*Neuroscience* 351 (2017) 1–14

# DEPLETION OF ROSTRAL VENTROLATERAL MEDULLARY CATECHOLAMINERGIC NEURONS IMPAIRS THE HYPOXIC VENTILATORY RESPONSE IN CONSCIOUS RATS

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**Abstract—The stimuli that commonly activate the catecholaminergic C1 neurons (nociception, hypotension, and hypoxia) also increase breathing. Pharmacogenetic evidence suggests that catecholaminergic neurons regulate breathing. Therefore, we evaluated whether the loss of C1 cells affects cardiorespiratory control during resting, hypoxic (8% $O_2$) and hypercapnic (7% $CO_2$) conditions. A bilateral injection of the immunotoxin anti-dopamine β-hydroxylase-saporin (anti-DβH-SAP; 2.4 ng/100 nl) or saline was performed in adult male Wistar rats (270–300 g, *N* = 5–8/group). Histology revealed a 60–75% loss of C1 neurons in anti-DβH-SAP-treated rats, but no significant changes or C1 cell loss was observed in sham-treated rats or those with off-target injection sites. Bilateral depletion of C1 neurons did not alter cardiorespiratory variables during rest and hypercapnia (7% $CO_2$), but it did affect the response to hypoxia. Specifically, the increase in ventilation, the number of sighs, and the tachycardia were reduced, but unexpectedly, the mean arterial pressure increased during hypoxia (8% $O_2$). The present study indicates that C1 neurons contribute to cardiorespiratory control during hypoxia rather than at rest or during hypercapnia.** 

**Key words: breathing, hypoxia, C1 cells.**

## INTRODUCTION

Several brainstem nuclei could be the catecholaminergic source for respiratory modulation. For example, A6 noradrenergic neurons modulate the hypercapnic ventilatory response, project to the pre-Bötzinger complex (preBötC) and regulate respiratory frequency (Hilaire et al., 2004; Biancardi et al., 2008; Viemari et al., 2011). The A5 neurons regulate sympathetic outflow and are involved in inspiratory activity during chemoreflex activation (Koshiya and Guyenet, 1994; Taxini et al., 2011). In addition, the A7, A1/C1, and A2/C2 can also be involved in the control of breathing (Li et al., 2008; Abbott et al., 2012; Bruinstroop et al., 2012; Oliveira et al., 2016). Together, these studies support the hypothesis that catecholaminergic neurons are important modulators of cardiorespiratory function (St-John and Leiter, 2008; Viemari et al., 2011).

The catecholaminergic C1 neurons, located in the rostral aspect of the ventrolateral medulla (RVLM), are well-organized in a viscerotopic manner, and most are involved in the control of sympathetic outflow by releasing glutamate as a neurotransmitter (Guyenet et al., 2013). These neurons are strongly activated by hypoglycemia, glucoprivic responses, inflammation, nociception and hypoxia (Koshiya et al., 1993; Reis et al., 1994; Erickson and Millhorn, 1994; Sun and Reis, 1996; Hirooka et al., 1997). A large proportion of C1 neurons express Fos in conscious mammals exposed to hypoxia (Erickson and Millhorn, 1994; Hirooka et al., 1997), and the sympathetic activation elicited by the hypoxia stimulus is severely depressed after selective ablation of the C1 neurons (Schreihofer and Guyenet, 2000; Wenker et al., 2013). Recently, it was demonstrated that optogenetic activation of C1 cells produces an intense increase in breathing rate and amplitude in anesthetized and conscious mammals (Abbott et al., 2012, 2013; Burke et al., 2014). The C1 neurons project to the forebrain, hindbrain, spinal cord, and to several regions involved in cardiorespiratory control, including the ventral respiratory column (Hökfelt et al., 1984; Berridge and Waterhouse, 2003; Guyenet et al., 2013; Burke et al., 2014; Stornetta et al., 2015; Kang et al., 2016).

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*Abbreviations:* ANOVA, analysis of variance; Anti-DH-SAP, saporin conjugate-dopamine beta hydroxylase; AP, arterial pressure; AUC, area under curve; BötC, Bötzinger complex; CNS, central nervous system; $CO_2$, carbon dioxide; DAP, diastolic arterial pressure; E1, passive expiration; E2, active expiration; $f_R$, Respiratory frequency; GFAP, glial fibrillary acidic protein; HF, high frequency; HR, heart rate; I, inspiration; I.P., intraperitoneal (small capitals); LF, Low frequency; MAP, mean arterial pressure; NTS, nucleus of the solitary tract; $N_2$, Nitrogen; $O_2$, oxygen; PI, pulse interval; PBS, phosphate-buffered saline; preBötC, pre-Bötzinger complex; RTN, retrotrapezoid nucleus; RVLM, rostral aspect of ventrolateral medulla; SAP, systolic arterial pressure; SEM, standard error of the mean; TH, Tyrosine hydroxylase; TrpOH, Tryptophan hydroxylase; $T_E$, Expiratory time; $T_I$, Inspiratory time; $V_E$, ventilation; VRC, ventral respiratory column; $V_T$, tidal volume.

http://dx.doi.org/10.1016/j.neuroscience.2017.03.031

Given these findings, we hypothesized that loss-of-function experiments specifically ablating C1 neurons would substantiate their role in not only cardiovascular but also respiratory control during resting, hypoxic and hypercapnic conditions. Thus, the goal of this study is to further examine the hypothesis that C1 cells are involved in the control of breathing under hypoxic (8% $O_2$) and hypercapnic (7% $CO_2$) conditions in conscious rats.

## EXPERIMENTAL PROCEDURES

### Animals

Animal care and experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of São Paulo (protocol number: 07/2014). All experiments were conducted using adult male Wistar rats weighing 270–300 g at the time of surgery.

### Immunotoxin lesions

Surgical procedures were performed on rats anesthetized with an intraperitoneal (ip) injection of a mixture of ketamine and xylazine (100 and 7 mg/kg of body weight, respectively). Postsurgical protection against infection included intramuscular injections of two antibiotics (benzylpenicillin, 160,000 U/kg, and dihydrostreptomycin, 33.3 mg/kg). For selective chemical lesions of C1 cells, the rats were fixed to a stereotaxic frame and the coordinates used to locate the RVLM (−2.8 mm from lambda; ±1.8 mm from midline; −8.4 mm from the skull surface) were based on the stereotaxic atlas for rats (Paxinos and Watson, 1998). The tip of a pipette, connected to a Hamilton syringe, was inserted directly into the RVLM for bilateral injections of saporin conjugate-dopamine beta hydroxylase (Anti-DβH-SAP; Advanced Targeting Systems, San Diego, CA) (2.4 ng in 100 nl of saline per side; $N = 8$) containing 1% fluorescent latex microbeads (Lumafluor, New City, NY, USA). Based on a previous publication from our laboratory and the present study, we did not notice any differences in neuroanatomical or physiological experiments in animals that received IgG-saporin or saline in the C1 region (Taxini et al., 2011, present results). Thus, in the present study, the sham-operated rats were injected with saline (0.15 M; $N = 5$).

After surgery, the animals were kept in recovery for 2 weeks before they were used in physiological experiments. The dose of anti-DβH-SAP used in the present study was selected based on previous experiments investigating the cardiovascular and sympathetic effects of anti-DβH-SAP administration into the RVLM region (Schreihofer and Guyenet, 2000; Wenker et al., 2013; Barna et al., 2016).

### Determination of pulmonary ventilation

The ventilatory response was assessed using barometric, unrestrained whole-body plethysmography (EMKA Technologies, France). Freely moving rats were kept in a 5-L plethysmography chamber with room air for 45–60 min before the ventilatory parameters were recorded. The plethysmography chamber was continuously flushed with 1.5 L/min and regulated by computer-driven mass flow controllers for $O_2$, $N_2$, and $CO_2$ (Alicat Scientific, Inc., Tucson, AZ, USA). The flow controllers were adjusted to 21% $O_2$ balanced with $N_2$ in the normoxia condition (N), 8% $O_2$ balanced with $N_2$ in the hypoxia condition (8% $O_2$) and 7% $CO_2$, 21% $O_2$ and 72% $N_2$ in the hypercapnia condition (7% $CO_2$). The ambient temperature (23–26 °C) and humidity (50–60%) were continuously recorded inside the plethysmography chamber and used to calculate the tidal volume. Rectal temperature was used as a core body temperature index. Rectal temperature was measured twice: before and at the end of the experiments. The values were averaged. The ventilatory parameters measured by the plethysmography system included respiratory frequency ($f_R$, bpm), inspiratory time ($T_I$, sec), expiratory time ($T_E$, sec), tidal volume ($V_T$, ml/kg), and ventilation ($V_E$, ml/min/kg).

### Arterial blood pressure recording

In a second set of experiments approximately 3 days after the ventilatory measurements, we recorded blood pressure in unanesthetized freely moving rats through a chronically indwelling catheter. To instrument the rats, they were anesthetized as before and a catheter made from polyethylene tubing (PE-10 connected to PE-50; Clay Adams, Parsippany, NJ, USA) was inserted into the descending aorta through a femoral artery. The other end of the catheter was tunneled subcutaneously and emerged on the rat's back for access. The rats were given a 24-h recovery period at which time the rats resumed normal food and water consumption with no impairment of motor activity or locomotion and no apparent differences in reactivity during handling. Mean, systolic and diastolic arterial pressures (MAP, SAP, and DAP; mmHg, respectively), pulse interval (PI; ms) and heart rate (HR; bpm) were measured from the AP recording using LabChart 8.0 (model Powerlab 8SP ADInstruments). The mean and standard deviation of PI and SAP were calculated for each exposure. The double product (SAP × HR) was calculated and used as the heart work index (Darrah and Engen, 1982; Joannides et al., 1998). Systolic blood pressure and heart rate variabilities and power spectral analyses of SAP and HR were performed as previously described (da Palma et al., 2016). Briefly, the recorded AP signal was sampled at 4 kHz. The complete 10-min time series of PI and SAP were cubic-spline-interpolated (250 Hz) and decimated to be equally spaced in time. Following linear trend removal, power spectral density was obtained using Fast Fourier Transformation. The spectral power components for low- (LF, from 0.20 to 0.75 Hz) and high-frequency (HF, > 0.75 Hz) bands were obtained through power spectrum density integration within each frequency bandwidth (Cardioseries v.2.2, São Paulo, SP, Brazil). These bandwidths were previously used to analyze the spectrum of blood pressure and HR variability in rats (da Palma et al., 2016).