ROLE OF A5 NORADRENERGIC NEURONS IN THE CHEMOREFLEX CONTROL OF RESPIRATORY AND SYMPATHETIC ACTIVITIES IN UNANESTHETIZED CONDITIONS

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Abstract—The A5 area at the ventrolateral pons contains noradrenergic neurons connected with other medullary areas involved in the cardiorespiratory control. Its contribution to the cardiorespiratory regulation was previously evidenced in anesthetized conditions. In the present study, we investigated the involvement of the A5 noradrenergic neurons to the basal and chemoreflex control of the sympathetic and respiratory activities in unanesthetized conditions. A5 noradrenergic neurons were lesioned using microinjections of anti-dopamine β-hydroxylase saporin (anti-DβH-SAP). After 7–8 days, we evaluated the arterial pressure levels, heart rate and minute ventilation in freely moving adult rats (280–350 g) as well as recorded from thoracic sympathetic (tSN) and phrenic nerves (PN) using the arterially perfused *in situ* preparation of juvenile rats (80–90 g). Baseline cardiovascular, sympathetic and respiratory parameters were similar between control ($n = 7$–8) and A5-lesioned rats ($n = 5$–6) in both experimental preparations. In adult rats, lesions of A5 noradrenergic neurons did not modify the reflex cardiorespiratory adjustments to hypoxia

(7% O_2) and hypercapnia (7% CO_2). In the *in situ* preparations, the sympatho-excitation, but not the PN reflex response, elicited by either the stimulation of peripheral chemoreceptors (ΔtSN: 110 ± 12% *vs* 58 ± 8%, $P < 0.01$) or hypercapnia (ΔtSN: 9.5 ± 1.4% *vs* 3.9 ± 1.7%, $P < 0.05$) was attenuated in A5-lesioned rats compared to controls. Our data demonstrated that A5 noradrenergic neurons are part of the circuitry recruited for the processing of sympathetic response to hypoxia and hypercapnia in unanesthetized conditions. © 2017 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: sympathetic activity, respiration, hypoxia, hypercapnia, noradrenergic neurons.

INTRODUCTION

Endogenous noradrenaline release is required for adequate control of the cardiorespiratory functions (Schreihofer et al., 2000; Dawid-Milner et al., 2001; Swoap et al., 2004; Viemari et al., 2004b; Li and Nattie, 2006; Doi and Ramirez, 2010; Oliveira et al., 2016). During development, an increase in the tyrosine-hydroxylase activity in pontine-medullary neurons is an important step for the maturation of respiratory system (Bezin et al., 1994; Lagercrantz et al., 1994). In adults, lesions of the brainstem catecholaminergic neurons reduce resting arterial pressure and breathing frequency, impair hypoxia-induced sympathoexcitatory response and depress the ventilatory response to hypercapnia (Schreihofer and Guyenet, 2000; Li and Nattie, 2006). It has been evidenced that pontine catecholaminergic neuronal groups are essential for the appropriate basal and reflex regulation of cardiorespiratory functions (Guyenet et al., 1993; Biancardi et al., 2008; Taxini et al., 2011; Lopez Gonzalez et al., 2013). Among them, the A5 area, located within the ventrolateral pons, is suggested as an important nucleus involved in cardiorespiratory control (Loewy et al., 1979b; Byrum and Guyenet, 1987; Dobbins and Feldman, 1994).

The A5 noradrenergic cells project to the pre-sympathetic neurons of the rostral ventrolateral medulla (RVLM) as well as to the pre-ganglionic sympathetic neurons of the spinal cord (Loewy et al., 1979a; Byrum and Guyenet, 1987; Huangfu et al., 1991). However, functional evidence about the contribution of the A5 noradrenergic neurons to the regulation of sympathetic outflow is dubious, with studies reporting that A5 stimulation

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Abbreviations: Anti-DβH-SAP, saporin conjugated to anti-dopamine beta-hydroxylase; bpm, bursts per minute; E2, second stage of expiration; f_R, respiratory frequency; GABA, γ-aminobutyric acid; IgG-SAP, saporin conjugated to immunoglobulin G; KCN, potassium cyanide; MAP, mean arterial pressure; PAP, pulsatile arterial pressure; PN, phrenic nerve; post-I, post-inspiration; RVLM, rostral ventrolateral medulla; TH, tyrosine hydroxylase; tSN, thoracic sympathetic; V_E, pulmonary ventilation; V_T, tidal volume.

produces complex cardiovascular responses, including renal sympathetic activation, increases and decreases in the arterial pressure and muscle vasodilatation or no effect (Loewy et al., 1979b; Neil and Loewy, 1982; Stanek et al., 1984; Woodruff et al., 1986; Kanbar et al., 2011). Moreover, although studies reported that stimulation of peripheral chemoreceptors promotes the activation of A5 cells (Huangfu et al., 1991; Hirooka et al., 1997; Kanbar et al., 2011), lesions of the A5 noradrenergic neurons did not modify the sympathoexcitatory response to hypoxia (Taxini et al., 2011). These contrasting data may be associated with the fact that some of these studies were performed on anesthetized animals – an experimental condition that may affect the mechanisms responsible for the control of sympathetic activity and the processing of reflex responses (Machado and Bonagamba, 1992; Accorsi-Mendonca et al., 2007; Massey et al., 2015), or due to the lesion protocol, which was not limited to the A5 noradrenergic neurons.

With respect to breathing regulation, anatomical studies reported that the A5 cells present respiratory-related pattern of discharge (Guyenet et al., 1993) and are connected to the bulbospinal respiratory premotor neurons (Dobbins and Feldman, 1994). At early life, the A5 area appears to exert a tonic inhibitory control on breathing frequency, at least in in vitro reduced preparations (Hilaire et al., 1989). In adult anesthetized and vagotomized rats, it was evidenced that pharmacological inhibition of the cells of dorsolateral pons, including the A5 area, promotes an apneustic phrenic burst pattern (Jodkowski et al., 1994) while the stimulation of the same region prolongs the expiratory time (Jodkowski et al., 1997). In addition, it was demonstrated that respiratory-modulated neurons of the A5 are stimulated by hypoxia (Guyenet et al., 1993) and hypercapnia (Kanbar et al., 2011) and the inhibition of the A5 area attenuates the chemoreflex respiratory responses (Taxini et al., 2011). These studies indicate that the A5 region is involved in the control of respiratory frequency, at least under certain experimental conditions. However, there is no evidence whether the A5 noradrenergic neurons play a role in the control respiratory rhythm in unanesthetized conditions.

Previous studies have attempted to investigate the role of A5 neurons in the cardiovascular control of unanesthetized conditions (Maiorov et al., 2000; Madden and Sved, 2003; Kvetnansky et al., 2006; Vianna and Carrive, 2010). However, there is a lack of selectivity regarding the neuronal phenotype targeted in the A5 region. Moreover, none of these previous studies have explored the possible involvement of A5 neurons in the respiratory control. Accordingly, the present study was designed to investigate the role of the A5 noradrenergic neurons in the control of respiratory rhythm and sympathetic outflow in the absence of depressant effects of anesthesia. To reach this goal, we performed chemical lesions of the A5 noradrenergic cells using the ribosomal toxin saporin conjugated with a monoclonal antibody associated to the catecholamine-specific enzyme dopamine β-hydroxylase (Anti-DβH-SAP) and evaluated resting and chemoreflex control of cardiorespiratory function in unanesthetized, freely moving adult animals as well as in the decerebrated arterially perfused in situ preparations of juvenile rats.

EXPERIMENTAL PROCEDURES

Animals and Ethical approval

Experiments were performed on male Wistar adult rats (280–360 g, in vivo experiments) and on male Holtzman juvenile rats (75–80 g, in situ experiments). All protocols were performed in accordance with the Ethical Principles of Animal Experimentation and were approved by the Ethics Committee on Animal Experimentation of the School of Agriculture Sciences of Jaboticabal (Protocol: 004449/10) as well as of the School of Dentistry of Araraquara (Protocol: 18/2014). The animals received food and water ad libitum and were maintained at conditions of controlled temperature (22 ± 1 °C) and humidity (50–55%), with light–dark cycles of 12 h (lights on at 06:30 am).

Chemical lesions of A5 noradrenergic neurons

Lesions of the A5 noradrenergic neurons were achieved using anti-dopamine β-hydroxylase saporin (anti-DβH-SAP), as previously described (Taxini et al., 2011). Adult and juvenile rats were anesthetized with ketamine (80 mg kg^{-1}, intraperitoneally, ip) and xylazine (7 mg kg^{-1}, ip) and positioned in a stereotaxic apparatus (Kopf Instruments, Kent, England). After a longitudinal incision in the skin and subcutaneous tissue, the cranial bone was exposed and the sutures were identified for reference to target the A5 region using the following stereotaxic coordinates: 0.8 or 1.2 mm caudal to lambda, 1.5 or 2.3 mm lateral to midline and 9.0 or 9.9 mm below the bone surface, respectively in juvenile and adult rats. Bilateral microinjections of anti-DβH-SAP (4.2 ng; Advanced Targeting Systems, San Diego, CA, USA) or IgG-SAP (control; Advanced Targeting Systems, San Diego, CA, USA) were performed using a 30-G stainless needle coupled to a Hamilton syringe. The microinjection volumes were 100 nL/side in adult rats and 50 nL/side in juvenile rats, which were performed slowly (during 2 min) with the aid of a pump (Model 310, Stoelting Co., IL, USA) to minimize drug diffusion. After the injections, the subcutaneous and muscular tissues were sutured and the animal received antibiotic (enrofloxacin, 10 mg kg^{-1}, intramuscularly, im) and analgesic (flunixinmeglumine, 2.5 mg kg^{-1}, subcutaneously, sc). At the end of surgical procedures, the animals were monitored until regain consciousness and received water and food ad libitum. The animals were then kept for a period of one week for recovery and toxin action (Taxini et al., 2011).

Determination of pulmonary ventilation (V$_E$)

Whole body plethysmography (Bartlett and Tenney, 1970) was used to measure V$_E$ in adult unanesthetized animals, as previous described (Biancardi et al., 2008; Taxini et al., 2013). One day before the experiments, animals were anesthetized with ketamine (80 mg kg^{-1}, ip) and xylazine (7 mg kg^{-1}, ip) and a small incision was

performed in the abdominal wall to implant a temperature recorder (data-loggers; SubCue, Calgary, AT, CA) for body temperature measurements – used to calculate the tidal volume. After surgery, animals were treated with antibiotic (enrofloxacin, 10 mg kg^{-1}, im) and analgesic (flunixin meglumine, 2.5 mg kg^{-1}, sc) and monitored until regain consciousness. Twenty-four hours after, the animals were kept in a 5-L chamber ventilated with room air, hypercapnic gas mixture (7% CO_2, 21% O_2 and N_2 for balance; White Martins, Sertãozinho, Brazil) or a hypoxic gas mixture (7% O_2 and N_2 for balance) in low noise conditions. The flow rate (1 L m^{-1}) of gases into the chamber was monitored by a flowmeter (Sierra Instruments, Monterey, CA, USA). During measurements, the flow was interrupted and the chamber was sealed for short periods of time (approximately 2 min) to record the respiratory-related pressure oscillations using a differential pressure transducer (TSD 160A, Biopac Systems, Santa Barbara, CA, USA). The signals were amplified (DA 100C, Biopac Systems) and acquired on a microcomputer (MP100A-CE, Biopac Systems) at a sampling rate of 1 kHz (Acqknowledge, v. 4.2.3, Biopac Systems). Tidal volume (V_T) and respiratory frequency (f_R) were calculated and used to determine minute ventilation (V_E). V_T was determined based on the calibration volume (obtained in every experiment by injecting 1 mL of air into the chamber) and using an appropriated formula, as previously described (Bartlett and Tenney, 1970).

Measurements of arterial blood pressure, heart rate and body temperature in unanesthetized animals

In a subgroup of animals, cardiovascular parameters were measured simultaneously to ventilation. To this end, one day before the experiments, the adult rats were anesthetized with ketamine (80 mg kg^{-1}, ip) and xylazine (7 mg kg^{-1}, ip) and a polyethylene catheter (PE-10 connected to PE-50; Clay Adams, Parsippany, NJ, USA) was inserted into the abdominal aorta through the femoral artery for measurements of pulsatile arterial pressure (PAP). The arterial catheter was then tunneled subcutaneously and exteriorized at the animal dorsum. After that, a temperature recorder was also implanted into the abdominal cavity, as previously described. After surgery, animals were treated with antibiotic (enrofloxacin, 10 mg kg^{-1}, im) and analgesic (flunixin meglumine, 2.5 mg kg^{-1}, sc) and monitored until recovered from anesthesia. On the next day, when the animals were recovered from surgical procedures, the arterial catheter was connected to a pressure transducer (TSD 104A; Biopac systems) and to an amplifier (DA 100C; Biopac systems) for the monitoring of PAP in unanesthetized conditions. PAP was recorded using a data acquisition system (MP100 ACE; Biopac systems) at a sampling rate of 1 kHz. Mean arterial pressure (MAP) and heart rate (HR) were derived from PAP signals (Acqknowledge, v 4.2.3, Biopac Systems).

In situ working heart-brainstem preparation

Working heart-brainstem preparations (Paton, 1996) were surgically prepared one week after the microinjections in

the A5, as previously described (Zoccal et al., 2008). The animals were initially deeply anesthetized with halothane (AstraZeneca, Cotia, SP, Brazil) until the loss of the paw withdrawal reflex, transected caudal to the diaphragm, submerged in a chilled Ringer solution (in mM: NaCl, 125; $NaHCO_3$, 24; KCl, 3; $CaCl_2$, 2.5; $MgSO_4$, 1.25; KH_2PO_4, 1.25; dextrose, 10) and decerebrated at the precollicular level. Lungs were removed the preparations were then transferred to a recording chamber. The descending aorta was cannulated and perfused retrogradely using a roller pump (Watson-Marlow 502 s, Falmouth, Cornwall, UK) via a double-lumen cannula. The perfusate consisted of Ringer solution containing 1.25% Polyethylene glycol (an oncotic agent, Sigma, St Louis, USA) and a neuromuscular blocker (vecuronium bromide, 3–4 μg mL^{-1}, Cristália Produtos Químicos Farmacêuticos Ltda., São Paulo, Brazil). This solution was gassed continuously with 5% CO_2–95% O_2, warmed to 31–32 °C and filtered using a nylon mesh (pore size: 25 μm, Millipore, Billirica, MA, USA). The perfusion pressure was maintained in the range of 50–70 mmHg by adjusting the rate flow to 21–25 mL min^{-1} and by adding vasopressin to the perfusate (0.6–1.2 nM, Sigma, St. Louis, MO, USA).

Nerves recording and data analyses

Sympathetic and phrenic nerves (PN) were isolated and their activity recorded simultaneously using bipolar glass suction electrodes held in micromanipulators (Narishige, Tokyo, Japan). Left phrenic nerve discharges were recorded from its central end and its rhythmic ramping activity was used to monitor preparation viability. The sympathetic activity was recorded from the thoracic sympathetic chain (tSN) at the level of T10–T12. The signals were amplified, band-pass filtered (0.1–3 kHz; Grass Technologies, Middleton, USA), and acquired with an A/D converter (CED 1401; Cambridge Electronic Design, Cambridge, UK) to a computer using Spike2 software (version 7; Cambridge Electronic Design, Cambridge, UK) at a sampling rate of 5 kHz. All nerves were recorded in absolute units (μV). At the end of the experiments, the perfusion pump was turned off to determine the electrical noise (after the death of the preparations).

The analyses were carried out on rectified and smoothed signals (time constant of 50 ms), and performed off-line using Spike 2 software (CED, Cambridge, UK) after noise subtraction. PN activity was evaluated by its burst frequency and amplitude. PN burst frequency was derived from the time interval between consecutive integrated phrenic peak bursts and expressed in bursts per minute (bpm). Integrated PN burst amplitude was determined (in μV) by the value difference between the burst peak and the minimal activity observed during expiratory period. Baseline tSN activity was calculated as the mean values (in μV) of integrated signals. Phrenic-triggered averages of tSN were also generated from 1–3 min epochs to analyze the pattern of tSN across the respiratory cycle. To this, mean tSN activity was measured during inspiration (period coincident with phrenic burst), post-inspiration

(post-I, first half of expiratory period) and second stage of expiration (E2, second half of expiratory period). The values were then normalized by the maximal tSN observed during late-inspiratory/post-inspiratory phases (100%), as previously described (Zoccal et al., 2008). The analyses were applied for each preparation and data obtained were pooled together and compared between groups. The changes in the PN amplitude and tSN activity induced by potassium cyanide (KCN) and hypercapnia (see bellow) were expressed as percentage values in relation to basal values prior to the stimuli. The changes in the other parameters were expressed in their original units.

Experimental design

Unanesthetized adult rats – Seven days after surgery, each animal was individually placed into the plethysmoghraphic chamber (5 L) and acclimatized for at least 1 h, while the chamber was flushed with humidified room air at 25 °C. After this period, baseline recordings of V_T, fR, MAP and HR were obtained for 10–15 min. Subsequently, hypercapnic (7% CO_2, 21% O_2, 81% N_2) or hypoxic (7% O_2, 93% N_2) gas mixture was flushed separately in the chamber for 30 min. Cardiovascular and ventilatory parameters were evaluated at 10 and 30 min of exposure. Animals were randomly exposed to both hypercapnia and hypoxia, with a time interval of 30 min between stimuli.

In situ preparations of juvenile rats – After a stabilization period of 20–30 min, baseline sympathetic and phrenic activities were recorded for 15 min. After that, peripheral and central chemoreceptors were stimulated KCN and hypercapnia, respectively, as previously described (Costa-Silva et al., 2010; Molkov et al., 2011). KCN (0.05%) was injected intra-arterially (50 μL) while the hypercapnia was achieved by bubbling the perfusate with 7% and 10% CO_2 (balanced in O_2) for 5 min. The diving reflex was also tested by topical administration of cold Ringer's solution (0.5 mL) into the animal's snout (Kawakami et al., 1967; Khurana et al., 1980). This reflex was evaluated to verify the integrity of a sympathoexcitatory reflex that is stimulated independently on the activation of central and peripheral chemoreceptors. The tests were performed randomly with a time interval of at least 10 min between consecutive stimuli.

Tyrosine hydroxylase immunohistochemistry

Adult animals were deeply anesthetized (2,2,2-tribromoetanol, 250 mg/kg, ip,Sigma- Aldrich, St Louis, EUA) at the end of experiments and perfused through the heart with phosphate buffer saline (PBS 0.1 M, pH 7.4), followed by 4% paraformaldehyde in phosphate buffer (PB 0.1 M). The *in situ* preparations were perfused only with 4% paraformaldehyde in PB (0.1 M) at the end of experiments (after electrical noise detection). After fixation, the brainstems were then carefully removed and stored in the same fixative at 4 °C for 4 h and thereafter kept in 30% sucrose solution dissolved in 0.1 M PB, pH 7.4, at 4 °C for 48 h. The tissue was then placed in 2-methylbutane (Sigma-Aldrich, St Louis, EUA), frozen in dry ice (1 min), embedded in Tissue Tek OCT and cut in the cryostat (Leica CM 1850) at 25 μm. The brain slices were submitted to tyrosine hydroxylase (TH) immunostaining to verify the effectiveness of the lesions of noradrenergic neurons in the A5 areas. To this, the coronal sections were incubated with polyclonal primary mouse antibody anti-TH (1:10000, Sigma–Aldrich) for 48 h, and then incubated with biotinylated secondary rabbit antibody (1:1000, Vector Laboratories, Burlingame, CA, USA) for 1 h. The biotinylated antibody was complexed with avidin DH-biotinylated horseradish peroxidase (Vector Laboratories, Burlingame, CA, USA, code PK-4001) and revealed with peroxidase substrate 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma). The sections were placed on gelatinized slides, dried, dehydrated through graded concentrations of alcohol, cleared in xylene, and sealed with a coverslip for later examination.

The TH immunoreactive cells (TH-IR) were analyzed by bright-filed microscopy, and labeled neurons were registered with the use of an image analysis system (Zeiss KS 300). For quantification, A5 noradrenergic neurons were bilaterally counted over the entire length of this cell group (9.50–11.05 mm caudal from Bregma, divided into five sections) and a total average value was obtained. The data obtained in each animal were pooled and an average value was calculated in each experimental group. Images were captured and analyses were carried out using a computerized image analysis system (NIH System, Image J developed at the U.S. National Institutes of Health and available on the Internet at http://www.rsb.info.nih.gov/nih-image/). TH-IR cells in each section were counted by setting a size range for cellular nuclei (in pixels) and a threshold level for staining intensity. Representative sections in control and experimental groups were acquired at the same level, with the aid of the Adobe Photoshop Image Analysis Program, version 6.0. The counting was repeated at least twice for each section analyzed, which ensured that the number of profiles obtained was similar.

Statistical analyses

The values are reported as mean ± SEM. The effectiveness of the lesions was analyzed using Student's unpaired t test. For *in vivo* experiments, V_E, V_T, fR, MAP and HR data were compared using repeated measurement two-way ANOVA. When interactions between the factors were observed, groups were compared using Bonferroni's post hoc test. The data obtained in the *in situ* preparations were compared using repeated measurement one-way ANOVA and Student's unpaired t test. The statistical analysis was carried out using SigmaStat (Systat Software, Point Richmond, CA. USA), considering $P < 0.05$ as significantly different. Graphs were generated using Prism (GraphPad, La Jolla, USA).

RESULTS

Effectiveness of the noradrenergic neuron lesions in the A5 of adult and juvenile rats

A5 noradrenergic neurons were identified as the group of TH-positive cells in the ventral pons, located laterally to the rostral edge of the facial motor nucleus, in between the exit of the facial nerve and the superior olive, as previously described (Byrum and Guyenet, 1987; Taxini et al., 2011). At the same coronal level, we also identified the TH-positive cells of the *Locus coeruleus* (LC), at the dorsal pons, as demonstrated in the Fig. 1A. The microinjections of anti-SAP-DβH in the A5 region (A5-lesioned group) reduced the number of noradrenergic neurons in adult (Fig. 1Bi, Bii) and juvenile rats (Fig. 1Ci, Cii). In adult animals, the total average number of bilateral TH-positive neurons in the A5 region of control group was 24 ± 1 while in the A5-lesioned group was 11 ± 1 ($P < 0.05$). In the juvenile rats, similar results were observed (24 ± 1 *vs.* 8 ± 1 neurons/section, respectively control and A5-lesioned groups, $P < 0.05$). Our data indicate that our procedures were effective to lesion 55–70% of noradrenergic neurons in the A5 area of both adult and juvenile rats.

Baseline cardiovascular and ventilatory parameters in animals with lesions of A5 noradrenergic neurons

At normoxic and normocapnic conditions, unanesthetized animals with chemical lesions of A5 noradrenergic neurons ($n = 9$) showed similar cardiovascular (MAP: 106 ± 3 *vs* 104 ± 4 mmHg; HR: 315 ± 11 *vs* 338 ± 5 bpm, respectively control and A5-lesioned groups) and ventilatory parameters (fR: 90 ± 4 *vs* 91 ± 7 breaths.min^{-1}; V$_T$: 5.0 ± 0.3 *vs* 4.6 ± 0.3 mL.kg^{-1}; V$_E$: 458 ± 31 *vs* 459 ± 83 mL.kg^{-1}.min^{-1}, respectively control and A5-lesioned groups) in comparison to control group ($n = 8$). The group data are shown in Fig. 2. Similar to unanesthetized animals, the *in situ* preparations of control ($n = 7$) and A5-lesioned juvenile rats ($n = 6$) exhibited similar values of PN burst amplitude (41 ± 10 *vs* 45 ± 20 μV, respectively control and A5-lesioned groups) and frequency (13 ± 1 *vs* 15 ± 2 bpm respectively control and A5-lesioned groups), and mean tSN levels (12 ± 2 *vs* 13 ± 2 μV, respectively control and A5-lesioned groups, Fig. 3A, 3D), as illustrated in Fig. 3. Regarding the sympathetic discharge pattern, tSN of control rats showed respiratory-related bursts with a ramping pattern that initiated during the inspiratory phase and reached a peak during the late part of inspiration or the beginning of post-I. This respiratory-modulated tSN peak was followed by a decline during the post-inspiratory phase, with lower levels during the E2 phase (Fig. 3A). A similar tSN discharge pattern was verified in the *in situ* preparations of A5-lesioned rats (Fig. 3A). The average values of normalized tSN during inspiration (54 ± 6 *vs* 63 ± 5%, respectively control and A5-lesioned groups), post-I (47 ± 4 *vs* 48 ± 4%, respectively control and A5-lesioned groups) and E2 phases (37 ± 4 *vs* 43 ± 3%, respectively control and A5-lesioned groups) were

similar between control and A5-lesioned groups respectively (Fig. 3E).

Cardiovascular and respiratory reflex responses to hypoxia in unanesthetized A5-lesioned rats

During hypoxic challenge (7% O$_2$ during 30 min), the values of MAP (at 10 min: 103 ± 4 *vs* 104 ± 9; at 30 min: 97 ± 2 *vs* 101 ± 4 mmHg; respectively control and A5-lesioned groups, Fig. 4A) and HR (at 10 min: 305 ± 32 *vs* 337 ± 15; at 30 min: 348 ± 28 *vs* 397 ± 19 bpm; respectively control and A5-lesioned groups, Fig. 4B) were similar between control ($n = 4$) and A5-lesioned group ($n = 4$). The exposure to hypoxia, at 10 and 30 min, also elevated V$_E$ in control group due to increases in fR and V$_T$ ($P < 0.05$, Fig. 4C–E). In the A5-lesioned rats, the hypoxic-induced increase in V$_E$, at 10 and 30 min during exposure, was mostly dependent upon an increase in fR ($P < 0.05$, Fig. 4C–E). In spite of the differences in the pattern of the ventilatory response to hypoxia, the average magnitudes of fR (at 10 min: 113 ± 9 *vs* 125 ± 21; at 30 min: 126 ± 11 *vs* 123 ± 12 breaths.min^{-1}; respectively control and A5-lesioned groups, Fig. 4C), V$_T$ (at 10 min: 7 ± 0.6 *vs* 6 ± 1; at 30 min: 8 ± 0.6 *vs* 6 ± 1 mL.kg^{-1}; respectively control and A5-lesioned groups, Fig. 4D) and V$_E$ (at 10 min: 802 ± 93 *vs* 737 ± 120; at 30 min: 1028 ± 131 *vs* 761 ± 93 mL.min^{-1}.kg^{-1}; respectively control and A5-lesioned groups, Fig. 4E) were comparable between control ($n = 8$) and A5-lesioned ($n = 4$) rats.

Sympathetic and phrenic responses to peripheral chemoreceptor activation in the *in situ* preparations of A5-lesioned rats

Activation of the peripheral chemoreceptors with KCN promoted marked increases in the tSN activity as well as in the PN burst frequency and amplitude of control *in situ* preparations ($n = 7$; $P < 0.05$), as demonstrated on Fig. 5. In juvenile rats with lesions of A5 noradrenergic neurons ($n = 6$), the magnitude of sympathoexcitatory response was attenuated in comparison to the control group (110 ± 12 *vs* 58 ± 8%, $P < 0.01$, respectively control and A5-lesioned groups, Fig. 5B). On the other hand, the magnitude of the increase in PN burst amplitude (20 ± 4 *vs* 17 ± 4%, respectively control and A5-lesioned groups, Fig. 5C) and frequency (20 ± 3 *vs* 21 ± 2 cpm, respectively control and A5-lesioned groups, Fig. 5D) was similar between control and A5-lesioned groups.

Cardiovascular and respiratory reflex responses to hypercapnia in unanesthetized A5-lesioned rats

The exposure to hypercapnia (7% CO$_2$, 30 min) did not elicit changes in MAP and HR of both experimental groups. Moreover, the values of MAP (at 10 min: 102 ± 2 *vs* 105 ± 6; at 30 min: 100 ± 1 *vs* 105 ± 6 mmHg) and HR (at 10 min: 285 ± 13 *vs* 317 ± 27; at 30 min: 302 ± 9 *vs* 301 ± 22 bpm) were similar between control ($n = 4$) and A5-lesioned groups ($n = 5$), as illustrated in Fig. 6A, B. Regarding the respiratory changes elicited by
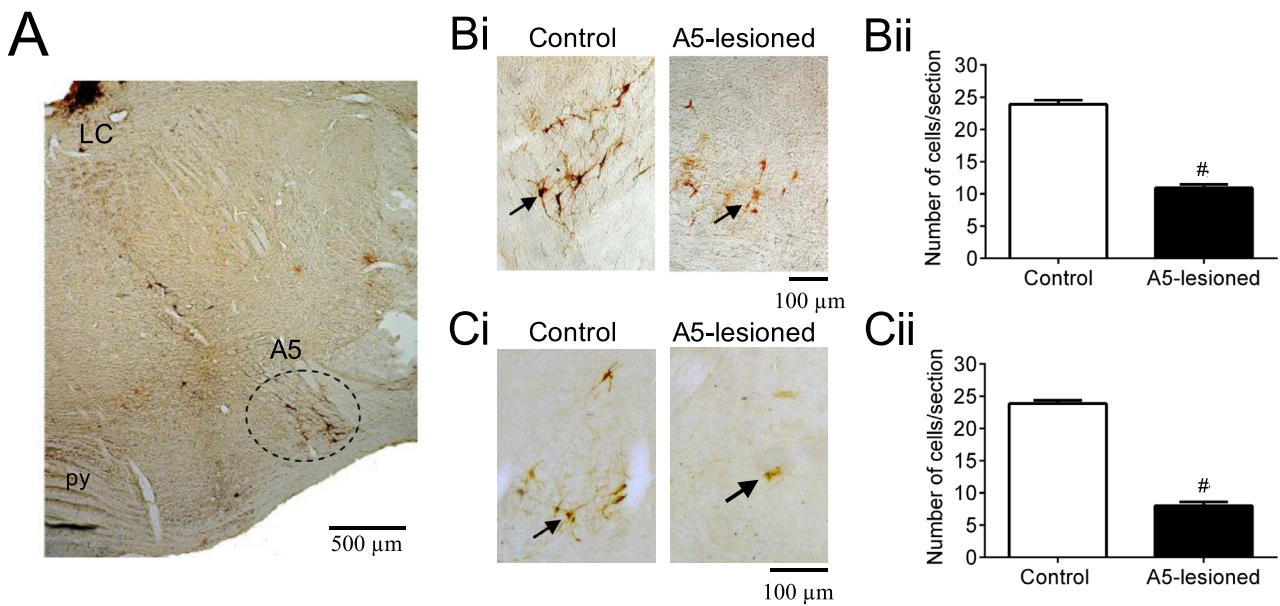
Fig. 1. Immunohistochemical identification of A5 tyrosine hydroxylase (TH) immunoreactive neurons. Panel A: coronal section of a rat illustrating the TH-positive cells in the ventrolateral pons, corresponding to the A5 area (dotted line). In this section, also note a great density of TH-positive cells at the dorsal pons, adjacent to the fourth ventricle, in the Locus Coeruleus (LC). Panel Bi: TH-immunoreactive neurons in the A5 region of adult rats, representative of their respective groups, which received either IgG-SAP (control) or anti-DβH-SAP microinjections (A5-lesioned) seven days prior to the experiments *in vivo*. The arrows indicate TH neurons in the A5 region. Panel Bii: average number of TH-positive neurons in the A5 area of adult control ($n = 13$) and A5-lesioned rats ($n = 13$). Panel Ci: TH-immunoreactive neurons in the A5 region of juvenile rats, representative of their respective groups, which received either IgG-SAP (control) or anti-DβH-SAP microinjections (A5-lesioned) seven days prior to the *in situ* experiments. The arrows indicate TH neurons in the A5 region. Panel Cii: average number of TH-positive neurons in the A5 area of control ($n = 7$) and A5-lesioned *in situ* preparations ($n = 6$). *Abbreviation:* py, pyramid. *Different from respective control group ($P < 0.05$).
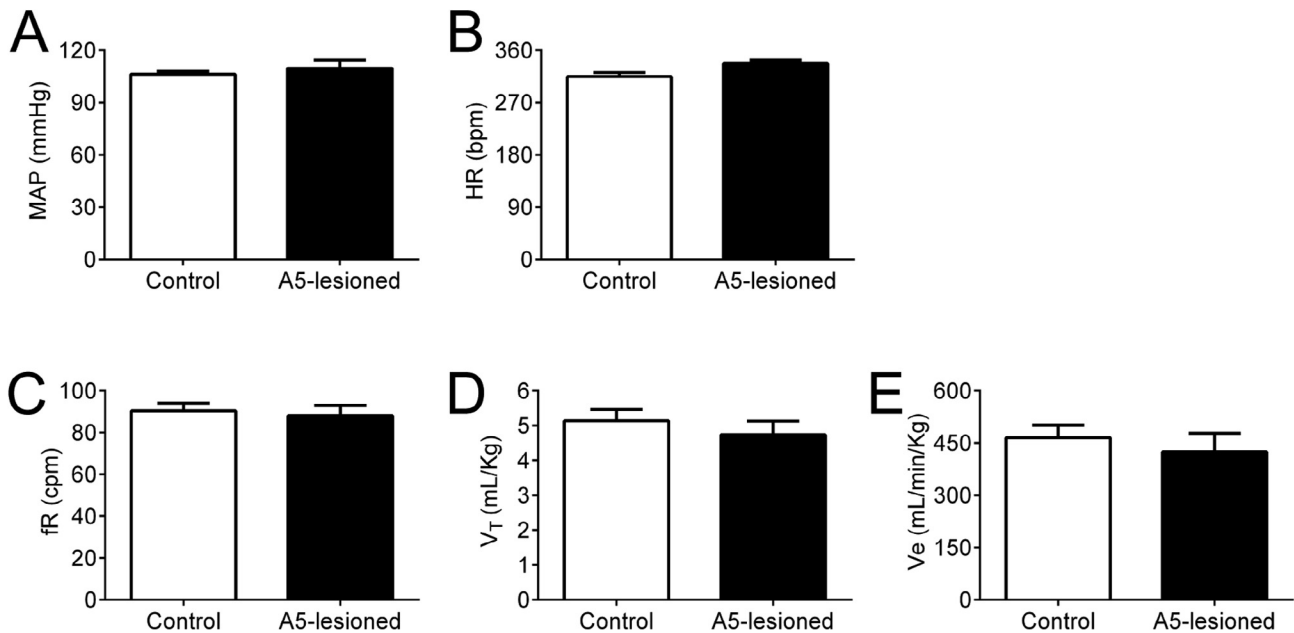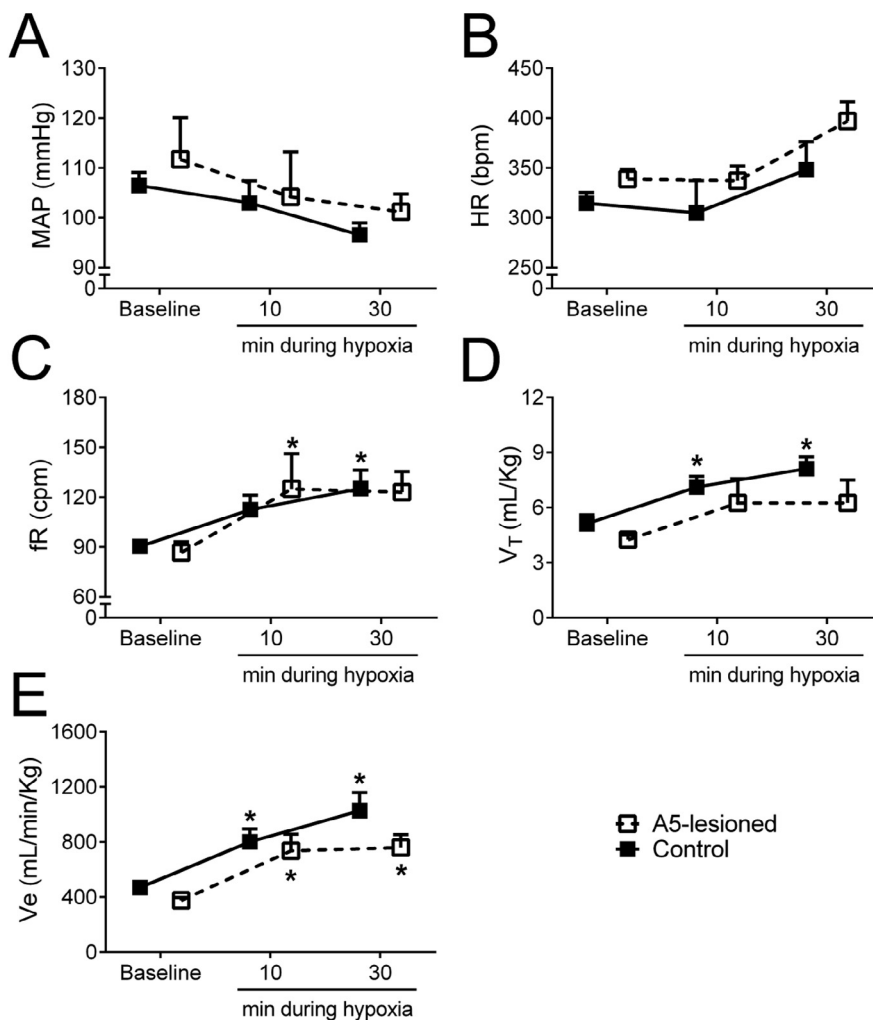

Fig. 2. Baseline cardiorespiratory parameters in unanesthetized adult rats with lesions of TH-positive neurons in the A5 area. Average values of mean arterial pressure (MAP, panel A), heart rate (HR, panel B), respiratory frequency (fR, panel C), tidal volume (V_T, panel D) and minute ventilation (V_E, panel E) of adult rats from control ($n = 8$) and from A5-lesioned groups ($n = 9$).

hypercapnia, both experimental groups showed significant increases in fR, V_T and V_E at 10 and 30 min in both groups ($P < 0.001$, Fig. 6C–E). The magnitude of reflex increases in fR (at 10 min: 128 ± 14 *vs* 140 ± 17; at 30 min: 138 ± 5 *vs* 149 ± 13 breaths.min^{-1}; respectively control and A5-lesioned groups, Fig. 6C),
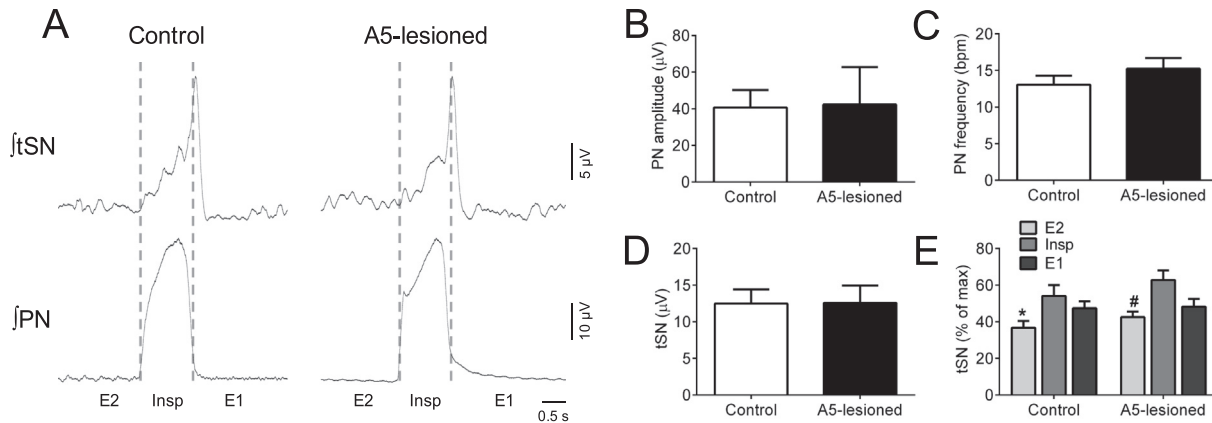
Fig. 3. Baseline phrenic and sympathetic nerve activities of *in situ* preparations of rats with lesions of TH-positive neurons in the A5 area. Panel A: cycle-triggered averages (3-min epochs) of integrated ($\int$) phrenic (PN) and sympathetic nerve (tSN) activities of control and A5-lesioned *in situ* preparations, representative from their groups. Panels B–D: average values of phrenic burst amplitude and frequency, and mean thoracic sympathetic activity, respectively, of control ($n = 7$) and A5-lesioned groups ($n = 6$). Panel E: percentage values of tSN activity during inspiration (insp), first (E1) and second stage of expiration (E2) in control ($n = 7$) and A5-lesioned *in situ* preparations ($n = 6$). *Different from control inspiratory phase. # Different from A5-lesioned inspiratory phase.


Fig. 4. Hypoxia-induced cardiorespiratory reflex responses in adult rats with lesions of the A5 TH-positive neurons. Average values of mean arterial pressure (MAP, panel A), heart rate (HR, panel B), respiratory frequency (fR, panel C), tidal volume (V_T, panel D) and minute ventilation (V_E, panel E) of adult rats from control ($n = 8$) and from A5-lesioned groups ($n = 4$), before (baseline) and during the exposure to hypoxia (7% O_2) for 30 min. *Different from respective baseline.

V_T (at 10 min: 8 ± 0.4 *vs* 7 ± 1; at 30 min: 9 ± 0.5 *vs* 8 ± 1 mL kg^{-1}; respectively control and A5-lesioned groups, Fig. 6D) and V_E (at 10 min: 1011 ± 65 *vs* 1087 ± 256; at 30 min: 1297 ± 95 *vs* 1204 ± 226 mL kg^{-1} min^{-1}; respectively control and A5-lesioned groups, Fig. 6E), was similar between control ($n = 8$) and A5-lesioned group ($n = 5$).

Sympathetic and phrenic responses to hypercapnia in the *in situ* preparations of A5-lesioned rats

The rise in the fractional concentration of CO_2 in the perfusate of *in situ* preparations (7% and 10% CO_2) increased the tSN and PN activities in control ($n = 7$) and A5-lesioned groups ($n = 6$), as illustrated in Fig. 7. The tSN response to 7% CO_2 was attenuated in A5-lesioned rats compared to controls (10 ± 1 *vs* $4 \pm 2\%$, $P < 0.05$, Fig. 7Bi); however, at 10% CO_2, the magnitude of tSN response was similar between groups (16 ± 3 *vs* $11 \pm 2\%$). The increase in PN burst amplitude in response to 7% CO_2 (8 ± 4 *vs* $7 \pm 2\%$, Fig. 7Bi) and 10% CO_2 (15 ± 4 *vs* $20 \pm 4\%$, Fig. 7Cii) were similar between control ($n = 7$) and A5-lesioned group ($n = 6$). No significant changes were observed in the PN burst frequency in response to the two levels of CO_2 in both
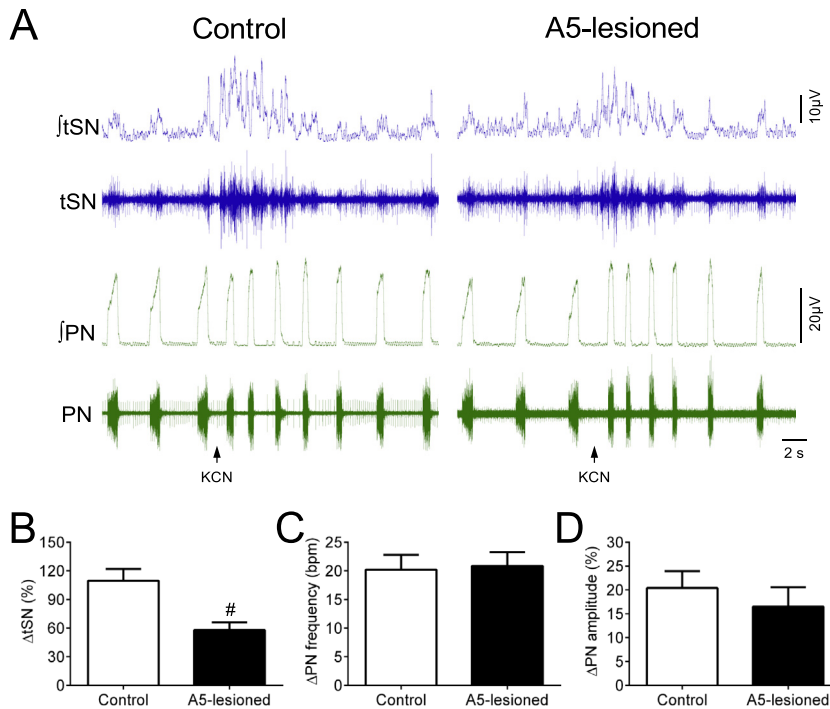
Fig. 5. Sympathetic and phrenic reflex responses to stimulation of peripheral chemoreceptors in the *in situ* preparations of rats with lesions of the A5 TH-positive neurons. Panel A: raw and integrated (∫) recordings of thoracic sympathetic (tSN) and phrenic (PN) activities, illustrating the sympathetic and phrenic changes elicited by peripheral chemoreceptor stimulation with potassium cyanide (KCN, arrow) in a control and A5-lesioned *in situ* preparation. Panels B–D: KCN-induced changes (Δ) in the tSN activity and in the PN frequency and amplitude, respectively, of *in situ* preparations from control ($n = 7$) and A5-lesioned groups ($n = 6$). *Different from control group.

experimental groups (ΔPN frequency – 7% CO_2: 1 ± 1 vs 1 ± 1 cpm; 10% CO_2: 1 ± 1 vs 0 ± 1 cpm; respectively control and A5-lesioned animals).

Diving reflex responses in A5-lesioned rats

The activation of the diving reflex evoked responses of sympatho-excitation (75 ± 14 *vs* 60 ± 14%, respectively control and A5-lesioned rats, $P < 0.01$) and expiratory time lengthening (2 ± 0.4 *vs* 2 ± 0.7 s, respectively control and A5-lesioned rats, $P < 0.01$) of similar magnitude in control ($n = 7$) and A5-lesioned *in situ* preparations ($n = 6$).

DISCUSSION

In the present study, we report that lesions of A5 noradrenergic cells did not modify resting arterial pressure and minute ventilation of unanesthetized rats. In agreement with these findings, we also show that baseline sympathetic and phrenic activities, as well as the respiratory-sympathetic coupling pattern, were not altered in the *in situ* preparations of A5-lesioned rats. On the other hand, we found that A5 noradrenergic neuron lesion depressed the sympatho-excitation, but not ventilatory response, elicited by hypoxia and, to a less extent, hypercapnia. Our findings support the notion that the maintenance of resting sympathetic vasomotor tonus and respiratory rhythm of rats, at least

in juvenile/adult ages, does not require the noradrenergic neurons of the A5 area. In contrast, A5 cells contribute to the complete expression of sympathetic chemoreflex response in unanesthetized conditions.
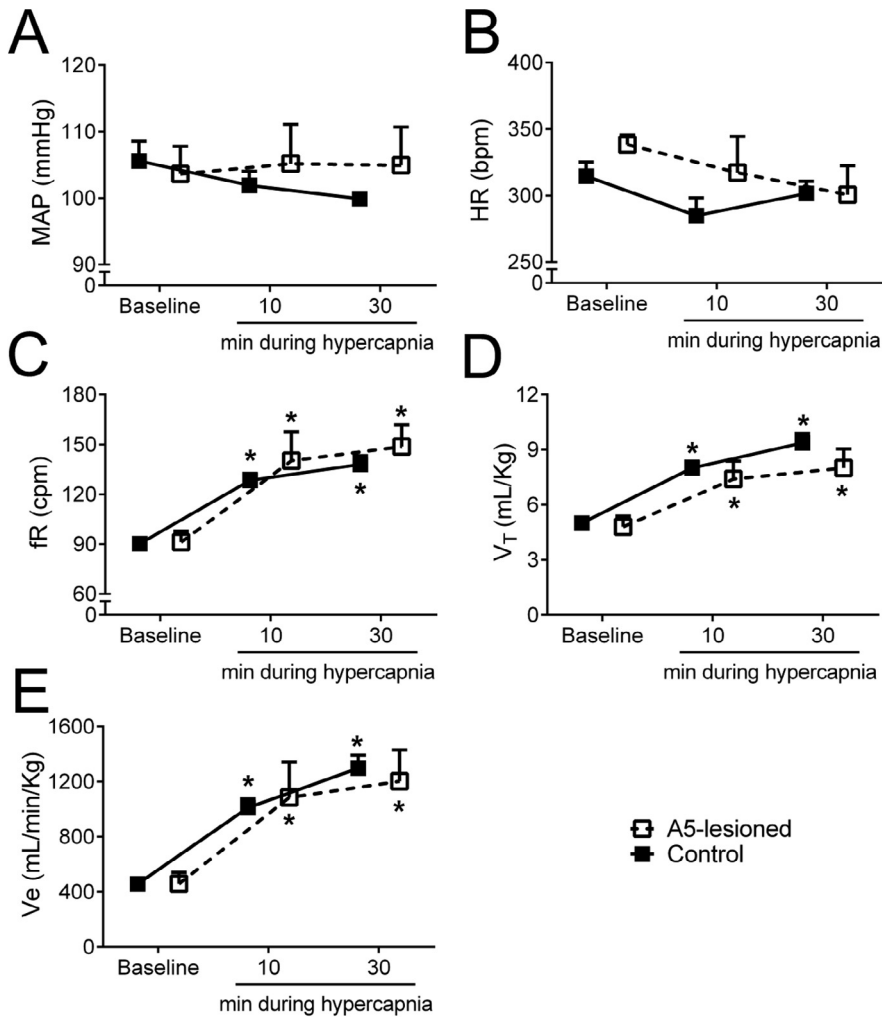
Contribution of A5 noradrenergic neurons to the maintenance of baseline arterial pressure and respiratory rhythm

Anatomical and electrophysiological evidence demonstrates that the A5 noradrenergic neurons that exhibit low activity at rest, are inhibited during baroreflex activation (Huangfu et al., 1991; Kanbar et al., 2011), interact with cardiorespiratory centers of the medulla oblongata and spinal cord (Loewy et al., 1979b; Byrum and Guyenet, 1987; Huangfu et al., 1991), and evoke a robust sympathoexcitatory response when stimulated (Kanbar et al., 2011). These observations suggest that A5 neurons are an important excitatory component of sympathetic nervous system. However, acute pharmacological inhibition and chronic lesion of noradrenergic cells in the A5 did not reduce baseline arterial pressure levels (Koshiya and Guyenet, 1994a; Schreihofer and Guyenet, 2000; Taxini et al., 2011). It is important to mention that all these previous studies were performed on anesthetized rats – an experimental condition that may interfere with the normal functioning of sympathetic nervous system (Machado and Bonagamba, 1992; Accorsi-Mendonca et al., 2007; Massey et al., 2015). In this regard, we demonstrated that lesions of the noradrenergic neurons in the A5 did not alter baseline arterial pressure of conscious rats and the sympathetic activity levels of unanesthetized *in situ* rat preparations. The arterially perfused, decerebrated *in situ* preparations may also represent a technical limitation; however, data from literature support that the functioning of brainstem network, the pattern of sympathetic discharge and vasomotor control mechanisms in the *in situ* preparations resemble to the mechanisms *in vivo* (Zoccal et al., 2009; McBryde et al., 2013; Moraes et al., 2013). Therefore, our results agree with previous observations, and support the concept that A5 noradrenergic neurons do not contribute to the maintenance of baseline sympathetic activity in unanesthetized conditions.

In addition to its tonic nature, the sympathetic activity displays oscillatory components that are important for the control of vascular tonus (Barman and Gebber, 2000; Simms et al., 2009). Part of the sympathetic rhythmicity is entrained with the breathing cycle and depends on inputs from respiratory network to premotor sympathetic neurons (Malpas, 1998; Molkov et al., 2011; Moraes

Fig. 6. Hypercapnia-induced cardiorespiratory reflex responses in adult rats with lesions of the A5 TH-positive neurons. Average values of mean arterial pressure (MAP, panel A), heart rate (HR, panel B), respiratory frequency (fR, panel C), tidal volume (V_T, panel D) and minute ventilation (V_E, panel E) of adult rats from control ($n = 8$) and from A5-lesioned groups ($n = 5$), before (baseline) and during the exposure to hypercapnia (7% CO_2) for 30 min. *Different from respective baseline.
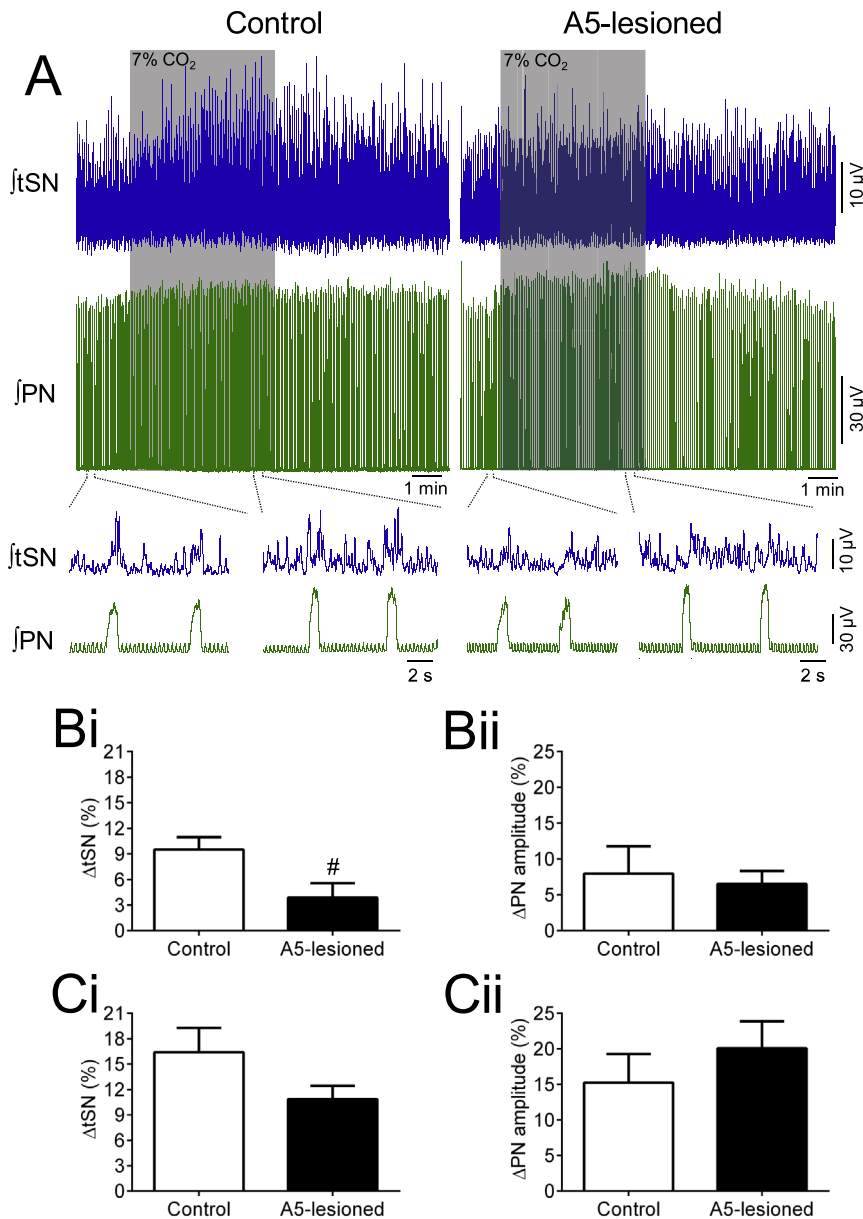
network (Dobbins and Feldman, 1994). In neonates, it has been shown that A5 noradrenergic neurons tonically inhibit the ventromedullary respiratory neurons and reduce the f_R (Viemari and Hilaire, 2003; Hilaire et al., 2004; Viemari et al., 2004a). In contrast, we found that baseline ventilation of adult unanesthetized rats and the phrenic activity of *in situ* preparations of juvenile rats were similar between control and A5-lesioned groups. Our findings agree with previous suggestion that the strength of the A5-dependent inhibitory effect on f_R may be age dependent (Hilaire et al., 2004), and indicate that A5 noradrenergic neurons do not play a role in the generation of respiratory rhythm in juvenile/adult animals.

A5 noradrenergic neurons and the autonomic and respiratory responses to hypoxia and hypercapnia

In our experimental conditions, we found that the increase in ventilation during hypoxia and hypercapnia in A5-lesioned unanesthetized rats was similar to control rats. Moreover, the values of arterial pressure during hypoxia and hypercapnia were similar between control and A5-lesioned rats. However, during prolonged hypoxia or hypercapnia exposure *in vivo*, a prominent response of vasodilatation is also observed in conjunction to the sympatho-excitatory response, which affects the evaluation of sympathetic-mediated pressor response (Barros et al., 2002). For that reason, the experiments with *in situ* preparations were important to perform direct measurements of the chemoreflex control of sympathetic activity in the absence of anesthesia. In these experiments, while central chemoreceptors were activated using a perfusate with elevated levels of CO_2, the peripheral chemoreceptors were activated with KCN in order to produce consistent respiratory and sympathetic reflex responses among preparations (Lemes et al., 2016).

Similar to the *in vivo* data, the phrenic responses to KCN and hypercapnia were similar between *in situ* preparation of control and A5-lesioned rats, indicating that A5 noradrenergic neurons do not contribute to the processing of chemoreflex ventilatory responses. On the other hand, the sympatho-excitation elicited by peripheral chemoreceptor stimulation and hypercapnia *in situ* was significantly depressed in A5-lesioned rats. Importantly, the sympathetic response to stimulation of diving reflex was not affected in A5-lesioned rats,

et al., 2013). Previous studies demonstrated that the pons critically contributes to the generation of respiratory-sympathetic coupling, since the pontine-medullary disconnection abolished the sympathetic oscillations entrained with respiration (Baekey et al., 2008). By the fact that the A5 cells received projections from ventral respiratory group (Guyenet et al., 1993; Rosin et al., 2006) and some of the A5 neurons exhibit respiratory-related pattern of discharge (Guyenet et al., 1993), this region could potentially contribute to the generation of rhythmical respiratory oscillations in the sympathetic outflow. However, we found that lesion of A5 neurons did not alter the baseline pattern of respiratory modulation of sympathetic activity of the *in situ* preparations, indicating other neurons in the pons than the A5 noradrenergic neurons contribute to the rhythmical control of sympathetic activity to the cardiovascular system.

With respect to the respiratory activity, previous studies suggest the existence of descending projections from the A5 area to the ventromedullary respiratory

Fig. 7. Sympathetic and phrenic reflex responses of *in situ* preparations of rats with lesions of the A5 TH-positive neurons exposed to hypercapnia. Panel A: integrated ($\int$) recordings of thoracic sympathetic (tSN) and phrenic (PN) activities, illustrating the sympathetic and phrenic changes elicited by hypercapnia (7% CO_2, shaded area) in a control and A5-lesioned *in situ* preparation. Panels Bi and Bii: Reflex changes (Δ) in the tSN activity and in the PN amplitude, respectively, of *in situ* preparations from control ($n = 7$) and A5-lesioned groups ($n = 6$) exposed to 7% CO_2. Panels Ci and Cii: Reflex changes (Δ) in the tSN activity and in the PN amplitude, respectively, of *in situ* preparations from control ($n = 7$) and A5-lesioned groups ($n = 6$) exposed to 10% CO_2. *Different from control group.

suggesting that the processing of other sympatho-excitatory reflexes was preserved. Our data, therefore, indicate that the A5 noradrenergic neurons are recruited during chemoreflex activation and contribute mostly to the sympatho-excitatory response in anesthetic-free conditions. On the base of anatomical evidence (Byrum and Guyenet, 1987), we hypothesize that synapses from the NTS neurons that receive peripheral chemoreceptor inputs stimulate the A5 neurons in conditions of hypoxia. Moreover, by the fact that A5 noradrenergic neurons dis-

play reduced sensitivity to pH *in vitro* (Kanbar et al., 2011), we consider that the activation of A5 neurons during hypercapnia, and the generation of sympatho-excitatory reflex response, may depend on excitatory inputs from CO_2-sensitive regions that are connected to A5, such as retrotrapezoid nucleus (RTN) and nucleus tractus solitarii (NTS) (Ritucci et al., 1997; Rosin et al., 2006). Koshiya and Guyenet (1994b) described that intrathecal administration of α- or β-adrenergic antagonists did not affect the magnitude of sympatho-excitatory response to peripheral chemoreflex stimulation, at least in anesthetized conditions. These observations raise the possibility that the contribution of A5 neurons to the chemoreflex sympathetic response may rely on either the release of non-catecholaminergic fast-acting transmitter at the level of IML, similarly to C1 neurons (Holloway et al., 2013; Abbott et al., 2014), or the activation of pre-sympathetic neurons of the RVLM (Byrum and Guyenet, 1987). Both possibilities require additional experiments to be elucidated.

CONCLUSIONS

In conclusion, we demonstrated that A5 noradrenergic neurons do not play a relevant role in the maintenance of baseline sympathetic vasomotor tonus and f_R as well as in the processing of phrenic response to hypoxia and hypercapnia. In contrast, A5 noradrenergic cells contribute to the emergence of sympathetic response to hypoxia and hypercapnia in experimental conditions free of the depressant effects of anesthesia. Our data, therefore, support the notion that A5 noradrenergic neurons are an important component of the sympathetic nervous system, recruited mainly during conditions of metabolic challenges.

Perspectives

Based upon the evidence that A5 neurons are activated during hypoxia and hypercapnia, and by the fact that brainstem noradrenergic neurons are an important component of wake-promoting system and contribute to increase sympathetic tone upon awakening (Li and Nattie, 2006; Burke et al., 2015; Bellesi et al., 2016), additional experiments will be important to investigate the

involvement of A5 noradrenergic cells in the arousal responses linked to chemoreflex activation. Moreover, previous studies have evidenced that severe loss of A5 neurons in the A5 area contributes to the manifestation of autonomic disorder associated with the neurodegenerative disease of multiple system atrophy (Benarroch et al., 2008). These observations open the possibility that changes in the functioning of A5 neurons may underpin the development of augmented sympathetic levels, especially in pathological conditions associated with hyperactivity of peripheral chemoreceptors, such as obstructive sleep apnea (Narkiewicz et al., 1998), heart failure (Toledo et al., 2017) and neurogenic hypertension (Pijacka et al., 2016).

CONFLICT OF INTERESTS

Nothing to disclose.

AUTHOR CONTRIBUTION

CLT, TSM, ACT, KB, LHG and DBZ designed the research. CLT, LHG and DBZ performed the experiments, analyzed and interpreted the data. All authors provided critical and intellectual inputs during the preparation of the manuscript and approved the final version.

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