Appendix

COMMENTARY:

JUXTACELLULAR LABELLING OF IDENTIFIED NEURONS:

KISS THE CELLS AND MAKE THEM DYE

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A huge literature has been generated over the past twenty years using a variety of techniques to label single functionally identified neurons. Up until recently this has been achieved by sharp intracellular or patch microelectrode recording followed by iontophoretic ejection of a dye such as lucifer yellow (Shen and Dun, 1990; Pilowsky et al., 1990; Brandon and Criswell, 1991; Pilowsky et al, 1992), horseradish peroxidase (Dembowsky et al., 1985; Somogyi and Soltész, 1986; De Zeeuw et al., 1990; Ball et al., 1990; Forehand, 1990; Örnung and Ulfhake, 1990), biocytin (Kawaguchi et al., 1990; Bryant et al., 1993; Pilowsky et al, 1994a; Sun et al, 1994)or biotinamide (Kita and Armstrong, 1991; Pilowsky et al., 1994b; Sun et al, 1996)(originally known as Neurobiotin when first marketed by Vector inc.). Other imaginative approaches included injection of l-DOPA followed by a histofluorescence reaction to reveal single catecholamine neurons in the A10 nucleus (Grace and Bunney, 1980). Labelled cells can then be retrieved after fixation. By a judicious combination with a range of immunohistochemical techniques it then becomes possible to learn a great deal about the way that functionally identified neurons interact with other neurons that can be identified on the basis of their neurotransmitter content (Pilowsky et al, 1993; Pilowsky et al., 1997), projection patterns or inputs to the identified neuron (Pilowsky et al., 1992; Pilowsky and Llewellyn-Smith, 1993; Sun et al, 1994; Sun et al, 1997).

A major difficulty that we and others have found with this approach in the past is that when making recordings from adult animals *in vivo* the instability of the preparation prevents recordings for long periods. This in turn means that functional characterisation, beyond a few rapid tests, can be quite difficult. Thus, although there

is certainty about the nature of the neuron being recorded from, it is quite arduous to obtain recordings from large numbers of cells. This is especially so when the cell size is small. How can this problem be solved?

The recent introduction by Didier Pinault (Pinault, 1996) of the technique known as juxtacellular labelling helps considerably. This approach, which works by an as yet unknown mechanism, allows one to make recordings from the extracellular position using recording electrodes containing biotinamide. The cell is labeled by applying anodal (electrode is negative, ground is positive) current pulses through the recording electrode that in turn causes the firing of the unit to become entrained to the depolarising pulses (see Fig. 1). The recording electrode contains a combination of electrolyte solution and cell label, commonly 1.5% Neurobiotin® in 0.5M NaCl. Remarkably, most users report a success rate of 85% or greater with very rare instances of multiple cells being labelled. This is comparable to the success rate with intracellular labelling *in vivo*. Since the electrode is in the extracellular position recordings may be made for very long periods before labelling, allowing considerable characterisation.

Publications from Guyenet and colleagues in the context of cardiovascular and respiratory neurons have certainly demonstrated the utility of juxtacellular labelling combined with electrophysiological characterisation. He and his colleagues have shown that the inhibitory expiratory Bötzinger neurons in the rostral ventrolateral medulla are glycinergic and not GABAergic (Schreihofer et al., 1999), have determined the relative proportion and characteristics of bulbospinal baroreceptor sensitive neurons that are adrenaline synthesising (Schreihofer and Guyenet, 1997;

Verberne et al., 1999) and have determined which bulbospinal baroreceptor sensitive neurons produce neuropeptide Y (Stornetta et al., 1999). In this issue they also demonstrate that barosensitive neurons in the rostral ventrolateral medulla contain immunoreactivity for the mu opioid receptor and that some of the inputs to barosensitive neurons are also mu receptor immunoreactive (Aicher et al., 2000). These findings bridge significant gaps between information available at a physiological level and at an anatomical level. Other groups have also demonstrated success in using this method to record and label cells in various regions of the nervous system (Bevan et al, 1998; Kirouac et al, 1999; Manns et al, 2000).

Given that the labelling electrode is in the extracellular position, there may be less than complete certainty that the labeled cell is in fact the cell from which the recording was made. There is, however, good evidence that the recorded cell is indeed the cell that is labeled. There has been evidence of physical microdamage to the labeled neuron from the recording electrode as well as occasional marking left by the final trajectory of the electrode at high power microscopy (Pinault, 1996). When attempts at juxtacellular labelling have broken down, electrophysiological evidence of cell damage occurs- characteristic changes in the amplitude, frequency, and broadness of recorded action potentials followed by irreversible cessation of action potential discharges. Histochemical staining following these instances demonstrated badly damaged neurons at the recording site (Pinault, 1996). Voluntary electrocution of the recorded neuron following juxtacellular labelling has been shown to lead to histochemical evidence of badly damaged neurons at the recording locus (Pinault, 1994; Pinault, 1996). Others have demonstrated that application of similar amounts of current in regions where a unit is not recorded leaves no histological trace of label

(Schreihofer et al, 1997). This paper also demonstrated that when two cells were labeled the second cell was more faintly labeled and its presence had been detected during the recording. The intensity of the labelling has also been shown to be roughly proportional to the duration of the cell entrainment by the current pulses (Pinault, 1996; Schreihoffer et al, 1997).

A significant trade off, apart from the lack of complete certainty about the cell labeled, is that it is not possible to record post-synaptic potentials from the extracellular position. This means that it is not possible to tell if an inhibitory effect is due to disfacilitation (removal of an excitation) or direct inhibition.

A further disadvantage is that the quality of the fill although high in many cases may not be as good as that in intracellular recordings – presumably because of the larger amount of label that can be introduced into the cell during intracellular labelling. The quality of labelling seems to roughly proportional to the length of time the neuron was entrained by the current pulses, so allowing for large variations in labelling intensity (Pinault, 1996: Schreihofer et al, 1997).

Although there are still relatively few publications using this approach, as more people become aware of its ease of use and utility, there is bound to be considerably greater uptake – both of the dye and the method!

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Figure legend

Figure 1. The four phases of juxtacellular labelling. Electrophysiological extracellular recording and labelling of neurons in the rostral ventrolateral medulla. **A:** Isolation and recording from a single unit. **B:** Recording of unit activity whilst applying anodal current pulses (200 milliseconds on, 200 milliseconds off). Note the transition of the cells firing to being strongly entrained to the anodal current pulses. **C:** Following cessation of the anodal current pulses, recovery of spontaneous unit activity is seen- albeit with increased noise of recording. **D:** After processing, the juxtacellularly labelled neuron can be visualised with a variety of standard histochemical techniques. In this case the neuron was juxtacellularly labelled with biotinamide and further processed with fluoroscein isothiocyanate (FITC) conjugated to ExtrAvidin® (dilution 1:200: Sigma Chemical Company, St. Louis, USA). This approach can then be combined with immunohistochemistry on the same section, in this case rabbit anti-substance P and then Texas Red® conjugated donkey anti-rabbit antibodies (dilutions 1:2500 and 1:500 respectively: Sigma Chemical Company, St. Louis, USA). Single arrows demonstrate close appositions of substance P immunoreactive terminals to the juxtacellularly labeled cell. The double arrows show the region enlarged in the inset. This image viewed with confocal microscopy. Scale bar in D = 25μm.

NK1 RECEPTOR AND THE VENTRAL MEDULLA OF THE RAT:

BULBOSPINAL AND CATECHOLAMINERGIC NEURONS

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Running head: NK1 receptor in the ventral medulla

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ABSTRACT

Bulbospinal C1 neurons are sympathoexcitatory and excited by substance P. However the substance P receptor (NK1 receptor), has been reported to be absent from the somata of C1 neurons. In this study, using double and triple labelling immunofluorescence and retrograde tracing, we provide evidence that the NK1 receptor is present on 5.3% of C1 neurons, and that 4.7% of C1 neurons receive close appositions from NK1 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. We also demonstrate the NK1 receptor on bulbospinal neurons of the ventral respiratory group, in a region overlapping the preBötzinger Complex.

KEY WORDS

C1 neurons, rostral ventrolateral medulla, retrograde tracing, phenylethanolamine-N-methyltransferase, dopamine-β-hydroxylase, substance P, preBötzinger complex

Introduction

The rostral ventrolateral medulla (RVLM) is a sympathoexcitatory region of the brainstem that is vital for the maintenance of arterial blood pressure [1,2]. 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. Sixty percent of these neurons are adrenergic and thus of the C1 cell group [1,2].

The undecapeptide substance P, a member of the mammalian tachykinin family, has sympathoregulatory effects when injected into multiple brainstem nuclei [3,4]. This is thought to be a result of activation of the neurokinin 1 receptor (NK1R), 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 [5]. RVLM slices exposed to substance P and specific NK1R agonists *in vitro* causes an increase in the spontaneous firing rate of all intracellularly recorded bulbospinal putative sympathoexcitatory neurons [6]. Immunoreactivity to the NK1R is present in the RVLM [7], and C1 neurons receive synapses from substance P containing terminals when examined ultrastructurally [8].

Curiously, however, several studies have noted an absence of NK1R on C1 neurons of the RVLM [9,10]. The NK1R has recently been demonstrated on spinally projecting neurons of the ventral medulla, but it was noted that the NK1R is not present on C1 neurons [10].

Immediately dorsal and caudal to the RVLM lies the preBötzinger Complex (preBötC) of the ventral respiratory group (VRG). Recently it has been suggested that the preBötC can be anatomically distinguished from the rest of the VRG by the presence of the NK1R [11]. The purpose of the present study was to investigate the distribution of the NK1R 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.

Materials and Methods

All experimental protocols have been approved by the Animal Care and Ethics committees of the Royal North Shore Hospital.

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 pentobarbitol (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.

Perfusion and Sectioning

12 male Sprague-Dawley rats (300-500g) were deeply anaesthetised with sodium pentobarbitol (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).

Immunohistochemistry

Dual immunofluorescence labelling for neurokinin-1 receptor and PNMT. 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).

Triple Immunofluorescence labelling for Neurokinin-1 receptor, DBH and CTB. 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-NK-1 (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).

Microscopy

Sections were viewed with a Leica DML fluorescence microscope (Leica, Wetzlar, Germany) with appropriate filter sets (A, L4, and TX; excitation filters BP340-380, BP450-490, and BP530-595; dichroic mirrors RKP400, RKP510, and RKP600; and suppression filters LP425, BP 515-560, and LP 615, respectively) 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) equipped with a krypton-argon laser. Fluorochromes were viewed separately in single channel mode using 488nm excitation for FITC-labelled secondary antibodies and 568nm excitation for Texas red-labelled secondary antibodies.

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 [12].

Results

The distribution of catecholamine synthetic enzymes DBH and PNMT were as described previously [13,14,15]. Somata were found in a longitudinal column throughout the ventral medulla.

NK1R immunoreactive somata and dendrites were all found within the ventral medulla as previously described [9,10].

C1 neurons and NK1R 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 NK1R immunoreactivity. Of these, 5.3% (98/1862 cells) were immunoreactive to the NK1R (see figure 1A-C). 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 NK1R immunoreactivity.

Close Appositions of NK1R immunoreactive terminals and C1 neurons: NK1R 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 NK1R immunoreactive terminals (Fig 1D-G). 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 NK1R immunoreactive close appositions were themselves immunoreactive to the NK1R. Some sections demonstrating close appositions were taken for further analysis using confocal microscopy. Figure 1D demonstrates a 40μm thick optical section taken with confocal microscopy. The section was stained for PNMT (green) and the NK1R (red). Figure 1E-G are 1μm thick single slice confocal images stained for DBH (green) and the NK1R (red) demonstrating the close apposition of NK1 immunoreactive terminals to C1 neurons.

Bulbospinal Neurons and NK1R 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 [16]. 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 (Fig1H).

Neurons in the ventral medulla defined as bulbospinal were then assessed for immunoreactivity to the NK1R. Many, 18.3% (201/1097; n=3), of the spinally projecting neurons were also immunoreactive to the NK1 receptor (Fig I-K). However, there was a clear rostro-caudal variation in the proportion of the bulbospinal neurons that displayed NK1R immunoreactivity (Fig1L) . At some rostro-caudal levels up to 58% of the CTB immunoreactive neurons were also labelled for the NK1 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. NK1R 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 NK1R. These were found in the region corresponding to the RVLM.

Discussion

Many studies have determined the distribution of catecholamine synthesizing enzyme containing neurons in the ventral mammalian brainstem [2,13,14,15]. Several studies have also combined immunohistochemistry for these enzymes in conjunction with immunoreactivity to the NK1 receptor [9,10]. 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* [5], and the increase in firing frequency of bulbospinal RVLM neurons exposed to substance P *in vitro* [6], it is curious that these studies failed to demonstrate the presence of NK1R immunoreactivity on neurons of the C1 cell group [9,10]. In the present study we demonstrate that a small proportion of the neurons of the C1 cell group are in fact immunoreactive to the NK1R. Although it is not known what proportion of C1 neurons are required for a physiological response, such a small amount of postsynaptic 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). The small number of C1 neurons that are immunoreactive to the NK1R may be an underestimate since low levels of the NK1R may not be detected by the methods employed here. In this study, the quality of the immunofluorescence for the NK1R was directly influenced by the duration of fixation after perfusion of the animal. Optimal immunofluorescence for the NK1R 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. Many bulbospinal sympathoexcitatory neurons of the RVLM are not C1 neurons [2]. We also looked at the spinally projecting neurons of this region, which are sympathoexcitatory [1,2,17,18]. Very few spinally projecting (CTB positive) neurons were immunoreactive to both DBH and the NK1R. This is unexpected given the high percentage of neurons that are spinally projecting in the RVLM [2]. Of the 18.3% (201/1097 cells;n=3) CTB neurons that were NK1R immunoreactive, all but 7 were found in the region corresponding to the rVRG, rather than the RVLM.

A new finding to arise from this study is that 4.7% of the C1 neurons of the RVLM appear to receive close appositions from NK1R 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 NK1R. 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 [12]. 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 depolarization of neurons after exposure to substance P either in the presence or absence of tetrodotoxin, a synaptic transmission blocker [6]. This suggested 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 NK1R, rather than the NK2 or NK3 receptors. Presynaptic effects of substance P were also noted in some cases. In the present study we have demonstrated evidence for pre and postsynaptic NK1 receptors on C1 neurons. Recent studies have suggested that there are long and short isoforms of the NK1R, differing in their carboxy-terminal tails [19,20,21]. Our commercially acquired antibody, 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 NK1R C-terminal and an antibody raised to the receptor region of the NKIR 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 [21]. It remains to be seen if such differential expression is also present in the ventral brainstem.

A further finding is the presence of the NK1R 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 [11,22]. The preBötC region consists of propriobulbar cells, whereas the rVRG found caudally to the preBötC contains bulbospinal cells [11,22,23,24]. It has been suggested recently that the NK1R is an anatomically defining characteristic of the preBötC [11]. A separate study suggested that less than 10% of the NK1R immunoreactive neurons of the VRG project to the phrenic motor nucleus [10]. In the present study, up to 58% of bulbospinal neurons in the rVRG are immunoreactive to the NK1R (Fig 1L). This suggests that the presence of the NK1R is not an anatomically defining characteristic

of the preBötC, as it can be found in other regions of the ventral respiratory cell column. The functional significance of this result remains unclear.

Conclusion

This study is the first to demonstrate the presence of the NK1R on the putative sympathoexcitatory neurons of the RVLM. Approximately 5% of neurons of the C1 cell group were NK1R immunoreactive. Our data also support a role for substance P acting on these neurons through NK1R immunoreactive terminals that synapse with C1 neurons. NK1R immunoreactivity was also demonstrated on the majority of spinally projecting neurons of the rVRG, suggesting that the presence of the NK1R in the VRG is not an anatomically defining characteristic of the preBötC.

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Figures

Figure 1 ***NK1R immunoreactivity on C1 and bulbospinal neurons of the Ventral Medulla***

(A)-(C) NK1R on C1 neuron (A) C1 neuron of the RVLM labelled for PNMT (green). (B) The same neuron labelled for the NK1R (red) (C) Merged image of (A) and (B) showing both labels are present in the same cell, resulting in a yellow colour. (D) Merged dual fluorescence 40µm thick confocal projection image demonstrating close appositions between NK1R immunoreactive terminals (red) and a DBH immunoreactive neuron (green) in the RVLM. (E,F,G) Single 1µm thick optical slice dual label confocal images demonstrating close appositions between NK1R immunoreactive terminals (red) and DBH immunoreactive C1 neurons (green). Some examples of close appositions are demonstrated by the arrows. (H) Dual label immunofluorescence indicating the location of the spinally projecting CTB labelled neurons of the rVRG (blue) and C1 neurons stained for DBH (green). (I) Enlargement of the boxed region of (H) demonstrating spinally projecting neurons of the rVRG labelled for CTB (blue). (J) The same region as (I) stained for NK1R immunofluorescence (red). (K) A merged image of (I) and (J). Note 3 of the many dually labelled neurons indicated by the arrows. (L) Graph indicating the percentage of CTB labelled spinally projecting cells of the ventral medulla that are also immunoreactive to the NK1R relative to the caudal distance from Bregma (n=3). Note that the highest percentage occurs at a rostro-caudal level corresponding to the rVRG. Bars A-G=10µm, H=50µm, I-K=10µm.

HYPERCAPNIA SELECTIVELY ATTENUATES THE SOMATO-SYMPATHETIC REFLEX

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ABSTRACT

The effects of hyperoxic hypercapnia (5%, 10% or 15% CO_2 in O_2) on splanchnic sympathetic nerve activity (sSNA) and sympathetic reflexes such as the somato-sympathetic reflex or baroreflex were studied in urethane anaesthetized, paralysed, artificially ventilated and vagotomized Sprague-Dawley rats. Hypercapnia caused a small increase in mean arterial blood pressure in the 10% CO_2 group and a fall in heart rate in all 3 groups. Splanchnic sympathetic nerve activity increased in all 3 groups. Phrenic frequency and amplitude increased during hypercapnia, with frequency adapting back towards baseline during the CO_2 exposure. The somato-sympathetic reflex was attenuated in the 5% CO_2 group and abolished in the 10% and 15% CO_2 groups, whereas there was little effect on the sSNA baroreflex. Hypercapnia significantly affects phrenic nerve activity, sSNA and selectively inhibits the somato-sympathetic reflex with little effect on the sSNA baroreflex.

Keywords: hypercapnia, rostral ventrolateral medulla, RVLM, baroreflex, somato-sympathetic reflex, brainstem, sympathetic nerve activity

1. Introduction

Hypercapnia has many effects on mammalian sympathetic and respiratory systems. Hypercapnia is known to directly relax vascular smooth muscle (Brickner et al, 1956) and constricts splenic vessels whilst causing vasodilation in other vascular beds (Pelletier et al, 1972). Hypercapnia, as well as hypoxia, is known to stimulate peripheral chemoreceptors (Angell-James et al, 1985) and also activates central chemoreceptors (Wang et al, 2002). Increases in sympathetic nerve activity during hypercapnia are well documented (Somers et al, 1991; Hirakawa et al, 1997; Xie et al, 2001). Variable changes in blood pressure in response to hypercapnia have been reported. Some studies find an increase (Seller et al, 1990; Hirakawa et al, 1997; Bernardi et al, 2001; Xie et al, 2001), while others report no change (Greenberg et al, 1999; Jordan et al, 2000).

Less well studied, however, are the effects of hypercapnia on reflexes integrated in the brainstem, for example the baroreflexes and somato-sympathetic reflex. The somato-sympathetic reflex is the characteristic peak in sympathetic nerve activity following stimulation of somatic afferent nerve fibres (Morrison et al, 1989). The heart rate and sympathetic nerve baroreflexes have been reported to interact with the chemoreflex (Somers et al, 1991; Groom et al, 1997; Bernardi et al, 2001), but most research has centred on the effects of baroreflex stimulation on the chemoreflex, rather than vice versa. The interaction between stimulation of chemoreceptors with systemic hypercapnia and the somato-sympathetic reflex is unknown. Hypoxia augments the somato-cardiac 'A' reflex (Li et al, 1996) whereas there is evidence of inhibition of the somato-sympathetic reflex by vertebral artery perfusion with Ringers solution exposed to CO_2 in cats (Seller et al, 1990). Microinjection of 5-HT_{1A} agonists into the rostral ventrolateral medulla (RVLM) abolishes the somato-sympathetic

reflex (Miyawaki et al, 2001). Since the major source of serotonin in the RVLM is the medullary raphé (Bago et al, 2002), serotonergic neurons of the medullary raphé are known to be chemosensitive (Richerson et al, 2001), and to have significant discharge rates in urethane anaesthetized rats (Viana DiPrisco et al, 2002), we sought to determine if stimulation of central chemoreceptors with increased Pa_{CO2} is sufficient for attenuation of the somato-sympathetic reflex.

In the current study we examined the responses of vagotomized, artificially ventilated rats to systemic hypercapnia caused by ventilation with 5%, 10% and 15% CO_2 in 95%, 90% and 85% O_2 respectively. Arterial blood pressure, heart rate, splanchnic sympathetic nerve activity and phrenic nerve activity were all recorded before, during and after CO_2 exposure. The effect of hypercapnia on brainstem reflexes such as the baroreflex and somato-sympathetic reflex was also determined before, during and after CO_2 ventilation.

2. Materials and Methods

All experimental protocols were approved by the Animal Care and Ethics committee of the Royal North Shore Hospital.

2.1 General Procedures

Male Sprague-Dawley rats (300-500g) were initially anaesthetized with halothane (3% in 100% O_2) followed by an intraperitoneal injection of urethane (1.25-1.3 g/kg). Atropine (90 µg i.p.) was given at induction to prevent excessive respiratory secretions. The trachea was cannulated and the right cervical vagus nerve cut. The right carotid artery and jugular vein were catheterised for the measurement of arterial blood pressure measurement and drug administration respectively. The left aortic depressor (ADN), phrenic, and splanchnic sympathetic nerves were dissected and cut

distally. All nerves were maintained in paraffin oil during recording or stimulation. The right tibial nerve was exposed for stimulation with a bipolar silver wire cuff electrode. The animals were then secured in a stereotaxic frame, paralysed with pancuronium dibromide (0.8 mg i.v.) and artificially ventilated with O_2 enriched air. For baseline measurements, the end tidal CO_2 was measured and maintained at 4.0% by varying the ventilator frequency. The left cervical vagus nerve was then cut. Adequacy of anaesthesia was determined by the stability of blood pressure and phrenic nerve discharge and the absence of a pressor response to firm toe pinch. Additional doses of urethane (20-30 mg i.v.) and pancuronium dibromide (0.2 mg i.v.) were given as required to maintain adequate anaesthesia and neuromuscular blockade. Rectal temperature was maintained between 36-38°C by a combination of a heating pad and infrared lamp.

2.2 Nerve Recording

Bipolar silver wire electrodes were used to record splanchnic sympathetic nerve activity (sSNA) and phrenic nerve activity (PNA). The signals were amplified, full wave rectified, filtered (100-3000 Hz band pass), and integrated using a Paynter filter with a 50-ms time constant. The zero level of sSNA was determined using supramaximal stimulation of the aortic depressor nerve (0.2 ms stimulation, 50 Hz for 5 seconds).

2.3 Activation of cardiovascular reflexes

To activate the baroreceptor afferent fibres, the aortic depressor nerve was stimulated electrically (Miyawaki et al, 2002). Maximal activation of baroreceptor afferents was determined by tetanic ADN stimulation (0.2 ms duration, 50 Hz for 5 seconds). The stimulation voltage was adjusted to achieve maximal inhibition of sSNA. This was

usually 0.5-4.0 volts. To assess baroreceptor function, the average sSNA inhibition in response to intermittent ADN stimulation was determined. The ADN was stimulated (0.2 ms duration, 2 pulses at 2.5 ms interval, 0.5 Hz) and the sSNA response was averaged at least 50 times.

Activation of the somato-sympathetic reflex was achieved by bipolar silver wire cuff electrode placed on the right tibial nerve. The nerve was stimulated at 0.5 Hz (1 ms duration, 10-20 volts) and the sSNA response was averaged at least 50 times. The voltage was adjusted to the highest level where the characteristic 2 peaks of the somato-sympathetic reflex could still be identified clearly.

During ventilation with different concentrations of CO_2 the number of sweeps averaged for determination of both intermittent ADN stimulation and the somato-sympathetic reflex was doubled to 100 times to minimize the effect, if any, of increased respiratory modulation of the sSNA.

2.4 Experimental Procedures

After a period of stabilisation, rats were exposed to a 5% CO_2 / 95% O_2 inspired gas mixture for 30 mins. Variables such as mean Arterial Blood Pressure (MAP), Heart Rate (HR), PNA, and sSNA were measured immediately prior to exposure, following a 5 min and 30min exposure and again after 15 mins recovery. Cardiovascular reflexes were also tested at these times in the following order- *1)* baroreceptor activation by tetanic ADN stimulation; *2)* baroreceptor activation by intermittent ADN stimulation; *3)* activation of cutaneous and muscle afferents by intermittent electrical stimulation of the tibial nerve. Arterial blood gas samples were tested at these times. After a 1 hour recovery period this protocol was repeated with a different inspired CO_2 level, either 10% CO_2 / 90% O_2 or 15% CO_2 / 85%O_2, but not both.

2.5 Data Analysis

Data were analysed during and after experiments using a CED 1401 data capture system and Spike 2 software (version 4.10,Cambridge, U.K.). The average value over a 20 second period was used to evaluate ABP, HR and sSNA. Phrenic frequency, Phrenic amplitude and inspiratory time were determined using a phrenic nerve triggered waveform average over a 100 second period. An initial maximum response for the above variables was also measured during the first 10 mins of CO_2 exposure. The sSNA responses to intermittent ADN stimulation and tibial nerve stimulation were analysed using peristimulus waveform averaging. The amplitude of the SNA from –200 to 0 ms prior to stimulation was taken as the baseline. The maximum reflex response to stimulation was then expressed as a percentage change from the baseline. Changes in nerve activity and reflex responses following CO_2 exposure were then expressed as percentage changes from this pre-stimulus normalized control level. Statistical significance was assessed by paired *t*-tests. To evaluate the effect of each CO_2 concentration at different time points repeated measures one-way ANOVA with Tukey's post-hoc test (if one-way ANOVA was significant) was performed. The Wilcoxon signed-rank test was used to compare changes of sSNA and PNA from the baseline after conversion with a percentage of change. Data are expressed as means and standard error of means (SEM). All statistical analysis was performed using GraphPad software.

3. Results

3.1 Arterial Blood Gases

Baseline arterial blood gases prior to CO_2 exposure were pH 7.35 ± 0.03 and Pa_{CO2} 40.3 ± 1.5 mmHg (*n*=10). For the 5% CO_2 group the pH at 5 mins and 30 mins was 7.22 ± 0.02 and 7.20 ± 0.05 respectively and the Pa_{CO2} was 52.2 ± 5.6 mmHg and

55.7 ± 7.5 mmHg respectively (n=4 for 5 min and n=3 for 30 min). For the 10% CO_2 group the pH at 5 mins and 30 mins was 7.11 ± 0.02 and 7.05 ± 0.04 respectively and the Pa_{CO2} was 63.2 ± 9.9 mmHg and 74.9 ± 8.1 mmHg respectively (n=3). For the 15% CO_2 group the pH at 5 mins and 30 mins was 6.99 ± 0.04 and 6.91 ± 0.07 respectively and Pa_{CO2} was 99.4 ± 13.7 mmHg and 119 ± 19.6 mmHg respectively (n=3). In all groups Pa_{O2} was greater than 350 mmHg**.**

3.2 Blood pressure and HR

Changing the inspired CO_2 to 5%, 10% or 15% had little effect on the mean arterial blood pressure at 5 min and 30 min relative to baseline (Table 1). Some animals showed an increase in blood pressure (Fig. 1), but others did not. There was a significant maximum increase in the MAP within the first 10 mins for only the 10% inspired CO_2 group from 105 ± 7 mmHg to 124 ± 12 mmHg (n=6, P<0.05).

Ten percent inspired CO_2 evoked a decrease in HR throughout the entire period of hypercapnia (Fig. 1). As shown in Table 1, there was a significant decrease in the HR for all 3 inspired CO_2 levels during the 30 min exposure, returning to a level not significantly different from baseline after 15 mins recovery.

3.3 SNA

Ten percent CO_2 inhalation also evoked an increase in sSNA throughout the period of hypercapnia (Fig. 1). The effects of different inspired CO_2 levels on sSNA are shown in Table 1. The 3 different inspired CO_2 levels caused significant increases in the sSNA that returned to baseline levels following the removal of the hypercapnic stimuli.

3.4 Phrenic Nerve Activity

The group data for changes in phrenic nerve activity is summarized in Table 1. Phrenic frequency (PNA frequency) was immediately increased above baseline following exposure to all 3 levels of inspired CO_2 but then decreased to levels not significantly different from baseline for 5% and 10% CO_2 despite continuing hypercapnia.

Phrenic amplitude was significantly increased during 15% CO_2 inhalation (Fig. 2). This was the case for all 3 CO_2 levels (Table 1). There was no significant adaptation of the amplitude response during the stimulus period (Fig. 2) to any of the inspired gas levels.

Inspiratory time was significantly reduced at all 3 CO_2 levels. As with phrenic amplitude, there did not appear to be any significant inspiratory time adaptation during the 30 mins CO_2 inhalation for any of the 3 CO_2 levels (Table 1).

3.5 Baroreflex

Baseline intermittent stimulation of the ADN resulted in an inhibitory potential in the averaged sSNA with a latency of 189 ± 5 ms (n=11; Fig. 3A). The latency of the inhibitory potential was not altered by any of the hypercapnic stimuli. The magnitude of the inhibitory potential was not significantly affected by inhalation of 10% or 15% CO_2 (Fig. 5 and Table 2). However, there was a small but significant attenuation of the inhibitory potential upon ventilation with 5% CO_2 (Table 2 and Fig. 3). At 5 min and 30 min the response to intermittent ADN stimulation was 72 ± 7% (n=6, P<0.05) and 73 ± 9% (n=6, P<0.05) baseline respectively. The response did recover, not significantly different from baseline at 77 ± 9% (n=6, *NS*) 15 mins following cessation of 5% CO_2. However, when data from all 3 CO_2 exposures (5%, 10% and 15% CO_2) were pooled together, there was a small but significant attenuation of the inhibitory potential following intermittent ADN stimulation at 5mins and 30mins,

being 81±5% and 81±7% (n=18, P<0.01) baseline respectively. This returned to 102±7% baseline (n=18, *NS*) at 15mins recovery.

3.6 Somato-sympathetic Reflex

Intermittent stimulation of the right tibial nerve resulted in 2 distinct excitatory peaks in the sSNA recording, with latencies of 117 ± 2 and 210 ± 2 ms (n=11; Fig. 4A). These latencies were not significantly altered by any of the hypercapnic stimuli. As shown in Fig. 4 and Table 2, ventilation with all levels of inspired CO_2 markedly attenuated the first excitatory peak at 5 min and 30 min. At 15mins recovery after 5%, 10% and 15% CO_2 the first excitatory peak returned to levels not significantly different from baseline. Similar responses to intermittent tibial nerve stimulation were seen in the second peak of the somato-sympathetic reflex except that recovery was more variable (Fig. 4B). At 15 mins recovery the second peak of the somato-sympathetic reflex after 10% CO_2 had returned to levels not significantly different from baseline. After 5% and 15%, however, the second peak had only partially recovered and was still significantly different from baseline at 56± 15% (n=5, P<0.05) and 64 ± 8% (n=5, P<0.05) respectively.

4. Discussion

The principal novel findings of the present study are first, that the somato-sympathetic reflex is markedly inhibited during hypercapnia, and secondly that there is only a small change in the sympathetic baroreflex during hypercapnia. In addition, the present data confirms previous studies that reported changes in blood pressure, heart rate, sympathetic nerve activity and phrenic nerve activity under hypercapnic conditions.

The present study does not directly address the question of whether the hypercapnic stimuli applied are stimulating peripheral or central chemoreceptors. Previous research has suggested that at Pa_{CO2} levels of up to 60-70 mmHg, the peripheral chemoreceptors contribute equally with the central chemoreceptors in the response to CO_2, with declining peripheral effects at higher levels (Hanna et al, 1981). However, in this study the animals were in hyperoxic conditions, which has been shown to reduce the responsiveness of peripheral chemoreceptors to hypercapnia (Lahiri et al, 1975) and to have effects comparable to peripheral chemoreceptor denervation (Hanna et al, 1981). It therefore is reasonable to propose that the responses seen in the present study are due predominantly to stimulation of central chemoreceptors.

In the present study the effect on arterial blood pressure of different degrees of hyperoxic hypercapnia was variable, with the maximum increase in MAP during the first 5 minutes only statistically significant for 10%CO_2. The most common finding in previous studies has been a small increase in MAP following normoxic hypercapnia (Greenberg et al, 1999; Xie et al, 2001), hyperoxic hypercapnia (Bernardi et al, 2001) and hypoxic hypercapnia (Hirakawa et al, 1997), although others have failed to demonstrate an increase (Greenberg et al, 1999; Jordan et al, 2000). These small differences are likely due to variations in the experimental procedures.

In this study the heart rate was significantly decreased during all levels of CO_2 exposure for the 30 min experimental period, returning to baseline levels after 15 mins recovery. Previous experiments investigating hypercapnia under varying Pa_{O2} conditions have reported conflicting results, often describing an increase in heart rate (Richardson et al, 1961; Bernardi et al, 2001; Xie et al, 2001) or no change (Greenberg et al, 1999; Jordan et al, 2000). Other studies have demonstrated a fall in HR (Marshall, 1986; Walker, 1987; Mills et al, 1988; Walker et al, 1990; Hirakawa et

al, 1997). These results may be attributable to the direct myocardial depressant activity of hypercapnia (Marshall, 1986). Under hyperoxic and anaesthetised conditions, a fall in HR has also been described (Wendling et al, 1967). Our results are in agreement with these latter findings.

In this study sympathetic nerve activity increased for all three levels of hypercapnia, returning to baseline at recovery. Previous studies have reported increases in sympathetic nerve activity following normoxic hypercapnia (Xie et al, 2001), hypoxic hypercapnia (Hirakawa et al, 1997) and hyperoxic hypercapnia (Somers et al, 1991). Superperfusing the ventral medulla with hypercapnic fluid increases the sympathetic nerve output to the kidney, forelimb and hindlimb (Lioy et al, 1981). Our results support these findings.

Phrenic nerve activity responded to different inhaled levels of CO_2 in several ways. Phrenic burst frequency increased initially to a maximum level that was significantly different from baseline for all three levels of inspired CO_2, as previously described (Nielsen et al, 1986; Coates et al, 1993; Coles et al, 2002). However, phrenic frequency then fell during the exposure to levels not significantly different from baseline for 5% and 10%. The phrenic frequency for 15% CO_2 remained significantly different from baseline during the 30 mins test period but did fall significantly from the initial maximum. This adaption of the frequency response has been described previously (Coles et al, 2002). Severe, episodic, acute hypercapnia under hyperoxic conditions has been reported to induce long-term depression (>60 mins) of respiratory frequency and amplitude (Bach et al, 1998). Others report long-term depression of respiratory burst frequency only following continuous severe hypercapnia (>20 mins), not episodic acute hypercapnia (Baker et al, 2001). In the present study, however, there was no evidence of long-term depression of respiratory frequency as following

cessation of the inspired CO_2, the phrenic frequency for all groups at 15mins was not significantly different from baseline.

The amplitude of the phrenic nerve bursts also increased significantly following exposure to increased CO_2, consistent with previous studies (Nielsen et al, 1986; Zhou et al, 1996). This rise persisted throughout the exposure period. In contrast to phrenic frequency, significant adaption of the amplitude response was not seen during the increased CO_2 period. Long-term depression following the cessation of the stimulus was also not seen. This is in contrast to previous findings where continuous severe hypercapnia under hyperoxic conditions resulted in long-term depression of phrenic burst amplitude (Bach et al, 1998; Baker et al, 2001). Combining phrenic frequency and amplitude results, this study does not support the findings of others that prolonged severe hypercapnia under hyperoxic conditions results in long-term depression of phrenic nerve output.

The aortic depressor nerve contains no functional chemoreceptor fibres (Kobayashi et al, 1999) and is therefore entirely barosensory (Numao et al, 1985). When the ADN is stimulated a characteristic inhibition in sympathetic nerve activity is evoked that reflects the sensitivity of the baroreflex (Miyawaki et al, 2001; Miyawaki et al, 2002). Previous experiments have demonstrated a link between CO_2 levels and the baroreflex although of course, this will depend upon whether or not the stimulus excites peripheral and/or central chemosensors. Some studies find an increase in baroreflex sensitivity during hypercapnia (Hirakawa et al, 1997), others a decrease (Bernardi et al, 2001) and others no change (Somers et al, 1991; Groom et al, 1997). Under hyperoxic conditions both a decrease (Bernardi et al, 2001) and no change (Somers et al, 1991) in baroreflex sensitivity were seen. In our experiment, we demonstrated a small but significant attenuation in the sympathetic response to ADN stimulation, but

only following administration of 5% CO_2. The 10% and 15% CO_2 groups were not significantly affected. Given the fact that the response to 5% CO_2 did not return to baseline levels following cessation of the stimulus, and the lack of response from 10% CO_2 and 15% CO_2 it is possible that this result is due to technical factors rather than a genuine difference in the response between the different inspired CO_2 mixtures. However, as mentioned previously, on pooling the data from all 3 inspired CO_2 levels, a small but significant attenuation of the baroreflex was detected. Thus it appears that activation of central chemoreceptors has only a small impact on the sympathetic baroreflex. It is uncertain whether this small effect has any physiological significance.

An interesting finding in the current experiment was the marked reduction in both the first and second peaks of the somato-sympathetic 'A' reflex. Stimulation of the sciatic nerve evokes characteristic peaks in sympathetic nerve activity (Morrison et al, 1989). The excitatory peaks found in this experiment were similar in latency and morphology to those described in previous work from our laboratory and elsewhere (Zanzinger et al, 1994; Nagata et al, 1995; Miyawaki et al, 2001; Miyawaki et al, 2002). The first peak is most likely mediated by A-δ fibres and the second peak by slowly conducting efferent fibres from bulbospinal RVLM neurons rather than afferent c-fibres (Miyawaki et al, 2001). A later peak in the sSNA response (at approximately 500ms) is most likely due to c-fiber activation but this was not analysed in the current experiment. Although hypercapnic conditions increased levels of sSNA in this study, the increased levels are unlikely to explain the reduction in the somato-sympathetic reflex, since at 5 min following exposure to 5%CO_2 sSNA levels were 120±6% yet the somato-sympathetic reflex was reduced by more than 50%. It is interesting to note that the response of the somato-sympathetic reflex to hypercapnia

is markedly different from that of the baroreflex, which is only marginally affected. Both reflexes are integrated in the RVLM. The fact that sympathetic nerve activity increases during CO_2 exposure, and the baroreflex is only marginally altered suggests that the inhibition of the somato-sympathetic reflex is not due to a general inhibition of sympathoexcitatory bulbospinal RVLM neurons. One possible mechanism by which there could be a reduction in somato-sympathetic reflex amplitude during CO_2 exposure may be due to loss of a co-ordinated response to somatic afferent stimulation. This may occur by reduction of the active neuronal population by occlusion, or by loss of synchrony via alteration in neuronal refractory periods, resulting in loss of the well synchronized discharge necessary for the generation of an excitatory peak.

A further possible mechanism for the inhibition of the somato-sympathetic reflex by hypercapnia involves serotonergic neurons within the medulla. Recently we reported that microinjection of a 5-HT_{1A} agonist bilaterally into the RVLM potently inhibits the somato-sympathetic reflex whilst leaving the baroreflex unaffected (Miyawaki et al, 2001); a similar result to the present study. The robust inhibition of the somato-sympathetic reflex seen in the earlier study, with almost no effect on other excitatory or inhibitory afferents or baseline sSNA, suggested that activation of RVLM 5-HT_{1A} receptors presynaptically inhibits release of an excitatory amino acid from somatic afferents that synapse with RVLM neurons, gating or blocking the excitatory somatic inputs (Miyawaki et al, 2001). The major source of serotonergic input to the RVLM has been shown to be the medullary raphé (Bago et al, 2002). There are two types of chemosensitive neurons in the medullary raphé, those excited by an increase in CO_2 and acidosis and those inhibited by an increase in CO_2 and acidosis (Richerson et al, 2001; Wang et al, 2002). Medullary raphé neurons that are excited by hypercapnia are

serotonergic whereas those that are inhibited are not (Richerson et al, 2001; Wang et al, 2002; Bradley et al, 2002). It must be noted that these experiments were conducted with *in vitro* preparations, and the role of medullary raphé serotonergic neurons in chemoreception in the urethane anaesthetized *in vivo* rat preparation is unknown. However, it is possible that hypercapnia and acidosis excite chemosensitive serotonergic neurons in the medullary raphé that project to the RVLM, stimulating presynaptic 5-HT_{1A} receptors and inhibiting the somato-sympathetic reflex without affecting the baroreflex. However, 5-HT_{1A} receptors have been demonstrated postsynaptically on catecholaminergic and non-catecholaminergic bulbospinal putative sympathoexcitatory neurons of the RVLM (Helke et al, 1997). Some of these are serotonergic neurons that also contain 5-HT_{1A} receptors (Helke et al, 1997). The role of serotonergic neurons and 5-HT_{1A} receptors in the RVLM is still far from certain.

In summary, we find that in vagotomized, paralysed and ventilated rats, hypercapnia under hyperoxic conditions substantially increases splanchnic sympathetic nerve activity, phrenic burst frequency and phrenic burst amplitude. Following the initial increase, there was adaptation of phrenic frequency during the hypercapnic stimulus to levels not significantly different from baseline but no adaptation of the increase in phrenic amplitude. There was also no evidence for long-term depression of phrenic nerve activity following severe hypercapnia, as has been reported by others (Bach et al, 1998; Baker et al, 2001). There was little change in arterial blood pressure, but a significant fall in heart rate for all three levels of hypercapnia. Most interestingly, this study provides evidence for marked inhibition of the somato-sympathetic reflex during hypercapnia with only marginal inhibition of the baroreflex. Although the precise mechanism by which hypercapnia inhibits the somato-sympathetic reflex

remains unclear, this separation of effects for reflexes that are integrated in the same area provides further evidence for functional specificity of neurons in the integration of cardiorespiratory and sympathetic reflexes in the medulla.

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Figure Legends and Tables

Fig. 1. Trace showing the response of expired CO_2, mean arterial blood pressure (MAP), heart rate (HR), and splanchnic sympathetic nerve activity (sSNA) following exposure to 10% CO_2 / 90% O_2 gas mixture for 30 mins. Note the increase in splanchnic sympathetic nerve activity and fall in heart rate. In this example, the blood pressure initially rises, falling back down to baseline during the exposure.

Fig. 2. Effects on Averaged Phrenic Nerve Activity (PNA), Phrenic Nerve Activity and Phrenic frequency of 15% CO_2 for 30mins. Note the increase in averaged phrenic nerve amplitude following hypercapnia, with little attenuation during the exposure. In contrast, there is an initial increase in the phrenic frequency, which then falls to a lower level during the remainder of the exposure. There is no evidence following cessation of the CO_2 stimulus of long-term depression. Note also the reduction in inspiratory time during the stimulus, returning to baseline levels at recovery.

Fig. 3. A: An example of the effect of 15% CO_2 on the averaged sSNA response to intermittent ADN stimulation (arrowheads). Note the inhibitory trough with a latency of approx. 190 ms. In this example there was no attenuation of the response.

B: Group data (n=5-7) for the effect of hypercapnia on the averaged sSNA response to intermittent ADN stimulation. The response to 5% CO_2 is the only response that

shows a significant attenuation, however this does not return to baseline with recovery (15 mins post stimulus). * $P<0.05$ vs. baseline.

Fig. 4. A: An example of the response of the somato-sympathetic reflex to 10% CO_2 for 30mins following tibial nerve stimulation (arrowheads). The characteristic response of 2 peaks is abolished during the stimulus period, returning with recovery. The baseline prior to stimulation during the hypercapnic period is increased due to the general increase in sSNA following hypercapnia.

B: Group data (n=5-8) for the effects of hypercapnia on the first and second peaks of the somato-sympathetic reflex after tibial nerve stimulation. Both peaks are virtually abolished during the hypercapnic stimulus for 5%, 10% and 15% CO_2. Recovery to levels not significantly different from baseline occurred for the first peak, but for the second peak only the 10% CO_2 group returned to levels not different from baseline. The levels for 5% and 15% recovered only partially. * $P<0.05$ vs. baseline; ** $P<0.01$ vs. baseline.

Table 1. The effects of hypercapnia on physiological variables

	5 % CO_2 (*n*=7)	10 % CO_2 (*n*=6)	15 % CO_2 (*n*=9)
MAP (mmHg)			
Baseline	114±6	105±7	106±5
Max.	124±8	124±12*	114±6
5 min	120±8	120±13	109±5
30 min	121±9	115±9	106±7
Recovery	112±10	100±7	97±7
HR (bpm)			
Baseline	426±6	448±16	434±8
5 min	-18±2*	-19±6*	-32±9**
30 min	-14±8	-25±9**	-36±9**
Recovery	-5±6	-13±6	-10±9
sSNA			
Baseline	100%	100%	100%
5 min	120±6%**	156±11%*	137±8%**
30 min	137±13%**	193±21%*	155±9%**
Recovery	115±9%	129±19%	116±3%
PNA frequency			
Baseline	100%	100%	100%
Maximum	133±6%**	133±8%*	142±5%**
5 min	120±6%**	113±5%*	117±4%**
30 min	111±5%	106±9%	113±4%**
Recovery	85±6%	94±3%	90±3%
PNA Amplitude			
Baseline	100%	100%	100%
5 min	215±30%**	215±34%*	205±19%**
30min	166±21%**	191±38%*	186±13%**
Recovery	131±17%	96±17%	95±9%
PNA Inspiratory Time			
Baseline	100%	100%	100%
5 min	73±6%**	66±6%*	68±5%**
30 min	68±7%*	62±5%*	70±7%**
Recovery	99±6%	89±5%	100±7%

Data are Mean ± SEM. MAP, mean arterial pressure; HR, heart rate; sSNA, splanchnic sympathetic nerve activity; PNA, phrenic nerve activity; * $P < 0.05$ vs. Baseline; ** $P < 0.01$ vs. Baseline.

Table 2. The effects of hypercapnia on the baroreflex and the somato-sympathetic reflex.

	5% CO_2	**10% CO_2**	**15% CO_2**
Baroreflex			
Baseline	100% (*n*=6)	100% (*n*=5)	100% (*n*=7)
5min	72±7%*	86±6%	84±10%
30min	73±9%*	100±10%	74±13%
Recovery	77±9%*	124±14%	107±7%
Somato-sympathetic Reflex			
Peak 1			
Baseline	100% (*n*=7)	100% (*n*=6)	100% (*n*=8)
5min	49±5%**	27±7%*	31±9%**
30min	55±10%**	28±8%*	22±3%**
Recovery	72±14%	70±13%	97±14%
Peak 2			
Baseline	100% (*n*=5)	100% (*n*=5)	100% (*n*=5)
5min	28±10%*	14±10%*	8±6%*
30min	27±15%*	2±14%*	8±8%*
Recovery	56±15%*	89±45%	64±8%*

Data are Mean ± SEM. * $P < 0.05$ vs. Baseline. ** $P < 0.01$ vs. Baseline.