Research Paper

Neurons of the A5 region are required for the tachycardia evoked by electrical stimulation of the hypothalamic defence area in anaesthetized rats

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New Findings

- **What is the central question of this study?**
  As the parabrachial complex plays a role in the cardiorespiratory response evoked from the hypothalamic defence area (pressor response and tachycardia) and the A5 region is involved in the responses evoked from the parabrachial complex, a possible interaction between the hypothalamic defence area and A5 is suggested. Accordingly, the present study was carried out to assess this hypothesis in rats.
- **What is the main finding and its importance?**
  The neuromorphological, neuropharmacological and electrophysiological results of our study demonstrate, for the first time, that the A5 region is an important component of those brainstem pathways known to be involved in mediating the autonomic changes associated with the electrically evoked defence response.

In order to assess the possible interactions between the pontine A5 region and the hypothalamic defence area (HDA), we have examined the pattern of double staining for c-Fos protein immunoreactivity (c-Fos-ir) and tyrosine hydroxylase, throughout the rostrocaudal extent of the A5 region in spontaneously breathing anaesthetized male Sprague–Dawley rats during electrical stimulation of the HDA. Activation of the HDA elicited a selective increase in c-Fos-ir with an ipsilateral predominance in catecholaminergic and non-catecholaminergic A5 somata ($P < 0.001$ in both cases). A second group of experiments was done to examine the importance of the A5 region in modulating the cardiorespiratory response evoked from the HDA. Cardiorespiratory changes were analysed in response to electrical stimulation of the HDA before and after ipsilateral microinjection of muscimol within the A5 region. Stimulation of the HDA evoked an inspiratory facilitatory response, consisting of an increase in respiratory rate ($P < 0.001$) due to a decrease in expiratory time ($P < 0.01$). The respiratory response was accompanied by a pressor response ($P < 0.001$) and tachycardia ($P < 0.001$). After muscimol microinjection within the A5 region, pressor and heart rate responses to HDA stimulation were reduced ($P < 0.01$ and $P < 0.001$, respectively). The respiratory response persisted unchanged. Finally, to confirm functional interactions between the HDA and the A5 region, extracellular recordings of putative A5 neurones were obtained during HDA stimulation. Seventy-five A5 cells were recorded, 35 of which were affected by the HDA (47%). These results indicate that

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neurones of the A5 region participate in the cardiovascular response evoked from the HDA. The possible mechanisms involved in these interactions are discussed.

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Introduction

The A5 region is located in the ventrolateral pons, between the root of the facial nerve and the superior caudal olivary nucleus. Classically, A5 has been defined as a catecholaminergic region, which extends from interaural to 2.0 mm caudally (Dahlström & Fuxe, 1964; Byrum *et al.* 1984). It is known to provide the major component of the noradrenergic input to the sympathetic preganglionic neurones of the intermediolateral cell column of the spinal cord (IML; Loewy *et al.* 1979*a*; Byrum *et al.* 1984; Bruinstroop *et al.* 2012), whereby it has been implicated in cardiovascular control (Loewy *et al.* 1979*b*; Huangfu *et al.* 1991; Lara *et al.* 2002; Pilowsky & Goodchild, 2002; Dampney *et al.* 2003; Dawid Milner *et al.* 2003; Guyenet, 2006). It also contains non-catecholaminergic neurons, which are mainly located at the level of the most caudal part of the A5 region (Goodchild *et al.* 2001). These neurons seem to have properties similar to the respiratory chemoreceptors identified previously in the rostral medulla oblongata (Mulkey *et al.* 2004).

The A5 region has connections with the nucleus tractus solitarii (NTS), caudal ventrolateral medulla (CVLM), rostral ventrolateral medulla (RVLM), caudal pressor area and the retrotrapezoid nucleus in the medulla oblongata, with the medial, Kölliker–Fuse and lateral parabrachial nuclei in the pons, and with the perifornical area, the paraventricular nucleus and the amygdala in the hypothalamus (Byrum & Guyenet, 1987; Tavares *et al.* 1997; Sun & Panneton, 2005; Rosin *et al.* 2006; Usunoff *et al.* 2006; Abbott *et al.* 2012). These connections with regions of the CNS involved in cardiorespiratory regulation are indicative for a role of the A5 region in the control of both sympathetic activity and cardiorespiratory function (Spyer, 1994; Dampney *et al.* 2003; Taxini *et al.* 2011).

Moreover, A5 neurons are activated during baroreceptor unloading (Dampney *et al.* 2003) and stimulation of carotid chemoreceptors (Guyenet *et al.* 1993; Erickson & Millhorn, 1994). Thus, it has been proposed that A5 neurons may play an important role in the carotid sympathetic chemoreflex triggered by hypoxia (Koshiya & Guyenet, 1994; Kanbar *et al.* 2011; Song *et al.* 2011). Furthermore, the A5 region plays an important role in respiratory control, modulating the activity of respiratory neurons (Hilaire *et al.* 2004). These cells are synaptically connected to phrenic motoneurons (Dobbins & Feldman, 1994) and contribute to the respiratory responses evoked by hypoxia and hypercapnia (Coles & Dick, 1996; Roux *et al.* 2000; Schlenker & Prestbo, 2003; Kanbar *et al.* 2011; Song *et al.* 2011).

A5 cells also modulate the cardiorespiratory response evoked by activation of the parabrachial nucleus (Dawid Milner *et al.* 2003), which is a critical component of the brainstem respiratory network required for eupnoea (St-John & Paton, 2004).

Stimulation of A5 neurons with glutamate produces cardiorespiratory and laryngeal responses characterized by an expiratory facilitatory response associated with an increase in blood pressure, heart rate (Dawid-Milner *et al.* 2001) and subglottic pressure (Lara *et al.* 2002). The cardiovascular response is similar to that obtained during electrical stimulation of the classical 'hypothalamic defence area' (HDA; Hilton & Redfern, 1986), which was so named because stimulation of this region evokes a pattern of autonomic and behavioural changes that are typically observed when an animal is confronted with a threatening stimulus (DiMicco *et al.* 2002). The defence response evoked on HDA electrical stimulation is characterized by hypertension, tachycardia and tachypnoea. The simultaneous increase of arterial blood pressure, heart rate and sympathetic vasomotor activity implies that the baroreceptor reflex is reset to higher levels of arterial pressure, but without attenuation in sensitivity of the reflex (Hatton *et al.* 1997; Schadt & Hasser, 1998). In this effect, there is known to be involved a potentiation of the chemoreceptor reflex (Silva-Carvalho *et al.* 1995*a*) and an activation of GABAergic mechanisms at the level of the NTS (Jordan *et al.* 1988; Silva-Carvalho *et al.* 1995*b*).

In previous studies, we have demonstrated a role of the parabrachial complex in the cardiorespiratory response evoked on electrical stimulation of the HDA (Díaz-Casares *et al.* 2009, 2012). We have also demonstrated the participation of the A5 region in the cardiorespiratory responses evoked on parabrachial electrical and chemical stimulation (Dawid Milner *et al.* 2003). These functional connections suggest a possible interaction between the HDA and the A5 region. Accordingly, the present study was carried out to assess this hypothesis in rats.

In order to achieve the objectives, we have examined the pattern of double staining for c-Fos protein immunoreactivity (c-Fos-ir) and tyrosine hydroxylase immunoreactivity (TH-ir), throughout the rostrocaudal extent of the A5 region of anaesthetized male Sprague–Dawley rats during electrical stimulation of the HDA. In

a second study, the cardiovascular response evoked on HDA electrical stimulation was analysed before and after inhibition of the activity of A5 cells with the microinjection of the GABA agonist muscimol. Finally, to confirm the interactions between these regions, extracellular recordings of putative A5 cells were made during HDA electrical stimulation.

Methods

Ethical approval

All experimental protocols were performed in accordance with the recommendations of the European Union directive (86/609/EU) for animal care and experimental procedures, and the experiments were approved by the Ethical Committee for Animal Research of the University of Malaga and the Junta de Andalucía. Every attempt was made to reduce animal suffering and discomfort and to reduce the number of animals needed to obtain reliable results.

Animals and housing

Studies were performed on 42 male specific pathogen-free Sprague–Dawley rats weighing 250–350 g (Charles River, Barcelona, Spain). Animals were housed, six per cage, in a temperature-controlled room (22–24°C) and maintained on a 12 h–12 h light–dark cycle (light on at 07.00 h) in the Animal House of the University of Malaga. Food and water were available *ad libitum*.

General procedures

Anaesthesia was induced with sodium pentobarbitone (initial dose 60 mg kg^{-1} I.P., supplemented as necessary with 2 mg kg^{-1} I.V.) Catheters were inserted into a femoral artery for the measurement of arterial blood pressure and a femoral vein for the administration of drugs. The trachea was cannulated below the larynx for the measurement of airflow through a Fleish pneumotachograph. An air-filled catheter was introduced into the oesophagus for the indirect measurement of pleural pressure. The animals breathed spontaneously a mixture of humidified O_2-enriched room air. End-tidal CO_2 was monitored during the experiment with a fast-response CO_2 analyser (ADC FM1, The Analytical Development Co. Ltd., Great Amwell, UK); values ranged from 3 to 5%. Rectal temperature was maintained at 37–38°C by a servo-controlled heating pad.

The depth of anaesthesia was assessed by observing the presence or absence of a significant withdrawal reflex to pinching a paw and the absence of alterations in arterial blood pressure and heart rate. Throughout the experiment, a stable level of these variables was used as an indication of the anaesthetic level, and any changes in resting conditions were countered by supplemental anaesthetic doses.

The animals were positioned in a stereotaxic frame, with the upper incisor bar 3.3 mm below the interaural line (Paxinos & Watson, 2005), and fixed by clamps on the spinous processes of C7 and L2.

Interaction of HDA and A5: c-Fos/TH-ir experiments

Electrical stimulation of HDA and expression of c-Fos. In eight animals, two burr holes were drilled into the skull to allow access to the right HDA and the right pons through the cerebellum. A concentric bipolar electrode (NE-100; Rhodes Medical Electrodes, Summerland, CA, USA) was positioned in the right HDA according to the co-ordinates described in the atlas of Paxinos & Watson (2005). The right HDA was stimulated once with 1 ms pulses of 30–50 μA given at 100 Hz for 5 s, to obtain the classical defence response (Yardley & Hilton, 1986). Then, guanethidine (10 mg kg^{-1} I.V.) was administered to avoid sympathetically mediated cardiovascular responses. In one group of animals ($n = 4$), the HDA was stimulated with trains of 1 ms pulses of 30–50 μA given at 100 Hz for 5 s, every 60 s during 1 h. The other group of animals ($n = 4$) was not stimulated and served as the control group.

One hour after the end of the experiments, the animals were given an overdose of sodium pentobarbitone and perfused via the ascending aorta with 300 ml of cold 0.9% NaCl, immediately followed by 500 ml of 4% paraformaldehyde in 0.1 M PBS (pH 7.4) as fixative. The brains were rapidly removed, postfixed by immersion in the same fixative solution for 2 h at 4°C and cryoprotected in 30% phosphate-buffered sucrose for 48 h. The brainstem was cut on a cryostat (HM 500M; Micron, Walldorf, Germany) into 20 μm coronal sections, sampled every five sections with a random start and processed for c-Fos-ir.

c-Fos immunohistochemistry. Briefly, sections were incubated overnight at 4°C with a rabbit polyclonal antibody against the c-Fos protein (1:10,000 dilution in PBS containing 0.3% Triton X-100 and 1% normal sheep serum; Santa Cruz Biotechnology, Inc., Heidelberg, Germany). This antibody was raised against a peptide corresponding to amino acids 3–16, mapping at the amino terminus of human c-Fos p62 (identical to the corresponding mouse sequence), and is specific for c-Fos p62, showing no cross-reactivity with Fos B, Fra-1 or Fra-2. After washing in PBS, sections were incubated for 1 h at room temperature with biotinylated secondary antibody (1:200 dilution; donkey anti-rabbit IgG; Amersham plc, Amersham, UK), then rinsed in PBS and incubated for 1 h at room temperature with Vectastain solution (Vector Laboratories, Burlingame, CA, USA; diluted 1:100 in the above-described solution). After washing in PBS and in 0.05 M Tris–HCl buffer (pH 7.4), staining was carried out with 3,3′-diaminobenzidine (DAB; 25 mg in 100 ml Tris–HCl, pH 7.4 and 0.002% H_2O_2).

In order to assure the validity of our procedure, several controls were implemented. Three of the non-selected sections from each rat, taken from different levels, were incubated omitting either the anti-c-Fos or the anti-rabbit biotinylated antibodies in their respective incubation steps. No immunohistochemical labelling was observed in any of these sections.

Double immunolabelling of c-Fos and tyrosine hydroxylase. In order to investigate the presence of c-Fos-ir in TH-ir neurons, previously c-Fos-labelled sections from each rat were incubated overnight at 4°C with mouse anti-TH polyclonal antibody (1:5000 dilution; Sigma Biosciences, St Louis, MO, USA). After washing in PBS, sections were incubated for 1 h at room temperature with biotinylated anti-mouse secondary antibody (1:200 dilution; Amersham plc) and for 1 h at room temperature with Vectastain solution (1:100 dilution). After washing in PBS and in 0.05 M Tris–HCl buffer (pH 7.4), the sections were stained with the 4-chloro-1-napthol method. Sections were washed, dried and coverslipped with glycerol–PBS solution on gelatin–chromalum-coated slides.

Cell counting and statistical studies. Every section was numbered according to the rostrocaudal level determined with the rat brain atlas of Paxinos & Watson (2005). The c-Fos-ir in noradrenergic cell bodies (dark nucleus and brownish cytoplasm double immunostaining) and total number of c-Fos-ir cell nuclei were counted bilaterally in the sections containing the A5 region in a Nikon Microphot-FX microscope. All data are expressed as means ± SEM. For statistical comparisons, one-way analysis of variance was used to compare the differences between groups. Significance was taken at a probability of $P < 0.05$.

Interaction of HDA and A5: stimulation experiments

In 24 animals, two burr holes were drilled into the skull to allow access to the right HDA and the right pons through the cerebellum. A concentric bipolar electrode (NE-100; Rhodes Medical Electrodes) was positioned in the right HDA, which was stimulated with 1 ms pulses of 30–50 μA given at 100 Hz for 5 s.

A glass microelectrode was positioned stereotaxically into the A5 region of the pons ipsilateral to the stimulated HDA. The microelectrode was filled with muscimol (4 mM) dissolved in a solution of sodium-PBS (pH 7.4 ± 0.1) with 0.05% Evans Blue. Microinjections of PBS–Evans Blue alone were used for control purposes. Evans Blue served to mark microinjection sites.

Microinjection volumes of 50 nl were programmed with a pump controller (Ultra Micro Pump II, Micro 4; World Precision Instruments, Inc., Sarasota, FL, USA.) driving 0.5 μl microsyringes attached to the microelectrode. The volume injected was measured by observing the displacement of the microsyringe plunger wire. Only one microinjection was delivered in each animal.

According to the atlas of Paxinos & Watson (2005), the stereotaxic co-ordinates to locate the HDA were from −2.2 to −2.8 mm caudal to bregma, 0.6–1 mm lateral to the mid-line and 8–9 mm deep from the surface of the calota. In order to locate the A5 region of the pons, the barrel was positioned according to the following parameters: from interaural line to −1.6 mm, 2–3 mm lateral to the mid-line and 1 to −0.5 mm deep to the interaural line.

The following protocol was used in each experiment.

(i) Responses to HDA activation were measured before PBS or muscimol was injected into the A5 region. The respiratory and cardiovascular changes were analysed during HDA electrical stimulation.
(ii) Responses to HDA activation were measured after PBS or muscimol was injected into the A5 region. The cardiorespiratory responses to electrical HDA stimulations were characterized 4 min after the injection of PBS (50 nl, pH 7.4 ± 0.1, 5 s duration) or muscimol (50 nl, 0.25 nmol, pH 7.4 ± 0.1, 5 s duration). Only one microinjection was delivered in each animal and only two HDA stimulations, separated by 15 min, were given.

Evans Blue was used to find the position of the pontine electrode. Electrical lesions (250 μA DC for 20 s) served to locate the HDA stimulation sites. Brains were perfused with formal saline and serially sectioned (50 μm thickness) at the level of the hypothalamus and the pons. The hypothalamus was counterstained with Neutral Red. Pontine series were processed for TH-ir to check that microinjection sites lay within the A5 region.

In summary, HDA electrical stimulation was elicited twice, separated by 15 min; the first stimulation was before the microinjections and the second one 4 min after ipsilateral A5 microinjection.

Airflow, respiratory volume, pleural pressure (as an index of inspiratory activity) and arterial pressure were monitored and stored on digital tape for offline analysis (Neuro-Corder DR-890, Neuro Data Instruments Corp., New York, NY, USA). Measurements were made of inspiratory time, expiratory time, instantaneous respiratory frequency, mean arterial blood pressure and instantaneous heart rate. Recordings were made for 3 min, starting 30 s before the beginning of a stimulus. The 3 min window used for data analysis allowed a complete recovery of the evoked response. In all experiments, baseline values for mean arterial blood pressure, heart rate and respiratory parameters were measured immediately prior to HDA electrical stimulation. Changes in mean arterial blood pressure or heart rate were assessed by measuring the peak rise in blood pressure or heart rate observed during the 5 s electrical stimulation of the hypothalamus.

Table 1. Relative distribution of A5 region tyrosine hydroxylase-immunoreactive (TH-ir) cells, c-Fos-immunoreactive (c-Fos-ir) and double-labelled c-Fos/TH-ir cell bodies before (control) and after hypothalamic defence area (HDA) stimulation (stimulated) in spontaneously breathing rats, in which sympathetically mediated cardiovascular changes were abolished with guanethidine

	Control ($n = 4$)		Stimulated ($n = 4$)	
A5	Ipsilateral	Contralateral	Ipsilateral	Contralateral
TH-ir cells	103 ± 12	98 ± 14	110 ± 13	104 ± 9
c-Fos-ir cells	26.25 ± 0.85	9.5 ± 0.29***	71.5 ± 1.7†††	39.75 ± 1.1***†††
c-Fos/TH-ir cells	25.5 ± 0.95	19.5 ± 0.75***	36. 5 ± 1.1†††	21.25 ± 0.85**

Values are means ± SEM. Asterisks show differences between ipsilateral and contralateral sides of control or stimulated animals. (**$P < 0.01$ and ***$P < 0.001$). Daggers show differences between control and stimulated ipsilateral sides or control and stimulated contralateral sides (†††$P < 0.001$).

Stimulus-evoked changes in respiratory parameters were measured as the average response observed during the 5 s stimulation of the defence area.

Only data from animals in which the histology showed that the microelectrodes were positioned within the HDA and the A5 region were used for statistical procedures. All data are expressed as means ± SEM. For statistical comparisons, once the statistical normality (Kolmogorov–Smirnov test) and the homocedasticity (Bartlett's test) of the data were verified, a paired-sample test was applied to compare the control with the evoked response period for each animal. One-way analysis of variance with Student–Newman–Keuls *post hoc* test was used to compare different groups of animals. Significance was taken at a probability of $P < 0.05$.

Interaction of HDA and A5: neuronal unit recording experiments

In 10 animals, a stimulation bipolar electrode (SNE-100; Rhodes Medical Electrodes) was stereotaxically positioned in the right HDA. For initial localization of the HDA, 1 ms pulses of 50 μA at 100 Hz were given to evoke the characteristic cardiorespiratory response of the defence reaction. During neuronal recording, the stimulus was reduced to short trains of up to 10 pulses (0.1 ms given at 1 Hz). This markedly reduced or abolished the changes in blood pressure evoked by continuous stimulation.

Extracellular recordings were made from ipsilateral neurons in the vicinity of the A5 region with tungsten electrodes (DC impedances between 8 and 10 MΩ; A-M Systems Inc., Carlsberg, WA, USA). Extracellular potentials were amplified conventionally (Neurolog, Digitimer Ltd. Welwyn Garden City, UK). All data were stored and the offline analysis was done using a computer A/D system with data capture and analysis software (CED 1401, Spike2, Sigavg, Cambridge Electronic Design Ltd., Cambridge, UK).

Extracellular recordings from single neurons were distinguished from those of multiple neurons by analysis of spike shape, height and successive spike intervals. Neurons were shown to be antidromically or orthodromically activated on the basis of short constant response latency, frequency following and the collision test. A more pronounced initial-segment somato-dendritic (IS–SD) break and failures of invasion of the somato-dendritic (SD) complex with high-frequency stimulation was considered an index of the antidromic activation. Orthodromic potentials were slightly larger and of shorter duration than antidromic potentials. The analysis of neuronal discharge was obtained with interspike interval histograms and represented as integrated rate histograms. Neuronal cardiovascular or respiratory patterns of discharge were inferred from peritrigger time histograms using systolic blood pressure or the maximal inflexion of expiratory flow as the event markers.

The recorded cells were identified as putative noradrenergic A5 neurons when they fulfilled the following criteria (Horiuchi *et al.* 2006): (i) they had a low and steady firing rate (<5 Hz) and long-duration (>0.6 ms) action potentials; (ii) their activity was inhibited by clonidine (10 μg kg^{-1} I.V.); and (iii) they were recorded in sites having co-ordinates corresponding to the location of the A5 group and subsequently, when processed for TH-ir, revealed to be within the A5 region.

During the experiments, stereotaxic co-ordinates and the patterns of the cardiorespiratory responses to electrical stimulation through the recording electrode were used as an index of the location of the A5 region. The last recording site in each experiment was marked with an electrical lesion (250 μA DC for 20 s); the other recording sites were deduced from their stereotaxic co-ordinates relative to the marked site.

Results

Interaction of HDA and A5: c-Fos/TH-ir experiments

Distribution of TH-ir. In both control and electrically stimulated animals, no differences were found in the number and distribution of ipsilateral or contralateral TH-ir neurons (Table 1).

Distribution of c-Fos-ir. In control animals, non-catecholaminergic cells located within the A5 region (no TH-staining cell bodies) showed a higher number of c-Fos-ir profiles in the ipsilateral than in the contralateral

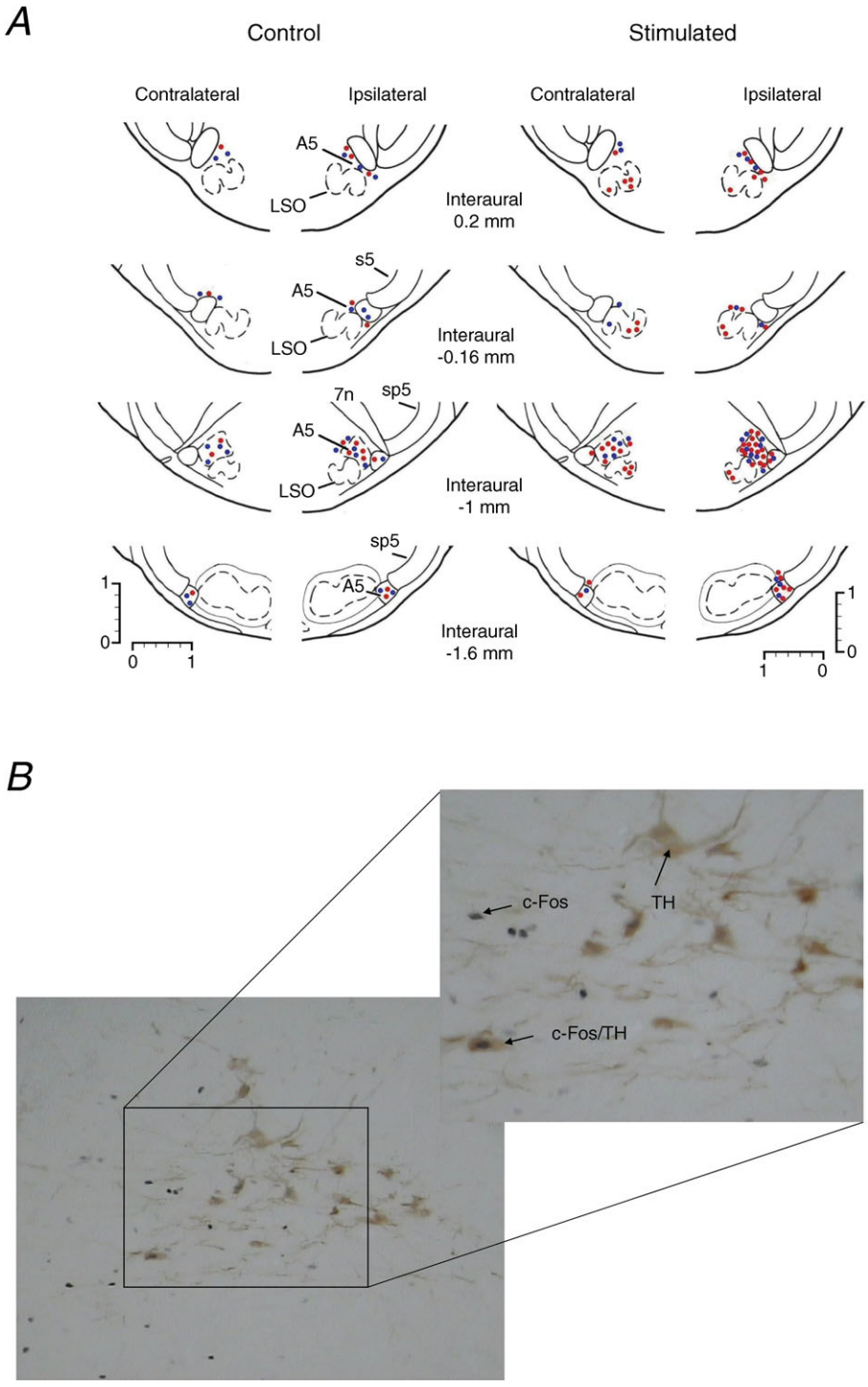

Figure 1. Distribution of c-Fos-and c-Fos/TH cell bodies through the A5 region in control and hypothalamic defence area stimulated rats
A, semi-schematic line drawings of ipsi- and contralateral coronal sections through the A5 region, from rostral (top) to caudal (bottom), showing the distribution of the expression of c-Fos-immunoreactive (c-Fos-ir; red dots), and double-labelled c-Fos/tyrosine hydroxylase (TH)-ir cell bodies (blue dots) in control animals (control) and hypothalamic defence area (HDA)-stimulated spontaneously breathing rats, in which sympathetically mediated cardiovascular changes were abolished with guanethidine (stimulated). Each dot represents two somata. *B*, photomicrograph of a coronal section showing c-Fos-ir, TH-ir and c-Fos/TH-ir cells within the A5 region of the ventrolateral pons (s5) sensory root trigeminal nerve. Abbreviations: LSO, lateral superior olivary nucleus; 7n, facial nerve or its root; and sp5, spinal trigeminal tract.

side ($P < 0.001$) in non-stimulated animals (Table 1 and Fig. 1).

In electrically stimulated animals, non-catecholaminergic cells located within the A5 region showed an ipsilateral predominance for c-Fos-ir staining ($P < 0.001$). Also, a greater number of c-Fos somata were observed in stimulated animals compared with control animals for both ipsilateral ($P < 0.001$) and contralateral sides ($P < 0.001$; Table 1 and Fig. 1).

Distribution of double-labelled c-Fos/TH-ir cell bodies. In control animals, the A5 region showed bilateral double staining. An ipsilateral predominance of double-labelled cells was observed ($P < 0.001$; Table 1).

In electrically stimulated animals, a higher number of double-labelled c-Fos/TH-ir neurons was found in the ipsilateral side compared with the contralateral side ($P < 0.01$). The ipsilateral side also presented a greater number of double-labelled c-Fos/TH-ir perikarya compared with the ipsilateral side of control animals ($P < 0.001$). No differences were observed for the contralateral side (Table 1 and Fig. 1).

Interaction of HDA and A5: stimulation experiments

Electrical stimulation of HDA. In the control group ($n = 7$), electrical stimulation within the HDA elicited a cardiorespiratory response characterized by a pressor response ($P < 0.001$) accompanied by an increase in heart rate ($P < 0.001$). The respiratory response consisted in an increase in respiratory rate ($P < 0.001$), due to a decrease in expiratory time ($P < 0.01$). No significant changes in inspiratory time were observed. Inspiratory activity, measured as pleural pressure, was also increased ($P < 0.05$; Figs 2, 4*A* and 5 and Tables 2 and 3).

Electrical stimulation of HDA before and after A5 microinjections. The microinjection of PBS within the A5 region ($n = 7$) failed to produce changes in the amplitude of the HDA-evoked cardiorespiratory response (Tables 2 and 3)

Muscimol microinjected into the A5 region ($n = 7$; Fig. 3) produced no changes in blood pressure or heart rate. A decrease in respiratory rate ($P < 0.05$) due to an increase in expiratory time ($P < 0.01$) appeared 3–4 min after the microinjection, although no changes were observed in inspiratory time or pleural pressure (Tables 2 and 3 and Figs 4*A* and *B* and 5).

Electrical stimulation of the HDA before and after the microinjection of muscimol into the A5 region evoked similar respiratory responses; therefore, the amplitude of the respiratory response to HDA stimulation after the microinjection of muscimol remained unchanged

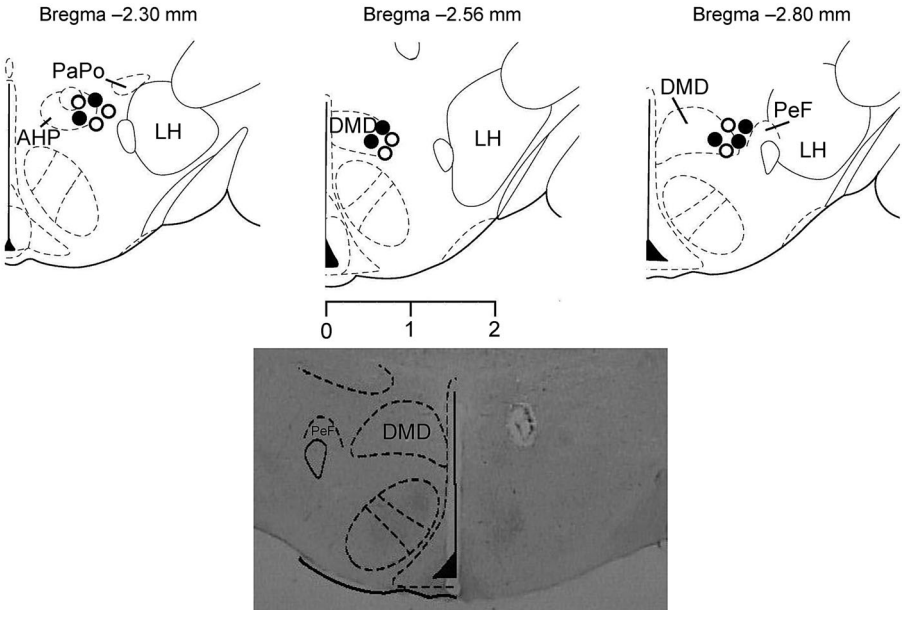

Figure 2. Semi-schematic line drawings of coronal sections through the HDA from rostral (top left) to caudal (top right), showing the sites where electrical stimulations were applied
Filled circles show the location of electrical stimulation in animals which received PBS microinjections within the A5 region. Open circles show the location of electrical stimulation in animals which received muscimol microinjections within the A5 region. Bottom panal is a photomicrograph of a coronal section through the HDA showing the location of stimulation. Abbreviations: AHP, anterior hypothalamic area, posterior part; DMD, dorsomedial hypothalamic nucleus, dorsal part; LH, lateral hypothalamic area; PaPo, paraventricular hypothalamic nucleus, posterior part; and PeF, perifornical nucleus.

Table 2. Respiratory and cardiovascular changes before (control) and during electrical stimulation of the HDA (stimulation), 4 min after the microinjection (PBS or muscimol) and during electrical stimulation of the HDA after PBS or muscimol microinjection (stimulation + PBS or stimulation + muscimol) within the A5 region

	Control	Stimulation	PBS	Stimulation + PBS
A5 ($n = 7$)				
t_I (s)	0.196 ± 0.01	0.199 ± 0.01	0.193 ± 0.01	0.203 ± 0.01
t_E (s)	0.536 ± 0.05	0.299 ± 0.03***	0.563 ± 0.04	0.321 ± 0.02***
RR (breaths min^{-1})	84.4 ± 5.2	123.4 ± 5.5***	81.5 ± 3.1	118.7 ± 4.1***
BP (mmHg)	92.4 ± 1.2	121.7 ± 3.7***	91.3 ± 0.7	122.0 ± 3.3***
HR (beats min^{-1})	352.0 ± 12.0	389.0 ± 12.0***	354.0 ± 14.0	386.0 ± 15.0***
	Control	Stimulation	Muscimol	Stimulation + muscimol
A5 ($n = 7$)				
t_I (s)	0.203 ± 0.02	0.195 ± 0.01	0.193 ± 0.01	0.209 ± 0.02
t_E (s)	0.494 ± 0.07	0.282 ± 0.03*	0.751 ± 0.12††	0.374 ± 0.04***†
RR (breaths min^{-1})	86.1 ± 9.4	125.8 ± 14.1***	63.5 ± 12.6†	102.8 ± 13.8***
BP (mmHg)	104.5 ± 1.1	135.1 ± 2.7***	102.3 ± 4.2	114.2 ± 4.6*
HR (beats min^{-1})	338.8 ± 13.3	382.7 ± 12.5***	335.6 ± 12.2	354.8 ± 15.9*

For statistical procedures, we have considered only those histologically verified sites of microinjection of muscimol within the A5 region in which cardiorespiratory changes were observed. Values are means ± SEM. Abbreviations: BP, mean arterial blood pressure; HR, heart rate; RR, respiratory rate; t_E, expiratory time; and t_I, inspiratory time. Asterisks show differences between control *versus* stimulation and PBS *versus* stimulation + PBS or muscimol *versus* stimulation + muscimol (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$). Daggers show differences between control *versus* PBS or control *versus* muscimol (†$P < 0.05$ and ††$P < 0.01$).

Table 3. Absolute cardiorespiratory changes during HDA stimulation before (control) and 4 min after the microinjection of PBS or muscimol (stimulation + PBS or stimulation + muscimol) within the A5 region

	Control	Stimulation +PBS	Control	Stimulation + muscimol
A5 ($n = 7$)				
RR (breaths min^{-1})	39.0 ± 8.0	44.0 ± 10.0	41.5 ± 8.2	37.7 ± 4.8
BP (mmHg)	30.0 ± 3.0	31.0 ± 4.0	30.7 ± 2.5	13.5 ± 2.1***
HR (beats min^{-1})	37.0 ± 4.0	34.0 ± 3.0	43.9 ± 6.2	19.4 ± 5.9***

Values are means ± SEM. Abbreviations: BP, mean arterial blood pressure; HR, heart rate; and RR, respiratory rate. Asterisks show differences between control *versus* stimulation + PBS or control *versus* stimulation + muscimol (***$P < 0.001$).

compared with control stimulations (Tables 2 and 3 and Figs 4*A* and *B* and 5).

Microinjection of muscimol into the A5 region decreased the cardiovascular response evoked by HDA stimulations. The increase in blood pressure and heart rate was still present ($P < 0.05$ in both cases), although the amplitude was smaller than before muscimol ($P < 0.01$ and $P < 0.001$, respectively; Tables 2 and 3 and Figs 4*A* and *B* and 5).

In the present report, only locations from histologically verified sites of microinjection of muscimol within the A5 region were considered for statistical procedures. However, in some experiments (10 rats) the microinjection of muscimol was delivered to sites outside the A5 region (Fig. 3).

In one animal, the microinjection of muscimol was delivered within the region of the accessory abducens nucleus. Muscimol decreased the resting respiratory rate, but no changes were observed in heart rate or blood pressure. During HDA stimulation, the intensity of the cardiovascular response remained unchanged, but the respiratory response was decreased.

Microinjections of muscimol within the rubrospinal tract (one rat), the lateral superior olivary nucleus (one rat), the facial nucleus or its root (two rats), the spinal trigeminal tract (one rat) or the sensory root of the trigeminal nerve (one rat) did not produce changes in resting cardiorespiratory parameters, and negligible changes of the intensity of the cardiorespiratory HDA-evoked response were observed.

In three animals, the microinjection of muscimol was delivered within the principal sensory trigeminal nucleus. In one case, an increase of blood pressure was observed with no changes in heart rate and respiratory frequency. The intensity of the respiratory response to HDA stimulation was decreased, but no changes were observed in the intensity of the cardiovascular response.

Interaction of HDA and A5: neuronal unit recording experiments

Seventy-five cells within the A5 region were recorded extracellularly (Fig. 6 and Table 4). These neurons showed patterns of discharge involving spontaneous

(61 neurons, 4.5 ± 0.3 spikes s^{-1}), silent (seven neurons), respiratory-modulated (five neurons; one postinspiratory, two inspiratory and two expiratory) and cardiovascular-modulated activity (two neurons). The activity of 35 out of 75 neurons was affected by the HDA (47%).

Seven cells, all silent, were orthodromically activated from the HDA (latency 10.7 ± 1.1 ms, ranging from 6.5 to 13.4 ms; Fig. 7*A*) and five, spontaneously active, were antidromically activated (latency 12.0 ± 0.5 ms, ranging from 10.4 to 13 ms). The inferred average conduction velocity of the orthodromic axons was 0.7–0.8 m s^{-1}, while for antidromic stimulation it was 0.6–0.7 m s^{-1} (the approximate straight-line distance between the HDA and the A5 region was considered to be 7.7 mm).

There was a very variable timing of the evoked excitatory responses to HDA stimulation (ranging from 10 to 40 ms), and some neurons showed a complex response involving both short- and long-latency responses ($n = 2$; Fig. 7*B*). The short-latency excitation presented a mean latency of 13.5 ± 0.9 ms (ranging from 10 to 19 ms, $n = 10$). The mean long-latency response was 35.5 ± 2.1 ms (range between 30 and 40 ms, $n = 4$). None of these excitatory responses presented constant latency.

Only one cell was inhibited during HDA stimulation. The duration of the inhibition lasted 60 ms (Fig. 7*C*).

The analysis of neuronal discharge rates in the form of integrated rate histograms during spontaneous activity and during HDA stimulation (0.1 ms given at 1 Hz) showed that seven cells which were not excited or inhibited during HDA stimulation changed the frequency of their spontaneously activity. In three cells, an increase of activity was observed (from 4.4 ± 0.4 to 5.6 ± 0.8 Hz, $P < 0.05$), while four cells decreased their spontaneous activity (from 4.8 ± 0.5 to 4.1 ± 0.5 Hz, $P < 0.001$; Table 4).

None of the respiratory- ($n = 5$) or cardiovascular-modulated cells ($n = 2$) was affected by HDA stimulation (Fig. 7*D*).

Ten spontaneously active neurons (one in each experiment), six activated from the HDA (four short latency, one long latency and one short and long latency) and three not activated were tested for their sensitivity to the I.V. administration of the α_2-adrenergic agonist, clonidine (10 μg kg^{-1}). All cells were inhibited by the drug. One inspiratory-modulated cell was also tested for clonidine, but failed to change the pattern of discharge.

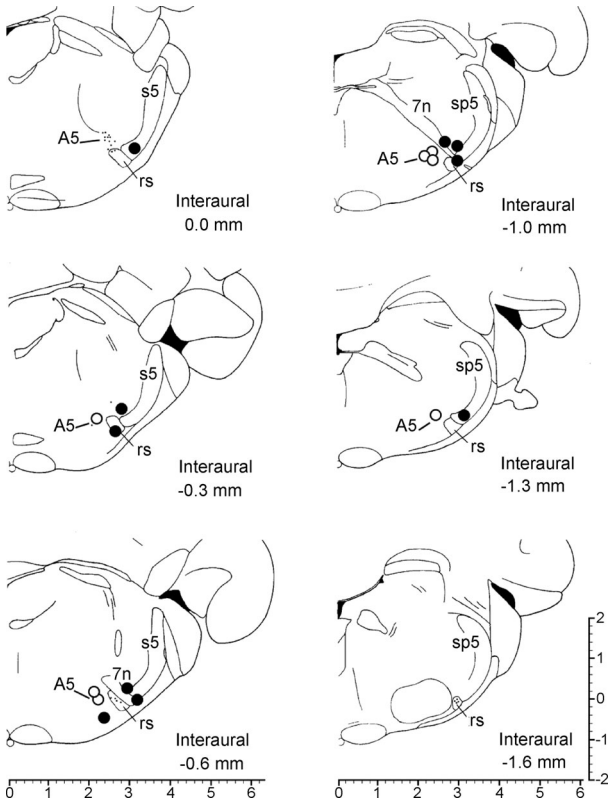

Figure 3. Semi-schematic line drawings of coronal sections through the A5 region from rostral (top left) to caudal (bottom right), showing the sites where microinjections were applied
The open circles show the location of muscimol microinjections within A5 region. Filled circles show the locations in which muscimol failed to produce changes in the HDA-evoked responses. Each circle corresponds to one animal. Abbreviations: 7n, facial nerve or its root; rs, rubrospinal tract; sp5, spinal trigeminal tract; and s5, sensory root of trigeminal nerve.

Discussion

The first observation of this paper indicates that stimulation of the HDA induces c-Fos-ir expression in both A5 catecholaminergic (TH-positive) and A5 non-catecholaminergic (TH-negative) cells of the pons. Second, the pattern of the cardiorespiratory response evoked from the HDA is modified by the microinjection of muscimol into the A5 region. Muscimol microinjected into the A5 region decreased the cardiovascular response to HDA electrical stimulation with no changes in the respiratory response. Finally, a high number of extracellulary recorded neurones within the A5 region were activated on electrical stimulation of the HDA.

These results suggest that, in our experimental conditions, catecholaminergic and non-catecholaminergic neurons located within the A5 region are involved in the cardiovascular evoked response from the HDA.

Methodological considerations

In the present study, we chose only electrical stimulation to activate the HDA. Electrical stimulation activates both neurons and fibres of passage. However, previous studies have demonstrated that either electrical or chemical stimulation of the HDA induces a similar cardiorespiratory response (Hilton & Redfern, 1986).

Electrical stimulation allowed us to regulate tightly the onset and offset of HDA activation. We selected the most usual stimulation parameters used in previous studies (Lara *et al.* 1994, 2002; Dawid-Milner *et al.* 1995, 2001, 2003; Silva-Carvalho *et al.* 1995*b*; Díaz-Casares *et al.* 2009, 2012).

To inhibit the A5 region, we have used muscimol, which acts specifically as a GABAergic agonist, inhibiting neuronal activity by sustained hyperpolarization due to an increase in potassium conductance. The usual volume of our muscimol injections was 50 nl given in 5 s. Volumes and injection times were selected in accordance with the

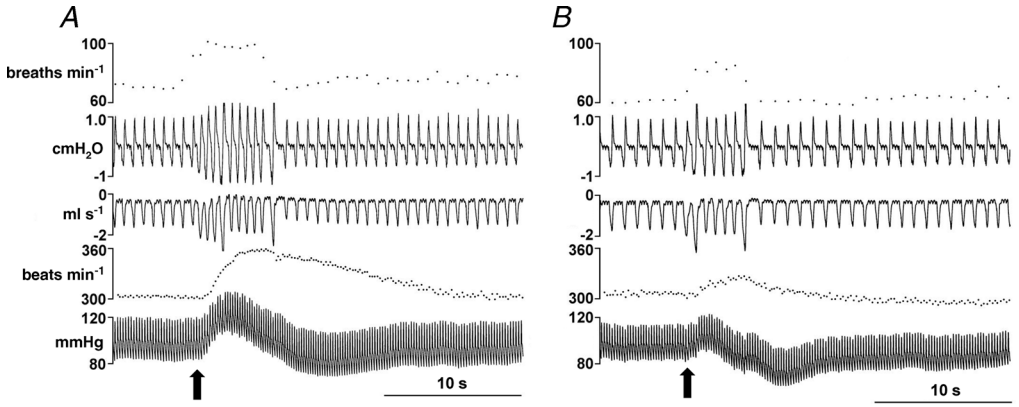

Figure 4. Instantaneous respiratory rate (upper trace), respiratory flow, pleural pressure, instantaneous heart rate and blood pressure in a spontaneously breathing rat, showing the cardiorespiratory response evoked on HDA stimulation before (*A*) and after the microinjection of muscimol (50 nl over 5 s) in the A5 region (*B*)
The arrows indicate show the onset of the HDA electrical stimulation.

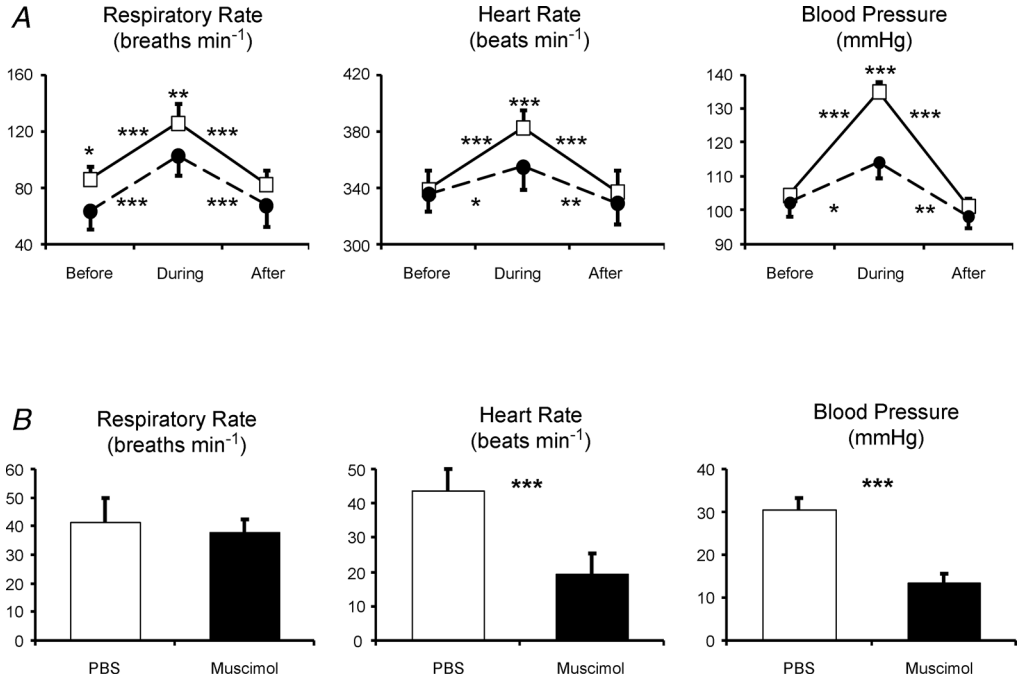

Figure 5. Effects of HDA stimulation on cardiorespiratory parameters before and after microinjection of PBS or muscimol within the A5 region
A, changes of respiratory rate, blood pressure and heart rate before, during and after HDA stimulation after microinjection of PBS ($n = 7$; open squares) or muscimol ($n = 7$; filled circles) within the A5 region. *B*, changes in the intensity of the cardiorespiratory response evoked on HDA stimulation (respiratory rate response, blood pressure response and heart rate response) after the microinjection of PBS (open bars) or muscimol (filled bars) within the A5 region ($n = 7$). Data are expressed as means ± SEM ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$).

observations of others involving their inhibitory effects (Jodkowski *et al.* 1994).

Another methodological consideration for the present study is that our experiments were performed in spontaneously breathing animals. We have shown that chemical blockade of the A5 region produced a significant decrease in baseline respiratory rate. This change in respiratory control may have independently altered baroreflex function (Dawid Milner *et al.* 2003).

Interaction of HDA and A5: c-Fos experiments

In the present study, once the HDA was located and to prevent secondary c-Fos expression due to changes in arterial blood pressure, the sympatholytic agent guanethidine was administered. Some studies have used electrical stimulation of the HDA to map systematically populations of neurons in the brainstem and other regions that are activated by changes in blood pressure in rats (Graham *et al.* 1995; Tassorelli & Joseph, 1995). The changes in blood pressure induced a consistent and specific pattern of c-*fos* expression within the A5 region.

In our study, the blockage of the cardiovascular changes with guanethidine shows that the A5 region presents a significant increase of c-Fos expression in both catecholaminergic and non-catecholaminergic neurons, after electrical stimulation of the HDA. The increase in the expression of c-Fos was higher in non-catecholaminergic than in catecholaminergic neurons. Therefore, both populations of neurons of the A5 region seem likely to be activated directly from the HDA and not secondarily to blood pressure changes evoked from the HDA during electrical stimulation. This result was further confirmed with neuronal recordings. We found that 93% of cells presenting c-Fos were probably influenced from cells or fibres passing through the HDA. These results need further investigation using specific neuronal chemical microstimulation. Also, the physiological role

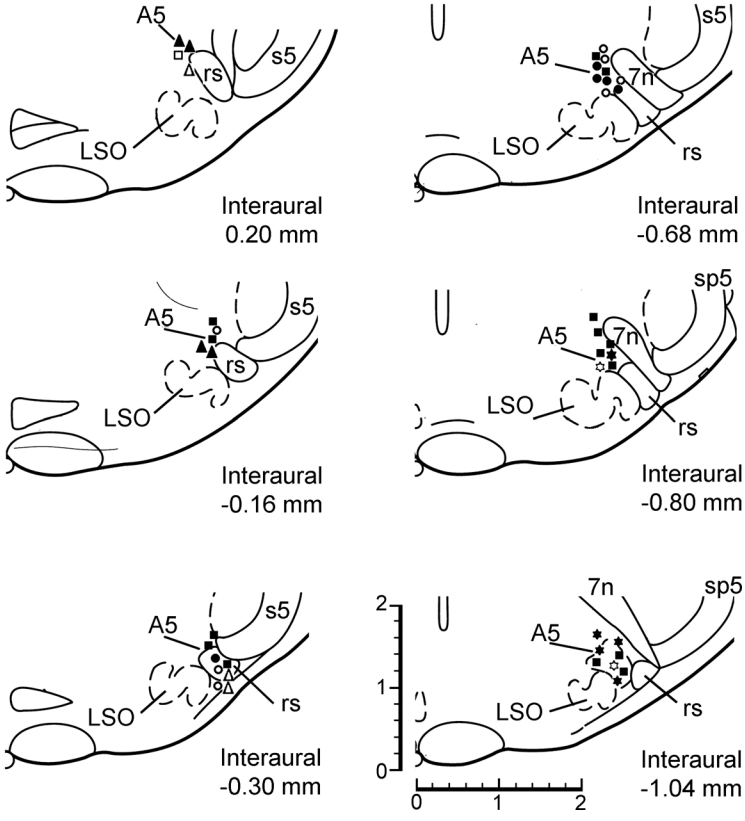

Figure 6. Semi-schematic line drawings of ipsilateral coronal sections through the A5 region from rostral (top left) to caudal (bottom right), showing a plot of neuronal inferred recording sites
Open circles show location of antidromically activated cells and filled circles show orthodromically activated cells. Filled squares indicate HDA-excited cells and open square shows the HDA-inhibited cell. Filled triangles show cells in which HDA stimulation decreased spontaneous neuronal activity, while open triangles show cells in which spontaneous neuronal activity increased during HDA stimulation. Open stars show location of cardiovascular-modulated neurons and filled stars the respiratory-modulated cells. Abbreviations: LSO, lateral superior olivary nucleus; 7n, facial nerve or its root; rs, rubrospinal tract; sp5, spinal trigeminal tract; and s5, sensory root of trigeminal nerve.

Table 4. Summary of results from extracellular neuronal recordings of putative cells located within the A5 region of the pons during HDA stimulation

	A5 region
Activation (orthodromic)	$n = 7$
	10.7 ± 1.1 ms
	6.5–13.4 ms
	All silent
Activation (antidromic)	$n = 5$
	12.0 ± 0.5 ms
	10.4–13 ms
Excitation	Short latency, $n = 10$
	13.5 ± 0.9 ms
	10–19 ms
	Long latency, $n = 5$
	35.5 ± 2.1 ms
	30–40 ms
Inhibition	$n = 1$
	60 ms duration
Decrease of spontaneous activity	$n = 4$
Increase of spontaneous activity	$n = 3$
Not affected	$n = 40$
	(5 respiratory modulated, 2 cardiovascular modulated)
Total	75

of the non-catecholaminergic neurons remains to be defined.

Interaction of HDA and A5: neuropharmacological experiments

This study deals with the notion that neurons located within the A5 region play a role in the cardiorespiratory response evoked from the HDA. We have previously shown that the stimulation of cell bodies located within the A5 region resembles the cardiovascular response elicited by HDA electrical stimulation, thus evoking tachycardia and hypertension (Dawid-Milner *et al.* 2001). Consequently, we have discussed the subject of a possible interaction between these cardiorespiratory regions.

In order to evaluate this possible interaction, microinjection of muscimol, a GABA agonist, was made into the A5 region. We have first analysed the effects of muscimol microinjections on resting cardiorespiratory parameters, followed by the effects on the cardiorespiratory response evoked by HDA electrical stimulation.

Resting conditions. The microinjection of muscimol within the A5 region produced, 4 min after its administration, a decrease in respiratory rate due to an increase of expiratory time; no changes were observed in inspiratory time or pleural pressure. Muscimol also failed to produce changes in blood pressure or heart rate. The primary observation of these results is that the A5 region, considered to be involved in cardiovascular regulation, produces only changes in respiration at rest.

It has been published that bilateral microinjections of muscimol within the A5 region did not produce changes in cardiovascular parameters or sympathetic activity at rest, but induced apneustic breathing in vagotomized rats (Jodkowski *et al.* 1994). Electrical lesions of the A5 region have similar effects, decreasing the baseline frequency of breathing (Schlenker & Prestbo, 2003). As muscimol inhibits both catecholaminergic and non-catecholaminergic neurons within the A5 region, is not clear which cells are responsible for the bradypnoea. Ablation of A5 catecholaminergic neurons with 6-hydroxydopamine produce similar effects (Koshiya & Guyenet, 1994), thus suggesting a role for catecholaminergic neurons. As A5 cells have direct connections with neurons of the retrotrapezoid nucleus, which are known to be involved in chemoreception, the inhibition of these projections could also lead to a decrease in respiratory rate. The mechanisms involved in this modulation need further investigation.

Cardiorespiratory response to HDA electrical stimulation after the muscimol microinjection within the A5 region. The microinjection of muscimol into the A5 region did not produce changes in the respiratory response to the electrical stimulation of the HDA; however, it produced a clear decrease of the cardiovascular response.

The tachycardia and the pressor response evoked during HDA stimulation involve a direct activation of the neurons of the RVLM. These neurons send direct projections to the preganglionic neurons of the IML that are ultimately responsible for the abrupt increase in blood pressure (Loewy, 1991). Also, a direct activation of the adrenal medulla contributes to a secondary increase in blood pressure due to liberation of adrenaline.

The activity of the RVLM can be also modulated via indirect forebrain projections. Furthermore, in a parallel pathway to the activation of the RVLM and the preganglionic neurons in the IML, the stimulation of the HDA increases the activity of the chemoreceptor reflex by means of the excitation or facilitation of chemoreceptor neurons in the NTS (Silva-Carvalho *et al.* 1995*a*). In a parallel circuit, an inhibition of the response to baroreceptor inputs is produced by disfacilitation or inhibition of baroreceptor neurons at the level of the NTS (Jordan *et al.* 1988; Mifflin *et al.* 1988). This inhibition seems to be mediated by GABAergic interneurons in the NTS (Jordan *et al.* 1988). Some studies have also demonstrate the involvement of serotoninergic receptors (5-HT_3) at the level of the NTS (Sévoz-Couche *et al.* 2003).

It has been also demonstrated in conscious rats that stress-evoked increases in arterial pressure and heart rate are accompanied by a resetting, rather than an inhibition, of the baroreceptor reflex. In these studies, the baroreceptor reflex control of heart rate was reset to higher levels of arterial pressure with no reduction in the gain of the reflex (Horiuchi *et al.* 2006; McDowall *et al.* 2006).

We have previously demonstrated that stimulation of A5 region neuronal cell bodies with glutamate, which specifically activates perikarya, consists mainly of an increase in both blood pressure and heart rate (Dawid-Milner *et al.* 2001). The simultaneous increase of arterial blood pressure, heart rate and sympathetic vasomotor activity implies a reset of the baroreceptor reflex but without attenuation in sensitivity of the reflex.

The inhibition of neurons located within the A5 region after the microinjection of muscimol reduces the tachycardia and the pressure response evoked by HDA stimulation. The decrease in the cardiovascular response to HDA stimulation could be an indication of an incomplete resetting of the baroreceptor reflex. This effect could explain the smaller increase of the heart rate component to HDA stimulation. It also explains, through an indirect pathway, the smaller increase in blood pressure, although the inhibition of the excitatory projections from the A5 region to the IML is probably the most relevant factor in this effect. These results suggest that the resetting of the baroreceptor response evoked by HDA stimulation could also be mediated through an indirect pathway via the A5 region.

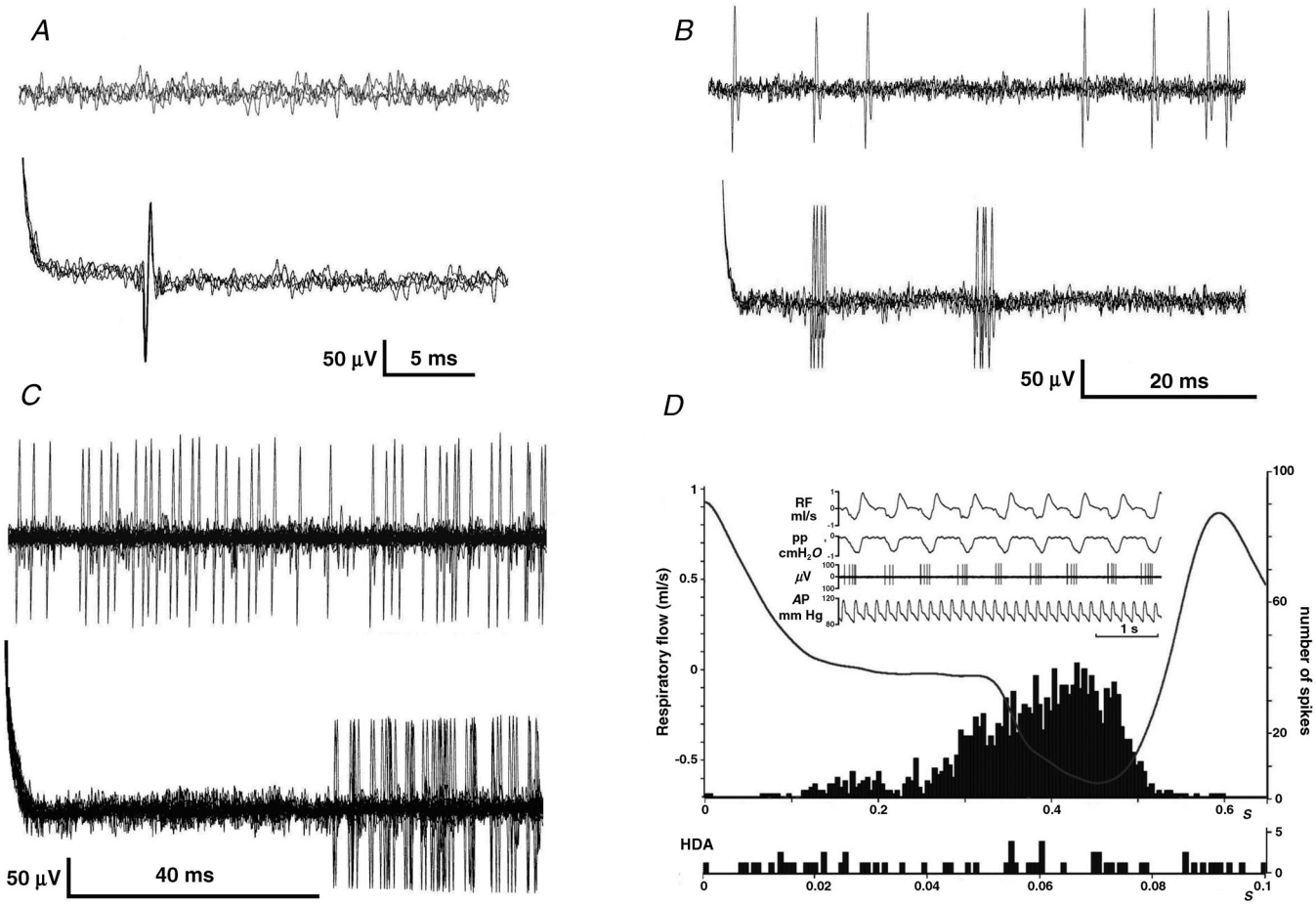

Figure 7. Extracellular recordings (superimposed sweeps) of four putative cells recorded form the A5 region
A, silent neuron (upper trace) with constant-latency responses to HDA stimulation (lower trace). The cell was demonstrated to be orthodromically activated from the HDA. *B*, spontaneously active cell (upper trace) excited with short- and long-latency responses to HDA stimulation (lower trace). *C*, spontaneously active cell (upper trace) inhibited by HDA stimulation (lower trace). *D*, inset shows recording of respiratory flow, pleural pressure, neuronal activity of a putative respiratory-modulated A5 cell and blood pressure. Main graph shows respiratory flow (inspiration downwards), and neuronal activity, while lower trace shows HDA-triggered histograms. This respiratory putative A5 neuron shows no modulation from the HDA.

Our results, therefore, suggest that the activity of neurons of the A5 region modulate the intensity of the cardiovascular response evoked on HDA stimulation through an indirect pathway to both the IML and NTS. The exact role of both pathways needs further investigation.

Interaction of HDA and A5: neural recording

Once the morphofunctional relationship between the HDA and the A5 region was demonstrated, we wanted to confirm the possible electrophysiological interactions between both regions. To this end, we recorded putative A5 cells extracellularly during HDA electrical stimulation.

Seventy-five putative neurons from the A5 region were registered; 91% were spontaneously active and their discharge rate was similar to that described in other studies (from 2 to 4 spikes s^{-1}; Andrade & Aghajanian, 1982; Byrum *et al.* 1984; Guyenet *et al.* 1993; Dawid Milner *et al.* 2003), whereas 9% were silent. We and others (Guyenet *et al.* 1993; Dawid Milner *et al.* 2003) have previously described the possible role of A5 neurons in respiratory modulation. Confirming these data, we found some respiratory-modulated neurons, but their discharge pattern was not modified during electrical stimulation of the HDA.

A significant number of these A5 neurones (57%) were activated from HDA stimulation, thus confirming the importance of the connections between both regions. Some A5 neurones were shown to be antidromically and orthodromically activated, on the basis of short, constant-latency responses and the collision test. Antidromically activated cells were spontaneously active, while orthodromically activated cells were silent, which is an indication of the origin of the somata. By definition, A5 cells were active, while hypothalamic fibres were, in all cases, silent. The presence of activated or facilitated cells is an indication of the existence of polysynaptic circuits acting on the A5 region. The association of these activations with inhibitions or disfacilitations illustrates the complexity of the different types of synaptic connections. All these data show the first electrophysiological evidence of interactions between HDA and A5 catecholaminergic neurons.

Final consideration

The results of our study demonstrate, for the first time, that the A5 region is an important component of those brainstem pathways known to be involved in mediating autonomic changes associated with the defence response. We have shown some preliminary electrophysiological evidence of interactions between HDA and A5 neurons, although it cannot be assumed that electrical stimulation is equivalent to activation of only cells within the HDA. The study must be extended to contribute to clarify the importance of the integrated control of this region on autonomic functions.

References

Abbott SB, Kanbar R, Bochorishvili G, Coates MB, Stornetta RL & Guyenet PG (2012). C1 neurons excite locus coeruleus and A5 noradrenergic neurons along with sympathetic outflow in rats. *J Physiol* **590**, 2897–2915.

Andrade R & Aghajanian GK (1982). Single cell activity in the noradrenergic A-5 region: responses to drugs and peripheral manipulations of blood pressure. *Brain Res* **242**, 125–135.

Bruinstroop E, Cano G, Vanderhorst VG, Cavalcante JC, Wirth J, Sena-Esteves M & Saper CB (2012). Spinal projections of the A5, A6 (locus coeruleus), and A7 noradrenergic cell groups in rats. *J Comp Neurol* **520**, 1985–2001.

Byrum CE & Guyenet PG (1987). Afferent and efferent connections of the A5 noradrenergic cell group in the rat. *J Comp Neurol* **261**, 529–542.

Byrum CE, Stornetta R & Guyenet PG (1984). Electrophysiological properties of spinally-projecting A5 noradrenergic neurons. *Brain Res* **303**, 15–29.

Coles SK & Dick TE (1996). Neurones in the ventrolateral pons are required for post-hypoxic frequency decline in rats. *J Physiol* **497**, 79–94.

Dahlström A & Fuxe K (1964). Localization of monoamines in the lower brain stem. *Experientia* **20**, 398–399.

Dampney RA, Polson JW, Potts PD, Hirooka Y & Horiuchi J (2003). Functional organization of brain pathways subserving the baroreceptor reflex: studies in conscious animals using immediate early gene expression. *Cell Mol Neurobiol* **23**, 597–616.

Dawid-Milner MS, Lara JP, Gonzaléz-Barón S & Spyer KM (2001). Respiratory effects of stimulation of cell bodies of the A5 region in the anaesthetised rat. *Pflugers Arch* **441**, 434–443.

Dawid Milner MS, Lara JP, López de Miguel MP, López-González MV, Spyer KM & González-Barón S (2003). A5 region modulation of the cardiorespiratory responses evoked from parabrachial cell bodies in the anaesthetised rat. *Brain Res* **982**, 108–118.

Dawid-Milner MS, Silva-Carvalho L, Goldsmith GE & Spyer KM (1995). Hypothalamic modulation of laryngeal reflexes in the anaesthetized cat: role of the nucleus tractus solitarii. *J Physiol* **487**, 739–749.

Díaz-Casares A, López-González MV, Peinado-Aragonés CA, González-Barón S & Dawid-Milner MS (2012). Parabrachial complex glutamate receptors modulate the cardiorespiratory response evoked from hypothalamic defense area. *Auton Neurosci* **169**, 124–134.

Díaz-Casares A, López-González MV, Peinado-Aragonés CA, Lara JP, González-Barón S & Dawid-Milner MS (2009). Role of the parabrachial complex in the cardiorespiratory response evoked from hypothalamic defense area stimulation in the anesthetized rat. *Brain Res* **1279**, 58–70.

DiMicco JA, Samuels BC, Zaretskaia MV & Zaretsky DV (2002). The dorsomedial hypothalamus and the response to stress: part renaissance, part revolution. *Pharmacol Biochem Behav* **71**, 469–480.

Dobbins EG & Feldman JL (1994). Brainstem network controlling descending drive to phrenic motoneurons in rat. *J Comp Neurol* **347**, 64–86.

Erickson JT & Millhorn DE (1994). Hypoxia and electrical stimulation of the carotid sinus nerve induce Fos-like immunoreactivity within catecholaminergic and serotoninergic neurons of the rat brainstem. *J Comp Neurol* **348**, 161–182.

Goodchild AK, Phillips JK, Lipski J & Pilowsky PM (2001). Differential expression of catecholamine synthetic enzymes in the caudal ventral pons. *J Comp Neurol* **438**, 457–467.

Graham JC, Hoffman GE & Sved AF (1995). c-Fos expression in brain in response to hypotension and hypertension in conscious rats. *J Auton Nerv Syst* **55**, 92–104.

Guyenet PG (2006). The sympathetic control of blood pressure. *Nat Rev Neurosci* **7**, 335–346.

Guyenet PG, Koshiya N, Huangfu D, Verberne AJ & Riley TA (1993). Central respiratory control of A5 and A6 pontine noradrenergic neurons. *Am J Physiol Regul Integr Comp Physiol* **264**, R1035–R1044.

Hatton DC, Brooks V, Qi Y & McCarron DA (1997). Cardiovascular response to stress: baroreflex resetting and hemodynamics. *Am J Physiol Regul Integr Comp Physiol* **272**, R1588–R1594.

Hilaire G, Viemari JC, Coulon P, Simonneau M & Bevengut M (2004). Modulation of the respiratory rhythm generator by the pontine noradrenergic A5 and A6 groups in rodents. *Respir Physiol Neurobiol* **143**, 187–197.

Hilton SM & Redfern WS (1986). A search for brain stem cell groups integrating the defence reaction in the rat. *J Physiol* **378**, 213–228.

Horiuchi J, McDowall LM & Dampney RA (2006). Differential control of cardiac and sympathetic vasomotor activity from the dorsomedial hypothalamus. *Clin Exp Pharmacol Physiol* **33**, 1265–1268.

Huangfu DH, Koshiya N & Guyenet PG (1991). A5 noradrenergic unit activity and sympathetic nerve discharge in rats. *Am J Physiol Regul Integr Comp Physiol* **261**, R393–R402.

Jodkowski JS, Coles SK & Dick TE (1994). A 'pneumotaxic centre' in rats. *Neurosci Lett* **172**, 67–72.

Jordan D, Mifflin SW & Spyer KM (1988). Hypothalamic inhibition of neurones in the nucleus tractus solitarius of the cat is GABA mediated. *J Physiol* **399**, 389–404.

Kanbar R, Depuy SD, West GH, Stornetta RL & Guyenet PG (2011). Regulation of visceral sympathetic tone by A5 noradrenergic neurons in rodents. *J Physiol* **589**, 903–917.

Koshiya N & Guyenet PG (1994). A5 noradrenergic neurons and the carotid sympathetic chemoreflex. *Am J Physiol Regul Integr Comp Physiol* **267**, R519–R526.

Lara JP, Dawid-Milner MS, López MV, Montes C, Spyer KM & González-Barón S (2002). Laryngeal effects of stimulation of rostral and ventral pons in the anaesthetized rat. *Brain Res* **934**, 97–106.

Lara JP, Parkes MJ, Silva-Carvhalo L, Izzo P, Dawid-Milner MS & Spyer KM (1994). Cardiovascular and respiratory effects of stimulation of cell bodies of the parabrachial nuclei in the anaesthetized rat. *J Physiol* **477**, 321–329.

Loewy AD (1991). Forebrain nuclei involved in autonomic control. *Prog Brain Res* **87**, 253–268.

Loewy AD, Gregorie EM, McKellar S & Baker RP (1979*a*). Electrophysiological evidence that the A5 catecholamine cell group is a vasomotor center. *Brain Res* **178**, 196–200.

Loewy AD, McKellar S & Saper CB (1979*b*). Direct projections from the A5 catecholamine cell group to the intermediolateral cell column. *Brain Res* **174**, 309–314.

McDowall LM, Horiuchi J, Killinger S & Dampney RA (2006). Modulation of the baroreceptor reflex by the dorsomedial hypothalamic nucleus and perifornical area. *Am J Physiol Regul Integr Comp Physiol* **290**, R1020–R1026.

Mifflin SW, Spyer KM & Withington-Wray DJ (1988). Baroreceptor inputs to the nucleus tractus solitarius in the cat: modulation by the hypothalamus. *J Physiol* **399**, 369–387.

Mulkey DK, Stornetta RL, Weston MC, Simmons JR, Parker A, Bayliss DA & Guyenet PG (2004). Respiratory control by ventral surface chemoreceptor neurons in rats. *Nat Neurosci* **7**, 1360–1369.

Paxinos G & Watson C (2005). *The Rat Brain in Stereotaxic Coordinates*. Academic Press, San Diego, CA, USA.

Pilowsky PM & Goodchild AK (2002). Baroreceptor reflex pathways and neurotransmitters: 10 years on. *J Hypertens* **20**, 1675–1688.

Rosin DL, Chang DA & Guyenet PG (2006). Afferent and efferent connections of the rat retrotrapezoid nucleus. *J Comp Neurol* **499**, 64–89.

Roux JC, Peyronnet J, Pascual O, Dalmaz Y & Pequignot JM (2000). Ventilatory and central neurochemical reorganisation of O_2 chemoreflex after carotid sinus nerve transection in rat. *J Physiol* **522**, 493–501.

St-John WM & Paton JF (2004). Role of pontile mechanisms in the neurogenesis of eupnea. *Respir Physiol Neurobiol* **143**, 321–332.

Schadt JC & Hasser EM (1998). Hemodynamic effects of acute stressors in the conscious rabbit. *Am J Physiol Regul Integr Comp Physiol* **274**, R814–R821.

Schlenker EH & Prestbo A (2003). Elimination of the post-hypoxic frequency decline in conscious rats lesioned in pontine A5 region. *Respir Physiol Neurobiol* **138**, 179–191.

Sévoz-Couche C, Comet MA, Hamon M & Laguzzi R (2003). Role of nucleus tractus solitarius 5-HT_3 receptors in the defense reaction-induced inhibition of the aortic baroreflex in rats. *J Neurophysiol* **90**, 2521–2530.

Silva-Carvalho L, Dawid-Milner MS, Goldsmith GE & Spyer KM (1995*a*). Hypothalamic modulation of the arterial chemoreceptor reflex in the anaesthetized cat: role of the nucleus tractus solitarii. *J Physiol* **487**, 751–760.

Silva-Carvalho L, Dawid-Milner MS & Spyer KM (1995*b*). The pattern of excitatory inputs to the nucleus tractus solitarii evoked on stimulation in the hypothalamic defence area in the cat. *J Physiol* **487**, 727–737.

Song G, Xu H, Wang H, Macdonald SM & Poon CS (2011). Hypoxia-excited neurons in NTS send axonal projections to Kölliker-Fuse/parabrachial complex in dorsolateral pons. *Neuroscience* **175**, 145–153.

Spyer KM (1994). Annual review prize lecture. Central nervous mechanisms contributing to cardiovascular control. *J Physiol* **474**, 1–19.

Sun W & Panneton WM (2005). Defining projections from the caudal pressor area of the caudal ventrolateral medulla. *J Comp Neurol* **482**, 273–293.

Tassorelli C & Joseph SA (1995). Systemic nitroglycerin induces Fos immunoreactivity in brainstem and forebrain structures of the rat. *Brain Res* **682**, 167–181.

Tavares I, Lima D & Coimbra A (1997). The pontine A5 noradrenergic cells which project to the spinal cord dorsal horn are reciprocally connected with the caudal ventrolateral medulla in the rat. *Eur J Neurosci* **9**, 2452–2461.

Taxini CL, Takakura AC, Gargaglioni LH & Moreira TS (2011). Control of the central chemoreflex by A5 noradrenergic neurons in rats. *Neuroscience* **199**, 177–186.

Usunoff KG, Itzev DE, Rolfs A, Schmitt O & Wree A (2006). Brain stem afferent connections of the amygdala in the rat with special references to a projection from the parabigeminal nucleus: a fluorescent retrograde tracing study. *Anat Embryol (Berl)* **211**, 475–496.

Yardley CP & Hilton SM (1986). The hypothalamic and brainstem areas from which the cardiovascular and behavioural components of the defence reaction are elicited in the rat. *J Auton Nerv Syst* **15**, 227–244.

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