Guyenet and Wang, 2001). A separate study suggested that less than 10% of the neurokinin-1 receptor immunoreactive neurons of the VRG project to the phrenic motor nucleus (Wang *et al.*, 2001). These studies suggest that the presence of the neurokinin-1 receptor is not an anatomically defining characteristic of the preBötC, as it can be found in other regions of the ventral respiratory cell column. The respiratory rhythm generating neurons of the preBötC most likely represent a subset of the neurokinin-1 receptor containing neurons of the VRG. The functional significance of this result remains unclear.

4.6 Conclusion

This study is the first to demonstrate the presence of the neurokinin-1 receptor on the putative sympathoexcitatory neurons of the RVLM. Approximately 5% of neurons of the C1 cell group were neurokinin-1 receptor immunoreactive. This study also supports a role for substance P acting on C1 neurons through pre-synaptic neurokinin-1 receptor immunoreactive terminals that synapse with a small (4.7%) non-overlapping population of C1 neurons. Approximately 10% of RVLM C1 neurons may be affected by neurokinin-1 receptor agonists or antagonists (5% pre-synaptic and 5% post-synaptic). Neurokinin-1 receptor immunoreactivity was also demonstrated on the majority of spinally projecting neurons of the rVRG, suggesting that the presence of the neurokinin-1 receptor in the VRG is not an anatomically defining characteristic of the preBötC, which consists of propriomedullary neurons.