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J. Physiol. published online Mar 9, 2006;

DOI: 10.1113/jphysiol.2005.103622

This information is current as of March 26, 2006

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Running head: Central respiratory modulation of GABAergic CVLM neurones

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key words:
cardiovascular, cardio-respiratory integration, phrenic nerve, sympathetic nerve

Category: iv. Integrative physiology
Abstract

The sympathetic nerves that maintain blood pressure are modulated by the central respiratory generator. Neurones in the rostral ventrolateral medulla (RVLM) that drive this sympathetic nerve activity (SNA) also display central respiratory drive (CRD)-related activity, suggesting integration of respiratory and cardiovascular regulatory systems within the brain stem. Whether CRD-related activity in the RVLM is due to direct inputs from central respiratory neurones or modulation of cardiovascular-related neurones that influence the RVLM is not known. The caudal ventrolateral medulla (CVLM) contains GABAergic neurones that tonically inhibit pre-sympathetic RVLM neurones and are essential for the production of numerous cardiovascular reflexes. The present study sought to determine whether cardiovascular-related GABAergic neurones in the caudal ventrolateral medulla (CVLM) display central respiratory drive (CRD)-related activity. The firing patterns of individual barosensitive CVLM neurones were examined in relation to phrenic nerve activity in chloralose-anaesthetized, ventilated, neuromuscular-blocked, vagotomized rats. Phrenic-triggered histograms of CVLM neuronal activity showed that all baro-activated CVLM neurones displayed 1 of 4 patterns of CRD-related activity: (I) inspiratory peak ($n=15$), (II) inspiratory depression ($n=15$), (III) inspiratory peak with post-inspiratory depression ($n=10$), and (IV) post-inspiratory peak ($n=9$). A subset of each type of CVLM neurone was identified as GABAergic by individually filling the recorded neurone with biotinamide and observing expression of GAD67 mRNA by in situ hybridization ($n=10$). These data suggest that the activity of
GABAergic neurones in the CVLM are regulated by cardiovascular and respiratory inputs, and baro-activated GABAergic CVLM neurones may contribute to CRD-related modulation of pre-sympathetic RVLM neurones and SNA.
Introduction

The central respiratory pattern generator has a marked influence upon the sympathetic nerves that regulate cardiovascular function (Pilowsky, 1995; Malpas, 1998; Jänig & Häbler 2003). Central respiratory drive (CRD)-related activity in sympathetic nerves has been observed in all species examined including rat (Numao et al. 1987; Guyenet et al. 1990; Häbler et al. 1996; Miyawaki et al. 1996), cat (Adrian et al. 1932; Cohen & Gootman, 1970; Häbler et al. 1994), rabbit (Adrian et al. 1932; Terui et al. 1986), dog (Okada & Fox, 1967), and human (Eckberg, 2003). The exact timing of the CRD-related activity varies with the species under study (Czyzyk et al. 1987; Häbler et al. 1994), from nerve to nerve within a subject (Numao et al. 1987; Boczek-Funcke et al. 1992a,b; Jänig & Häbler, 2003), or state of the preparation or individually recorded sympathetic neurones (Gilbey et al. 1986; Boczek-Funcke et al. 1992a; Koshiya & Guyenet, 1995; Dick et al. 2004). The predominant patterns include a peak in sympathetic nerve activity (SNA) coincident with inspiration, or a peak immediately after inspiration during the post-inspiratory/early expiratory period. This CRD-related regulation of SNA appears to make a significant contribution to sympathetic vasomotor tone and AP in normocapnic states, and likely contributes to the sympatho-excitation and increase in AP observed with hypercapnia (Bachoo & Polosa, 1985; Haselton & Guyenet, 1989).

Some of the CRD-related activity observed in SNA may arise from pre-sympathetic neurones in the brain stem. Indeed, in rats, cats, and rabbits CRD-related patterns have been observed in the pre-sympathetic neurones of the
rostral ventrolateral medulla (RVLM; Terui et al. 1986; McAllen, 1987; Haselton and Guyenet, 1989; Miyawaki et al. 1995), which provide the major drive to sympathetic vasomotor tone under resting conditions in anaesthetized animals (Guyenet & Brown, 1986; McAllen, 1986; Schreihofer et al. 2000). Some of the observed CRD-related patterns are likely to be produced by direct inputs to these RVLM neurones from central respiratory neurones. In addition, some CRD-related patterns observed in pre-sympathetic RVLM neurones may be due to the influence of central respiratory neurones upon cardiovascular-related neurones that regulate RVLM neuronal activity. For instance, inhibition of the caudal ventrolateral medulla (CVLM) alters CRD-related activity in pre-sympathetic RVLM neurones and SNA (Miyawaki et al. 1996), and these changes in SNA can be mimicked by direct blockade of GABA_A receptors in the RVLM (Guyenet et al. 1990; Miyawaki et al. 1996, 2002). If the GABAergic CVLM neurones that regulate the activity of pre-sympathetic RVLM neurones are modulated by the central respiratory pattern generator, this cardiovascular-related input may be an important source of CRD-related activity observed in pre-sympathetic RVLM neurones and SNA.

The CVLM contains GABAergic neurones that project to the RVLM and are essential for the production of many neural cardiovascular reflexes, including baroreflexes and the Bezold-Jarisch reflex (Gordon, 1987; Verberne & Guyenet, 1992). Extracellular unit recordings within the CVLM reveal neurones whose properties suggest they may be the critical inhibitory interneurones that convey this reflex information to the pre-sympathetic RVLM neurones. These CVLM
neurones are highly barosensitive (baro-activated) with activity that is modulated by each AP pulse (pulse-modulated), and their activity is inversely related to RVLM neuronal activity and SNA during the production of cardiovascular reflexes that require the CVLM (Terui et al. 1990; Agarwal & Calaresu, 1991; Gieroba et al. 1992; Jeske et al. 1993; Schreihofer and Guyenet, 2003). Neurones in the CVLM with these physiological properties have been shown to express GAD67 mRNA, suggesting that they are GABAergic, and have axons that project toward the RVLM (Terui et al. 1990; Agarwal & Calaresu, 1991; Gieroba et al. 1992; Jeske et al. 1993; Schreihofer and Guyenet, 2000c, 2003). To test the hypothesis that central respiratory and cardiovascular regulatory mechanisms interact within the CVLM, the present study determined whether baro-activated, pulse-modulated, GABAergic CVLM neurones display CRD-related activity. In addition, we recorded adjacent baro-inhibited neurones, which are likely to be rostrally projecting C1 adrenergic CVLM neurones (Verberne et al. 1999; Schreihofer & Guyenet, 2003), to determine whether they also display CRD-related activity as previously reported for the spinally projecting rostral C1 adrenergic neurones in the RVLM (Haselton & Guyenet, 1989). To ensure observation of CRD-related activity the rats were vagotomized, and phrenic nerve discharge was not phase locked to the ventilation cycle.
Methods

All experiments were conducted in agreement with the regulations of the National Institutes of Heath *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee at the Medical College of Georgia.

Surgical preparation

Male Sprague Dawley rats (270-350g; Harlan, www.harlan.com) were anaesthetized with 2.5% isoflurane in 100% oxygen for surgery. Catheters were inserted into the femoral vein to inject drugs and a brachial artery to measure upper body AP. Heart rate (HR) was monitored by triggering from the AP pulse. A tracheotomy was performed to provide artificial ventilation (Model 683, Harvard Apparatus, www.harvardapparatus.com). End tidal CO₂ was monitored using infrared spectroscopy (100-130 mS response rate; CapStar-100, Charles Ward Electronics, www.cwe-inc.com) by sampling expired air as it exited the tracheal tube. The end-tidal CO₂ levels ranged between 3.5 and 5.5% with a tidal volume of 1 ml/100g body weight and a ventilation rate of 60 strokes/min. If a prominent CRD-related activity was not observed in the SNA, ventilation rate was decreased slightly (~55 strokes/min) to raise CRD-activity immediately prior to recording of CVLM neurones (Haselton and Guyenet, 1989). Core temperature was maintained at 37°C (Harvard). The rat was subjected to neuromuscular blockade (pancuronium, 1mg/kg, iv, Abbott Labs), and then cervical vagus nerves were cut bilaterally. A snare was placed around the abdominal aorta to
rapidly increase upper body AP (Brown & Guyenet, 1985; Schreihofer & Guyenet, 2003). The rat was placed into a stereotaxic frame (David Kopf Instruments, www.kopfinstruments.com) in the supine position with the bite bar at -11mm. A partial occipital craniotomy was performed to expose the dorsal surface of the brainstem. The spinal column was clamped at the mid-thoracic level to reduce ventilatory-related movements. Upon completion of the surgical preparation, α-chloralose (30 mg/ml solution in 3% sodium borate) was infused slowly (60 mg/kg iv, with hourly supplements of 20 mg/kg as needed) as isoflurane was eliminated. Animals were allowed recover for 30 minutes before extracellular recordings were performed. All recordings were performed under α-chloralose anesthesia with 100% oxygen. Adequacy of anaesthesia was verified every 30 minutes and immediately before each supplement of pancuronium by evaluating the cardiovascular response to a firm toe pinch (<10 mmHg increase in AP).

**Peripheral nerve recordings**

Raw splanchnic SNA (sSNA) was measured as previously reported (Schreihofer & Guyenet 2000a,b; Schreihofer et al. 2000). The left splanchnic nerve was exposed by a retroperitoneal approach and placed on two Teflon-coated silver wires that were bared at the tips (250-um bare diameter, A-M Systems, www.a-msystems.com). The nerve and wires were embedded in polyvinylsiloxane impression material (www.darbydental.com), and the incision was closed around the recording wires. The sSNA was amplified and filtered
The baseline sSNA (100%) was arbitrarily defined as the activity during the resting state immediately preceding each physiological test, and the minimum sSNA (0%) was determined after injection of clonidine at the end of the experiment (10 µg/kg iv, Sigma Chemicals www.sigma.com; Schreihofer & Guyenet, 2000a).

The left phrenic nerve was dissected using a dorsolateral approach as described previously (Schreihofer et al. 1999). The nerve was cut distally, placed on a bipolar silver electrode, and coated with polyvinylsiloxane. The raw PND was amplified and filtered (30Hz-3kHz band pass with a 60 Hz notch filter; Differential AC amplifier 1700, A-M Systems). Artificial ventilation was offset from the PND to desynchronize CRD from ventilatory-related changes in AP (Figure 1).

**Extracellular recording and juxtacellular labelling of CVLM neurones**

Extracellular recordings from individual neurones in the CVLM were performed as previously described (Schreihofer & Guyenet, 2000c, 2003) using glass electrodes (World Precision Instruments; www.wpiinc.com) filled with 1.5% biotinamide (Molecular Probes, www.probes.invitrogen.com) in 0.5M sodium acetate. The optimal electrode resistance was 10-20 MΩ measured in vivo. Recordings were made with an intracellular amplifier in bridge mode (Neuroprobe 1600, A-M Systems) to allow monitoring of the recording during injection of current through the recording electrode. The neuronal activity was amplified and
filtered (300 Hz - 5 kHz, NeuroLog System, Digitmer Ltd, www.digitimer.com). The CVLM was located using stereotaxic coordinates in relation to calamus scriptorius: 1.2-1.5 mm rostral, 1.8-2.1 mm lateral, and 2.5-2.9 mm ventral to the dorsal surface of the brain stem. We selected cardiovascular-related, baro-activated CVLM neurones based upon the following criteria: 1) spontaneous activity under resting conditions with significant baroreceptor tone (decreasing AP evoked increased sSNA) 2) discharge rate briskly increased by raising AP with constriction of the aortic snare (at least 3 times above baseline firing rate) 3) discharge pattern strongly modulated by the AP pulse, especially when AP was raised 4) lack of obvious respiratory-related activity in the raw data, and 5) location within the CVLM often immediately ventral to cells with ON-OFF respiratory-related activity. For comparison, we selected cardiovascular-related, baro-inhibited CVLM neurones using the same criteria except that they were silenced by raising AP with constriction of the aortic snare.

A subset of individually recorded CVLM neurones was filled with biotinamide using a previously described juxtacellular labelling method (Pinault, 1996; Schreihofer & Guyenet, 1997, 2003; Schreihofer et al. 1999, 2000). Positive current pulses were delivered through the recording pipette (200 millisecond pulses of 1.0-3.0 nA, 2.5 Hz) while monitoring the activity of the isolated neurone. Successful entrainment (1-3 minutes) of the cell’s activity to the current pulses produces the label of a single cell in the vast majority of cases (Schreihofer & Guyenet, 1997, 2003). One to three neurones were recorded
from each rat, but only one attempt to label a neurone was performed on each side of the medulla.

**Physiological data analyses**

All analog physiological variables (AP, end-tidal CO\(_2\), sSNA, PND, and CVLM unit activity) were converted to digital signals (Micro 1401, Cambridge Electronic Design, [www.ced.co.uk](http://www.ced.co.uk)) and viewed on-line (Spike2 software, Cambridge). The raw PND and SNA were full-wave rectified using the Spike2 software rectify function and were averaged using the Spike2 smooth function with 0.1 sec bins. The CVLM unit activity was also counted in 0.1 sec bins (Spike trigger, Neurolog). All triggered histograms were constructed off-line using 180-300 sec of recorded data (Spike2 software). Event channels were created in Spike2 to trigger initiation of PND, peaks of CO\(_2\) and AP. The AP-triggered histograms were constructed to confirm pulse-modulated activity of CVLM neurones. We evaluated 1000-1500 sweeps of a 1-second window in 0.01-second bins, which usually contained 6-8 AP cycles. The PND-triggered histograms were constructed to determine CRD-related activity in CVLM units and sSNA and to show a desynchronization with ventilation. End tidal CO\(_2\) wave-triggered histograms were constructed to determine whether CVLM neurones displayed ventilatory-related modulation. For PND and CO\(_2\) we evaluated 100-150 sweeps of a 4-second window in 0.1-sec bins, which usually contained 2-3 cycles.
**Histology of labelled CVLM neurones**

At the end of the experiment, the rat was perfused transcardially with phosphate-buffered saline (pH 7.4, 250 ml) followed by 4% paraformaldehyde (pH 7.4, 500 ml). The brain was removed and stored in the same fixative for 48 hours at 4°C. The brain stem was cut coronally into 30-micron sections using a Vibratome (The Vibratome Company, [www.vibratome.com](http://www.vibratome.com)) and stored in a cryoprotectant solution at -20°C (Schreihofer & Guyenet, 1997).

Individual biotinamide-labelled neurones were processed to identify phenotype and confirm location within the CVLM. The baro-activated CVLM neurones were examined for expression of GAD67 mRNA to verify that they were GABAergic (Schreihofer & Guyenet, 2000c, 2003). Adjacent vagal motor neurones are also baro-activated, but do not express GAD67 mRNA (Schreihofer & Guyenet, 2003). The baro-inhibited CVLM neurones have previously been shown to be catecholaminergic (caudal C1 cells), so they were examined for tyrosine hydroxylase (TH) or phenylethanolamine N-methyl transferase (PNMT) immunoreactivity (Schreihofer & Guyenet, 2003; Stornetta *et al.* 1999; Verberne *et al.* 1999).

The biotinamide-filled neurones were revealed by incubating the tissue with strept-avidin Alexa 488 (Molecular Probes, [www.probes.invitrogen.com](http://www.probes.invitrogen.com) 1:200 with 0.1% Triton X-100, 2 hours) and mounting the sections onto sterile slides in sterile phosphate buffer (pH 7.4). Sterile cover slips were placed onto
the sections and the biotinamide-labelled neurone was located using an epifluorescence microscope (Olympus BX50, Olympus, www.olympusamericas.com). The section containing the labelled neurone was drawn, and the location of the labelled neurone was plotted using the Neurolucida system (Microbrightfield, www.microbrightfield.com). The section was gently removed from the slide and processed to reveal phenotype.

Unless otherwise noted, all incubations and rinses were performed on free-floating sections at room temperature on a shaker in Tris-buffered saline (TBS, pH 7.4). Immunoreactivity for TH was revealed by incubation with a monoclonal mouse anti-TH antibody (1:1000, 48 hours, 4°C, Immunostar, www.immunostar.com) followed by incubation with donkey anti-mouse IgG-Cy3 (1:200, 1 hour, Jackson ImmunoResearch, www.jacksonimmuno.com). Immunoreactivity for PNMT was revealed by incubation with a polyclonal rabbit anti-PNMT antibody (1:2000, 48 hours, Chemicon, www.chemicon.com) followed by incubation with donkey anti-rabbit IgG-Cy3 (1:200, 1 hour, Jackson). Sections were mounted onto uncoated slides, and coverslips were applied with Krystalon.

**In situ hybridization histochemistry for GAD67 mRNA**

Expression of GAD67 mRNA was detected using antisense digoxigenin-labelled cRNA probes as previously described (Schreihofer *et al.* 1999, Schreihofer & Guyenet, 2003). The riboprobes were generated from a full-length cDNA encoding GAD67 (2.7 kb, generously supplied by Dr. A.J. Tobin, University of California, Los Angeles, CA; Erlander *et al.* 1991) cloned into pBluescript SK+
Plasmids were linearized with SalI (Promega, www.promega.com) and transcribed using T3 polymerase (Promega) in the presence of digoxigenin-11-UTP (Roche Applied Science, www.ibuybiochem.com). The template DNA was digested with RQ1 DNase (Promega), and unincorporated nucleotides were removed by Probe Quant G-50 Micro Columns (Amersham Pharmacia Biotech, www.apbiotech.com).

The section containing the biotinamide-labelled soma was rinsed in RNAse and DNase-free saline and placed in a pre-hybridization solution (Schreihofer et al. 1999) at room temperature for 30 minutes and then at 37°C for 1 hour. Then, the riboprobe (50-100 pg/ml) was added directly to the solution containing the section to hybridize for 16 hours at 55°C. Sections were rinsed through decreasing concentrations of sodium citrate (SSC) at 37°C, treated with a solution of RNAase A (1 hour, 37°C), and then rinsed in 0.1 X SSC at 55°C for 1 hour.

The digoxigenin-labelled riboprobe was revealed by incubation with a sheep polyclonal anti-digoxigenin antibody conjugated to alkaline phosphatase (1:1000, overnight, 4°C, Roche) with 10% heat inactivated horse serum (Gibco, www.invitrogen.com) and 0.1% Triton X-100. The next day sections were rinsed and incubated in NMT (0.1 M NaCl, 50 mM MgCl₂, and 0.1M Tris, 10 minutes). The blue-brown reaction product was produced by incubation in NMT with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt (Roche) in the dark for 1-3 hours (Schreihofer et al. 1999; Schreihofer & Guyenet, 2003).
**Mapping and imaging of biotinamide-labelled neurones**

Epifluorescence was used to visualize biotinamide-labelled CVLM neurones incubated with strept-avidin Alexa 488, or TH-immunoreactivity revealed with Cy3 as previously described (Schreihofer & Guyenet, 2003). Brightfield illumination was used to visualize the GAD67 mRNA hybridization reaction product. Examples of biotinamide-labelled CVLM neurones were captured using a digital camera (MagnaFire, Optronics, www.olympusamerica.com). The resulting files were imported into Adobe Photoshop (6.0, Adobe Systems, www.adobe.com) where they were converted to grayscale with the levels and sharpness adjusted to optimize visualization of the labelled neurones.

**Results**

**CRD-related modulation of sSNA of vagotomized, chloralose-anaesthetized rats**

To ensure that the preparation yielded a prominent CRD-related modulation of sSNA, an ultimate target of recorded CVLM neurones, we recorded splanchnic nerve activity in a subset of rats (28 of the 48 rats). The ventilation, as indicated by end tidal CO$_2$, was not phase locked with the discharge of the phrenic nerve to ensure observation of CRD-related sSNA (Figs. 1 and 4). The average length of the ventilation cycle (1.10 ± 0.02 seconds; peak
to peak of end-tidal CO$_2$) was shorter than the average length of the centrally
driven respiratory cycle (1.68 ± 0.02 seconds; peak to peak of PND, $P < 0.05$). In
every case, sSNA had a prominent peak during inspiration, coincident with the
burst of PND (Figs. 1, 4A3 and B3). No post-inspiratory bursts were observed in
the sSNA of any rats.

**Baro-activated CVLM neurones display CRD-related activity**

In 35 rats, we recorded 49 neurones in the CVLM that were briskly
activated by raising upper body AP (baro-activated, Fig. 2A) and displayed no
obvious respiratory-related activity in the raw data (Fig. 2A). These CVLM
neurones, which were exquisitely sensitive to AP, showed activity that was
modulated by each pulse of AP (pulse-modulated, Fig. 2B). As shown previously
(Schreihofer & Guyenet, 2000c, 2003), CVLM neurones with these physiological
characteristics express GAD67 mRNA (Fig. 2C), demonstrating they are
GABAergic neurones. In this group of rats the mean AP was 123 ± 2 mmHg and
the average end tidal CO$_2$ was 5.7 ± 0.2% (4.8 - 6.5%) at the time of CVLM unit
recordings. The average basal firing rate of the CVLM neurones was 2.3 ± 0.3
spikes/sec, and no differences in basal firing rate were observed when CVLM
neurones were grouped by CRD-related pattern.

Phrenic-triggered unit histograms revealed 4 general patterns of CRD-
related activity in baro-activated CVLM neurones. All baro-activated CVLM
neurones displayed some pattern of CRD-related activity when triggered from the
PND. Each neurone was categorized by its most prominent pattern feature.
Type 1 (*inspiratory peak*) neurones were sharply activated at the onset of the PND and abruptly inhibited at the end of the phrenic burst. The activity between phrenic bursts was fairly stable, and considerably lower. \((n = 15, \text{Fig. 3A1 and A2})\). Three of these neurones were filled with biotinamide and found to express GAD67 mRNA (see example in Fig. 2C). Type II (*inspiratory depression*) neurones showed a decrease in activity during the PND, which was either abrupt in onset (Fig. 3B1) or gradually decreasing with a nadir during the PND (Fig. 3B2). There were no obvious peaks of activity in between phrenic bursts. \((n = 15)\). Two of these neurones were filled with biotinamide and found to express GAD67 mRNA. Type III (*inspiratory peak, post-inspiratory depression*) neurones were activated during the PND, usually with a ramp of increasing activity. In addition these neurones showed a decrease in firing rate at the end of the PND, which was lowest period of activity \((n = 10, \text{Fig. 3C1 and C2})\). Three of these neurones were filled with biotinamide and found to express GAD67 mRNA. Type IV (*post-inspiratory peak*) neurones showed an increase in activity at the end of the PND or immediately following the burst, which ended with or soon after the termination of the phrenic burst. \((n = 9, \text{Fig. 3D1 and D2})\). In addition some Type IV neurones showed a slight decrease in activity during the PND. Two of these neurones were filled with biotinamide and found to express GAD67 mRNA.

In addition to their pulse-modulated and CRD-related activity, the baro-activated CVLM neurones also displayed a ventilatory-related modulation of their firing rate. This pattern has previously been observed in the SNA of vagotomized, artificially ventilated rats and is produced by arterial baroreceptors...
sensing ventilation-mediated waves in AP (Häbler et al. 1996). Baro-activated CVLM neurones are extremely sensitive to changes in AP, and they fire more during each systole (Fig. 4A1 and B1) and when AP increases on the expiratory phase of the ventilation cycle (Fig. 4A2 and B2). These patterns related to AP were the same in all baro-activated CVLM neurones, and were not related to PND (Fig. 4A and B). The CRD-related patterns observed in baro-activated CVLM neurones were also independent from the pulse- and ventilation-modulation of activity (Fig. 4A3 and 4B3). In agreement, unloading arterial baroreceptors with infusion of nitroprusside abolished pulse-modulated activity (n=13; Fig. 5A1 versus B1), but did not alter the CRD-related pattern in baro-activated CVLM neurones (n=13; Fig. 5A2 versus B2).

**Baro-inhibited CVLM neurones display CRD-related activity**

In 22 rats, we recorded 30 neurones in the CVLM that were silenced by raising upper body AP (Fig. 6A). Baro-activated neurones were also recorded in 9 of these rats. The baro-inhibited CVLM neurones displayed no obvious respiratory-related activity in the raw data (Fig. 6A). As shown previously (Schreihofer & Guyenet, 2003), CVLM neurones with these physiological characteristics express TH-immunoreactivity (Fig. 6B), indicating they are catecholaminergic. The TH-immunoreactive neurones at this rostro-caudal level of the medulla also display PNMT-immunoreactivity, suggesting they are C1 adrenergic neurones. These C1 neurones do not likely project to the spinal cord, but instead probably project to the forebrain (Verberne et al. 1999). In this group
of rats the mean AP was $112 \pm 2$ mmHg and the average end tidal CO$_2$ was $5.2 \pm 0.1\%$. The average basal firing rate of the CVLM neurones was $1.8 \pm 0.2$ spikes/sec, and no differences in basal firing rate were observed when CVLM neurones were grouped by CRD-related pattern.

All baro-inhibited CVLM neurones displayed some pattern of CRD-related activity, and phrenic-triggered unit histograms revealed 3 general patterns. Type 1 (inspiratory peak) neurones were activated at the onset of the PND and had decreased activity at the end of the phrenic burst. The CVLM neuronal activity between phrenic bursts was fairly stable, but considerably lower. ($n = 7$, Fig. 7A1 and A2). Type II (late inspiratory-early expiratory depression) neurones had a decrease in activity during the end of the PND, which continued briefly after the termination of the phrenic burst ($n = 11$, Fig. 7B1 and B2). Type III (inspiratory peak, post-inspiratory depression) neurones were activated during the PND, with a decrease in activity immediately after the phrenic burst ($n = 12$, Fig. 7C1 and C2). A catecholaminergic phenotype was confirmed in a subset of each type of baro-inhibited CVLM neurone (5 TH- and 3 PNMT-immunoreactive neurones).

At resting AP, many baro-inhibited CVLM neurones displayed some pulse-modulated activity, with activation occurring during the troughs of the AP pulses (Fig. 8A1). Unloading arterial baroreceptors with infusion of nitroprusside increased firing rate and abolished pulse-modulated activity (Fig. 8B1), but did not alter the CRD-related pattern in baro-inhibited neurones (Fig. 8A2 versus B2).
Location of recorded CVLM neurones

All recorded barosensitive neurones were found where expected within the CVLM. The neurones were ventral to nucleus ambiguus, and in between the lateral wings of the rostral tips of the lateral reticular nucleus (Fig. 9). There was no difference in the locations of the baro-activated (Fig 9, right) and the baro-inhibited (Fig. 9, left) neurones, or locations examined by CRD-related firing pattern.

Discussion

The SNA that contributes to resting AP is prominently modulated by the central respiratory pattern generator (Adrian et al., 1932; Pilowsky, 1995; Malpas, 1998; Jänig & Häbler 2003). Pre-sympathetic neurones in the RVLM that drive sympathetic vasomotor tone also display CRD-related activity (Haselton & Guyenet, 1989; McAllen, 1987; Miyawaki et al. 1995), indicating that the RVLM is a site of integration of brain stem regulatory systems for respiratory and cardiovascular functions. The principal observation of the present study is that integration between these two regulatory systems also occurs within the CVLM. Baro-activated GABAergic CVLM neurones display one of four patterns of CRD-related activity, namely (I) inspiratory peak, (II) inspiratory depression, (III) inspiratory peak with post-inspiratory depression, and (IV) post-inspiratory peak. In addition, many of these patterns appear to be inversely related to those
observed in pre-sympathetic RVLM neurones. These data suggest that CRD-related activity observed in pre-sympathetic RVLM neurones and SNA may be shaped by GABAergic neurones in the CVLM.

Role of baro-activated GABAergic CVLM neurones in the regulation of SNA

An assumption of the present study is that at least some of the individually recorded CVLM neurones target the pre-sympathetic RVLM neurones to alter SNA and AP. One issue at hand is that adjacent vagal motor neurones in the CVLM display some physiological properties that are indistinguishable from GABAergic CVLM neurones (Schreihofer and Guyenet, 2003). Vagal motor neurones, which are immediately dorsal to the baro-activated GABAergic neurones, are also baro-activated and display CRD-related activity (Jordan et al. 1982; Gilbey et al. 1984; Rentero et al. 2002). However, vagal motor neurones are cholinergic and do not express GAD67 mRNA, making the phenotypic identification of the CVLM neurones critical in the present study. Furthermore, whereas multiple CRD-related patterns are observed in cardiovascular-related CVLM and RVLM neurones, cardiac vagal motor neurones display a consistent CRD-related pattern from neurone to neurone within a preparation (Jordan et al. 1982; Gilbey et al. 1984; Rentero et al. 2002; Neff et al. 2003). The uniform CRD-related pattern observed in vagal motor neurones likely contributes to respiratory sinus arrhythmia. In contrast, the variety of patterns observed in barosensitive pre-sympathetic CVLM and RVLM neurones probably reflects the diversity of their sympathetic targets and physiological functions. Although some
of the CVLM neurones in the present study that were not identified as GABAergic may be vagal motor neurones, a GABAergic phenotype was conclusively established for CVLM neurones displaying each of the four CRD-related patterns.

In addition, we cannot conclusively demonstrate that the recorded CVLM neurones make synaptic contacts with pre-sympathetic RVLM neurones. However, evidence from microinjection studies strongly suggests that the CVLM contains GABAergic neurones that tonically inhibit pre-sympathetic RVLM neurones to reduce SNA (Willette et al., 1984; Blessing, 1988; Schreihofer et al., 2005) and mediate sympathetic cardiovascular reflexes such as the arterial baroreflex and Bezold-Jarisch reflex (Gordon, 1987; Verberne & Guyenet, 1992). In agreement, GABAergic neurones in the CVLM that project to the RVLM express Fos after increased AP, suggesting they are baro-activated (Minson et al., 1997; Chan and Sawchenko, 1998). Extracellular recordings in the CVLM reveal neurones whose activity is inversely related to SNA and expected RVLM neuronal activity. Namely, these GABAergic CVLM neurones are activated by increased AP and phenyl biguanide (Fig. 2; Schreihofer and Guyenet, 2003) and show strong pulse modulation with firing occurring only during systole at higher pressures (Fig. 2; Schreihofer and Guyenet, 2003). Thus, although their role in cardiovascular regulation is putative, these recorded CVLM neurones are excellent candidates for the critical GABAergic interneurones that regulate the RVLM and SNA.

Role of the CVLM in the generation of CRD-related activity in the RVLM and SNA
Some CRD-related patterns of activity in pre-sympathetic RVLM neurones may be produced by baro-activated GABAergic CVLM neurones. Accordingly, in many cases CRD-related patterns observed in the CVLM appeared to be inversely related to previously reported patterns in pre-sympathetic RVLM neurones (Haselton & Guyenet, 1989; Miyawaki et al. 1995) and SNA. For example, type I (inspiratory-peak) CVLM neurones could yield the inspiratory-depression pattern observed in RVLM neurones and SNA (Haselton & Guyenet, 1989; Miyawaki et al. 1995). In agreement, inhibition of the CVLM abolishes the inspiratory-depression pattern in pre-sympathetic RVLM neurones (Miyawaki et al. 1996). Likewise, type II (inspiratory-depression, a.k.a expiratory-related activity) CVLM neurones could permit the inspiratory-peak observed in RVLM neurones by a reduction of GABA, and maintain low activity of RVLM neurones and SNA during expiration. Consistent with this notion, inhibition of glutamatergic receptors in the RVLM does not affect the inspiratory-peak in sSNA (Miyawaki et al. 1996), suggesting it may be mediated by disinhibition of the RVLM neurones. Furthermore, inhibition of the CVLM increases expiratory-related activity in some pre-sympathetic RVLM neurones and SNA (Miyawaki et al. 1996). These effects on lumbar SNA were mimicked by blockade of GABAergic receptors in RVLM (Guyenet et al. 1990), suggesting expiration-related activity in CVLM neurones actively suppresses such activity in the RVLM and SNA to ostensibly enhance inspiratory-related activity.

Although some of the CRD-related activity in pre-sympathetic RVLM neurones may be derived from the CVLM, other patterns may be produced by
excitatory inputs to the RVLM but modulated simultaneously by the CVLM. The post-inspiratory peak in sSNA is abolished by antagonizing glutamate receptors within the RVLM (Miyawaki et al. 1996), suggesting glutamatergic inputs to RVLM may produce the post-inspiratory peak of pre-sympathetic RVLM neurones and sSNA. In contrast, blockade of GABAergic receptors within the RVLM or the inhibition of the CVLM, a major source of GABAergic input, enhances the post-inspiratory peak of individually recorded RVLM neurones and SNA (Guyenet et al. 1990; Miyawaki et al. 1996), and yields a greater CRD-related modulation of sSNA (Miyawaki et al. 1996). These data suggest that the CVLM could act to limit the amplitude of the post-inspiratory peak of pre-sympathetic RVLM neurones and SNA. Indeed, the activity of some baro-activated CVLM neurones displayed a post-inspiratory peak (Fig. 3D). Interestingly, this was the only CRD-related pattern in CVLM neurones with CVLM neuronal activity coincident with the peak in sSNA (Fig. 4B3), suggesting this particular pattern may restrain CRD-related fluctuation of sSNA.

**CRD-related activity in baro-inhibited catecholaminergic CVLM neurones**

In the present study baro-inhibited, catecholaminergic CVLM neurones also displayed CRD-related activity. Whether the adjacent baro-activated GABAergic CVLM neurones are the source of baroreceptor-mediated inhibition or their CRD-related activity is not clear. The three CRD-related patterns observed in the baro-inhibited CVLM neurones could either be described as
similar or inverse to patterns observed in baro-activated CVLM neurones, depending upon which neurones are compared. For example, the baro-inhibited, inspiratory-depression neurones (Fig. 7B2) could viewed as inversely related to the baro-activated type I (inspiratory-peak) neurones (Fig. 3A2), or as similar to the baro-activated type II (inspiratory-depression) neurones (Fig. 3B1). The patterns observed in the baro-inhibited neurones are comparative to those observed in the spinally-projecting rostral C1 cells of the RVLM, except that no baro-inhibited neurones with clear post-inspiratory peaks were observed. This difference may be the result of a small sample size, a true distinction between caudal and rostral C1 cells, or the state of the preparation. Given that caudal C1 neurones project to autonomic regions of the forebrain (Verberne et al. 1999), they could provide respiratory-related modulation of long-loop autonomic responses.

Ventilatory-related modulation of CVLM neuronal activity

In addition to their pulse-modulated and CRD-related activity, the baro-activated CVLM neurones also displayed a ventilation-related modulation of their firing rate. This pattern has been observed in the SNA of vagotomized, artificially ventilated rats (Häbler et al. 1996). These ventilation-related fluctuations in SNA are eliminated by transecting arterial baroreceptor afferent nerves, suggesting ventilation-related fluctuations in AP are sensed by baroreceptors and transmitted to the SNA (Boczek-Funcke et al. 1992a; Häbler et al. 1996). All baro-activated CVLM neurones had increased neuronal activity coincident with
rises in end-tidal CO$_2$ and AP (Fig. 4A2 and B2). The strength of this modulation appeared to coincide with the degree of pulse-modulation (Fig. 4A1 and A2 versus 4B1 and B2), in agreement with the notion that both patterns arise from the same afferent signals. The ventilation-related modulation was independent of the CRD-related activity, which was not synchronized with ventilation (Fig. 4A3 and B3). However, in animals with intact vagus nerves, where ventilation and the central respiratory pattern generator are synchronized, this baroreceptor-mediated modulation likely enhances respiratory-related activity of barosensitive brain stem neurones and SNA.

*Modulation of SNA by central respiratory generator*

In the present study we recorded sSNA to ensure the preparation had significant CRD-related modulation of autonomic function. In all cases sSNA peaked during inspiration, with no post-inspiratory peak observed in any animals. This pattern has also been observed in splanchnic nerve of vagotomized, ventilated, paralysed rats anesthetized with halothane (Numao *et al.* 1987), urethane after clonidine (Koshiya & Guyenet, 1995), or pentobarbital (Miyawaki *et al.*1996), and in cats under urethane or after decerebration (Cohen & Gootman, 1970). Under comparable experimental conditions, sympathetic nerves to other targets, such as renal, adrenal, muscle constrictor, or cardiac also display a peak in activity during inspiration (Numao *et al.* 1987, Connelly & Wurster, 1985, Jänig & Häbler, 2003). In contrast, under other experimental conditions sSNA displays a prominent post-inspiratory peak (Miyawaki *et al.*
In addition, within the same preparation different sympathetic nerves may simultaneously display diverse CRD-related patterns. For example, in halothane-anesthetized rats while splanchnic nerve activity peaks during inspiration, cervical and lumbar nerve activities peak only during post-inspiration (Numao et al. 1987). These observations suggest that CRD-related activity observed in SNA is differentially regulated to distinct autonomic targets and the patterns observed in whole nerves are state-dependent. Whether different states also change the CRD-related patterns observed in individual medullary cardiovascular-related neurones or simply alter which neurones are active remains to be determined.

Summary

In summary, the present study provides evidence for the integration of cardiovascular and respiratory controls within the CVLM in rats. GABAergic CVLM neurones are believed to play a major role in regulating the activity of pre-sympathetic RVLM neurones and to have an essential role in the production of many autonomic reflexes. Data from previous microinjection studies and recordings from individual CVLM neurones in the present study suggest that the CVLM may also provide CRD-related modulation of pre-sympathetic RVLM neurones, SNA, and AP. The present study suggests the CVLM may shape CRD-related patterns in SNA by providing inspiratory-and expiratory-related depressions and restraint of prominent post-inspiratory peaks in SNA. In addition, the absence of CVLM neuronal activity may allow inspiratory-related
peaks in SNA. Further study will be necessary to determine the mechanisms underlying CRD-related modulation of baro-activated GABAergic CVLM neurones and to understand how or whether CVLM neurones with particular CRD-related patterns are connected to baro-inhibited neurones within the ventrolateral medulla.
References


Acknowledgements

This work was supported by NIH grant HL-075174 to Dr. Schreihofer. The authors thank Ms. Sherita James for her invaluable technical assistance in the histological processing.
Figure Legends

Figure 1. Example of relationships of phrenic nerve discharge (PND), end tidal CO2, and splanchnic sympathetic nerve activity (SNA)

The integrated SNA (int SNA) has prominent respiratory-related rhythm with most of the bursts occurring with the PND. In this vagotomized rat, the PND desynchronized with ventilation, as shown by a lack of relationship with changes in end tidal CO2.

Figure 2. Example of a typical baro-activated GABAergic CVLM neurone.

A: Elevating arterial pressure (AP) by constricting the abdominal snare (at bar under AP trace) increases the firing of this baro-activated neurone and reduces the splanchnic SNA. B: At high AP this baro-activated neurone displayed pulse synchrony, with firing occurring with each systole. C1: The baro-activated neurone was filled with biotinamide revealed with strept-avidin Alexa 488 (at arrow) C2: The same area shown in C1 but under brightfield to reveal the presence of GAD67 mRNA in the biotinamide-labelled neurone (at arrow). Scale bar, 25 µm.

Figure 3. Four patterns of CRD-related activity in baro-activated CVLM neurones.

A1 and A2: Type I: Phrenic-triggered histograms reveal a clear peak of CVLM neuronal activity during the PND. B1 and B2: Type II: Phrenic-triggered histograms reveal a depression of CVLM neuronal activity during the PND, which
often persisted after the termination of the phrenic burst (B1). C1 and C2: Type III: Phrenic-triggered histograms reveal increased CVLM neuronal activity during the PND that was followed by a depression at the termination of the phrenic burst. D1 and D2: Type IV: Phrenic-triggered histograms reveal an increase in CVLM neuronal activity immediately after the termination of the phrenic burst. Each histogram represents 150 sweeps with a 0.1-second bin size.

Figure 4. Examples of pulse-modulation, ventilation-related modulation, and CRD-related activity in baro-activated CVLM neurones.

A1: Arterial pulse-triggered histogram reveals a strong pulse-modulation of CVLM neuronal activity, with highest firing occurring during systole. PND and end tidal CO₂ waves were not related to the AP pulse. A2: In the same neurone shown in A1 a CO₂-triggered histogram reveals correlated waves in AP and CVLM neuronal activity, with the highest CVLM neuronal activity occurring with the coincident peaks in CO₂ and AP. PND was unrelated to ventilation-related waves in end tidal CO₂, AP, or CVLM neuronal activity. A3: Phrenic-triggered histogram of same neurone shown in A1 and A2 reveals inspiratory-depression, type II CRD-related CVLM neuronal activity that was inversely related to SNA but unrelated to AP or end tidal CO₂. B1: Pulse-triggered histogram of another baro-activated CVLM neurone reveals pulse-modulation of CVLM neuronal activity, which is weaker than the neurone shown in A1. B2: CO₂-triggered histogram of same neurone shown in B1 reveals ventilation-related waves in AP,
CVLM neuronal activity, and SNA, which is also weaker than the neurone shown in A2. B3: Phrenic-triggered histogram of same neurone shown in B1 and B2 reveals inspiratory-peak type I CRD-related CVLM neuronal activity. Phrenic- and CO₂-triggered histograms represent 150 sweeps with a bin size of 0.1 seconds. The AP-triggered histograms represent 1500 sweeps with a bin size of 0.01 seconds.

Figure 5. Example of the effects of unloading arterial baroreceptors with nitroprusside (5 mg/kg, iv) on the activity of a baro-activated CVLM neurone. A1: AP pulse-triggered histogram reveals strong pulse-modulation of CVLM neuronal activity. A2: A phrenic-triggered histogram reveals inspiratory-depression CRD-related activity in the same CVLM neurone shown in A1. B1: AP-triggered histogram after nitroprusside-induced hypotension reveals an abolition of pulse-modulation of CVLM neuronal activity. B2: Phrenic-triggered histogram of same neurone shown in B1 reveals nitroprusside does not alter inspiratory-depression in the CVLM neuronal activity. Phrenic-triggered histograms represent 100 sweeps with a bin size of 0.1 seconds. The AP pulse-triggered histograms represent 1000 sweeps with a bin size of 0.01 seconds.

Figure 6. Example of a typical baro-inhibited catecholaminergic CVLM neurone.
A: Elevating arterial pressure (AP) by constricting the abdominal snare (at bar under AP trace) silences the firing of this baro-inhibited neurone and splanchnic
SNA.  

**B**: The baro-inhibited neurone was filled with biotinamide revealed with strept-avidin Alexa 488 (at arrow)  

**C**: The same area shown in **B** to reveal the presence of tyrosine hydroxylase immunoreactivity (at arrow), demonstrating this neurone is catecholaminergic. Scale bar, 25 µm.

**Figure 7. Three patterns of CRD-related activity in baro-inhibited CVLM neurones.**

**A1 and A2**: Type I: Phrenic-triggered histograms reveal a clear peak of CVLM neuronal activity during the PND.  

**B1 and B2**: Type II: Phrenic-triggered histograms reveal a depression of CVLM neuronal activity during the PND, which often persisted after the termination of the phrenic burst.  

**C1 and C2**: Type III: Phrenic-triggered histograms reveal increased CVLM neuronal activity during the PND that was followed by a depression at the termination of the phrenic burst.  

Each histogram represents 150 sweeps, and the bin size for PND-triggered histograms was 0.1 seconds.

**Figure 8. Example of the effects of unloading arterial baroreceptors with nitroprusside (5 mg/kg, iv) on the activity of a baro-inhibited CVLM neurone.**

**A1**: AP pulse-triggered histogram reveals pulse-modulation of CVLM neuronal activity.  

**A2**: A phrenic-triggered histogram reveals inspiratory-peak CRD-related activity in the same CVLM neurone shown in **A1**.  

**B1**: AP-triggered histogram after nitroprusside-induced hypotension reveals an abolition of pulse-modulation
of CVLM neuronal activity.  \textit{B2:} Phrenic-triggered histogram of same neurone shown in \textit{B1} reveals nitroprusside does not alter inspiratory-peak in the CVLM neuronal activity.  Phrenic-triggered histograms represent 100 sweeps with a bin size of 0.1 seconds.  The AP pulse-triggered histograms represent 1000 sweeps with a bin size of 0.01 seconds.

\textbf{Figure 9. Location of recorded CVLM neurones that were filled with biotinamide.}

Two representative coronal brain stem sections contain the plotted locations of recorded barosensitive CVLM neurones.  The more rostrally located neurones are in \textit{A} (bregma -13.68 mm) and the more caudally located neurones are in \textit{B} (bregma -13.24 mm).  The baro-inhibited neurones are on the left side of the sections ($n = 8$ catecholaminergic neurones) and the baro-activated neurones are on the right side of the sections ($n = 10$ GABAergic neurones, $n = 5$ filled with biotinamide, but not successfully processed for GAD67 mRNA).  There was no difference in the location of baro-activated vs. baro-inhibited neurones.  Shapes represent location of barosensitive CVLM neurones categorized by CRD-related activity: Triangles are type 1, \textit{inspiratory-peak}, circles are type II, \textit{inspiratory-depression}, squares are type III, \textit{inspiratory-peak with post-inspiratory depression}, and asterisks the type IV, \textit{post-inspiratory peak}.  

Figure 1
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Central respiratory modulation of barosensitive neurones in rat caudal ventrolateral medulla

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J. Physiol. published online Mar 9, 2006;

DOI: 10.1113/jphysiol.2005.103622

This information is current as of March 26, 2006