Respiratory Physiology & Neurobiology 227 (2016) 9–22

Contents lists available at ScienceDirect

Respiratory Physiology & Neurobiology

journal homepage: www.elsevier.com/locate/resphysiol

Neuroanatomical and physiological evidence that the retrotrapezoid nucleus/parafacial region regulates expiration in adult rats

Josiane N. Silva [a], Fabiola M. Tanabe [a], Thiago S. Moreira [b], Ana C. Takakura [a,*]

[a] Department of Pharmacology, Institute of Biomedical Sciences, University of São Paulo (USP), 05508-000 São Paulo, SP, Brazil
[b] Department of Physiology and Biophysics, Institute of Biomedical Science, University of São Paulo (USP), 05508-000 São Paulo, SP, Brazil

ARTICLE INFO

Article history:
Received 12 August 2015
Received in revised form 11 February 2016
Accepted 11 February 2016
Available online 18 February 2016

Keywords:
Retrotrapezoid nucleus
Active expiration
Caudal ventral respiratory group
Breathing
Chemoreflex

ABSTRACT

The rostroventrolateral medulla contains two functional neuronal populations: (1) the parafacial respiratory group (pFRG) neurons and (2) the chemosensitive retrotrapezoid nucleus (RTN) neurons. Using anatomical and physiological techniques, we investigated the role of the RTN/pFRG in CO_2-induced active expiration (AE) in urethane-anesthetized rats. Anterograde tracing using biotinylated dextran amine (BDA) revealed dense neuronal projections emanating from the RTN/pFRG to the caudal ventral respiratory group (cVRG), 60% of which contained vesicular glutamate transporter-2. The minority (16%) of the RTN projections to the cVRG emanated from Phox2b positive neurons. Hypercapnia (10% CO_2) increased Dia_{EMG} and elicited Abd_{EMG} activity. Bilateral injections of muscimol (2 mM) into the RTN/pFRG reduced the activation of Dia_{EMG} ($23 \pm 4\%$) and abolished AE-induced by chemoreflex stimulation. Taken together, these results support the presence of direct excitatory projections from RTN/pFRG neurons to cVRG expiratory premotor neurons, playing a role in the generation/modulation of AE.

1. Introduction

The retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG) is a heterogeneous group of neurons that surround the facial motor nucleus, with at least two functional neuronal populations that may overlap, the presumptive rhythmogenic pFRG neurons and the chemosensitive RTN neurons (Marina et al., 2010; Abbott et al., 2011; Pagliardini et al., 2011).

The chemosensitive RTN contains approximately 2000 neurons in rat that have a well-defined phenotype, characterized by the presence of vesicular glutamate transporter 2 (VGLUT2, *Slc17a6*) mRNA, Phox2b-immunoreactivity, and the absence of both tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT) immunoreactivities (Stornetta et al., 2006). $Phox2b^+/TH^-$ neurons comprise one of the major brainstem regions that integrate chemosensory information in anesthetized and unanesthetized animals (Nattie and Li, 2002; Takakura et al., 2014; Abbott et al., 2009, 2013; Marina et al., 2010). Recent evidence suggests that the selective activation of $Phox2b^+/TH^-$ neurons in the RTN elicits an increase in inspiratory activity and active expiration (AE) (Marina et al., 2010; Abbott et al., 2011). However, other studies argue that a different population of neurons in the pFRG region are involved in the generation of AE (Pagliardini et al., 2011; Tupal et al., 2014; Huckstepp et al., 2015). Although neurons with expiratory properties were reported to be present in *in situ* RTN/Phox2B preparations (Abdala et al., 2009; Moraes et al., 2012), the *in vivo* role of chemosensitive RTN/Phox2B neurons in late expiratory activity of anesthetized adult rats is largely unexplored (Marina et al., 2010; Abbott et al., 2013; Huckstepp et al., 2015).

Based on the information described above, our main objective in the present study is to use standard anatomical and physiological technologies to functionally analyze the role of the RTN/pFRG, which contains key elements of the central pattern generator for breathing, on the chemosensory control of AE.

2. Methods

2.1. Animals

The experiments were performed with a total of 20 male Wistar rats, weighing 250–400 g ($N = 12$ rats for the anatomical experiments; $N = 8$ for the physiological experiments). The procedures were done in accordance with guidelines approved by the University of São Paulo Animal Care and Use Committee.

2.1.1. Physiological experiments

Surgery and anesthesia. The surgical procedures and experimental protocols were similar to those described previously

* Corresponding author at: Department of Pharmacology, Institute of Biomedical Science, University of São Paulo, 1524, Prof. Lineu Prestes Avenue, 05508-900 São Paulo, SP, Brazil. Fax: +55 11 3091 7285.
E-mail address: takakura@icb.usp.br (A.C. Takakura).

http://dx.doi.org/10.1016/j.resp.2016.02.005
1569-9048/

(Takakura et al., 2006, 2011; Takakura and Moreira, 2011). Briefly, general anesthesia was induced with 5% isoflurane in 100% O_2. A tracheostomy was made, and the isoflurane concentration was reduced to 1.4–1.5% until the end of surgery. The femoral artery was cannulated (polyethylene tubing; 0.6 mm outer diameter, 0.3 mm inner diameter; Scientific Commodities, Lake Havasu City, AZ, USA) for arterial pressure (AP) measurement. The femoral vein was also cannulated for the administration of fluids and drugs. The occipital plate was removed, and a micropipette was placed in the brainstem using a dorsal transcerebellar approach for the microinjection of drugs. The right diaphragm and transverse abdominal muscles were dissected, and a recording electrode was inserted. To prevent any influence of artificial ventilation on breathing parameters, the vagus nerve was cut bilaterally as described previously (Takakura et al., 2006, 2011).

Upon completion of the surgical procedures, isoflurane was replaced with urethane (1.2 g/kg, slowly administered intravenously). In the physiological experiments, all rats were ventilated with 100% O_2 throughout the experiment. Rectal temperature was maintained at 37 °C. End-tidal CO_2 was monitored throughout each experiment with a capnometer (CWE, Ardmore, PA, USA) that was calibrated twice per experiment with a calibrated CO_2/N_2 mixture. This instrument provided a reading of <0.1% CO_2 during inspiration in animals that breathed 100% O_2 and provided an asymptotic, nearly horizontal reading during expiration. The adequacy of anaesthesia was monitored during a 20 min stabilization period by testing for the absence of withdrawal responses, pressor responses, and changes in diaphragm activity in response to a firm toe pinch. Approximately hourly supplements of one-third of the initial dose of urethane were needed to satisfy these criteria throughout the recording period (2–3 h).

In vivo recordings of physiological variables and muscle activity. As previously described (Moraes et al., 2013), AP, diaphragm (Dia_{EMG}) and abdominal (Abd_{EMG}) muscle activity, and end-expiratory CO_2 ($etCO_2$) were digitized with a micro1401 (Cambridge Electronic Design, Cambridge, UK), stored on a computer, and processed off-line with Spike 2 v.6 software (Cambridge Electronic Design, Cambridge, UK). Integrated diaphragm activity ($\int Dia_{EMG}$) and integrated abdominal activity ($\int Abd_{EMG}$) were obtained after rectifying and smoothing ($\tau = 0.03$) the original signal, which was acquired with a 30–300 Hz bandpass filter. Muscle activity was rectified, averaged over 1-s intervals, and stored on a hard disk for subsequent analysis. A direct physiological comparison of the absolute level of muscle activity across muscles was not possible because of the absence of any physiological factors (*e.g.*, size of muscle bundle) and ambiguity in interpreting the way in which a given increase in voltage in one muscle is related to an increase in voltage in another muscle. Thus, all muscle activity was defined as their baseline physiological state just prior to their activation. This activity was normalized to 100%, and the percent change was used to compare the magnitude of increases or decreases across muscles from these physiological baselines. Dia_{EMG} amplitude (Dia_{EMG} amp) and Dia_{EMG} frequency (Dia_{EMG} freq) were normalized in each experiment by assigning to each of the two variables a value of 100 at saturation of the chemoreflex (high CO_2) and a value of 0 to periods of apnea. Before starting the experiment, ventilation was adjusted to lower end-expiratory CO_2 to 4% at steady state (60–80 cycles/s; tidal volume, 1.2–1.4 ml/100 g). These conditions were selected because 4% end-expiratory CO_2 was typically below the firing threshold of active expiration. Variable amounts of pure CO_2 were then added to the breathing mixture to activate central chemoreceptors and adjust end-expiratory CO_2 to the desired level (10%). Potassium cyanide (KCN; 40 μg/0.1 ml, i.v.) was used to activate peripheral chemoreceptors because *in vivo* responses to KCN are robust, reversible, and blocked by carotid body denervation (Takakura et al., 2006). Central and peripheral chemoreceptors stimulations were done in the same animal in a randomized sequence.

Intraparenchymal injections. All of the drugs were purchased from Sigma unless otherwise indicated. The γ-aminobutyric acid-A ($GABA_A$) agonist muscimol was diluted in 2 mM of sterile saline (pH 7.4) and injected into the RTN/pFRG using single-barrel glass pipettes (25 μm tip diameter) that were connected to a pressure injector (Picospritzer III, Parker Hannifin, Cleveland, OH, USA). For each injection, a volume of 30 nl was delivered over 5 s. Injections in the RTN/pFRG region were made according to the following coordinates: 2.6–2.8 mm caudal to lambda, 1.6–1.9 mm lateral to midline, and 8.5–8.8 mm below the dorsal surface of the cerebellum. The second injection was made 1–2 min later at the same level on the contralateral side. We included a 2% dilution of fluorescent latex microbeads (Lumafluor, New York, NY, USA) with all drug applications to mark the injection sites and verify spread of the injections.

2.1.2. Anatomical experiments

Tracer injections were made while the rats were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (7 mg/kg) administered intraperitoneally (i.p.). Surgery used standard aseptic methods. After surgery, the rats were treated with the antibiotic ampicillin (100 mg/kg, intramuscular [i.m.]) and analgesic ketorolac (0.6 mg/kg, subcutaneous [s.c.]). A group of four rats received injections (30–50 nl) of the anterograde tracer biotinylated dextran amine (BDA; lysine-fixable, MW 10000; 10% w/v in 10 mM phosphate buffer, pH 7.4; Molecular Probes, Eugene, OR, USA) in the RTN/pFRG. These injections were made 2.6–2.8 mm caudal to lambda, 1.6–1.9 mm lateral to midline, and 8.5–8.8 mm below the dorsal surface of the brainstem. These rats were allowed to survive seven to ten days, after which they were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and transcardially perfused with fixative as described below. Another group of eight rats received an injection of 2% FluorGold (FG) in sterile water in the left cVRG to retrogradely label RTN/pFRG neurons that innervate the cVRG. These injections were made according to the following coordinates: 0 mm caudal to the calamus scriptorius, 1.3–1.6 mm lateral to midline, and 1.6–2.0 mm below the dorsal surface of the brainstem. Seven to ten days following the FG application, four rats were exposed for 3 h to a hypercapnia breathing mixture (7% CO_2, 21% O_2, balanced with N_2) in a small flow-through environmental chamber. The remainder of the rats ($N = 4$) were exposed to room air under the same conditions. Following exposure to hypercapnia or normoxia, the animals were anesthetized with sodium pentobarbital and immediately perfusion-fixed.

2.1.3. Histology

The rats were deeply anaesthetized with 60 mg/kg sodium pentobarbital (i.p.), injected with heparin (500 U, intracardially), and perfused through the ascending aorta with 250 ml phosphate-buffered saline (pH 7.4) followed by 4% phosphate-buffered paraformaldehyde (0.1 M, pH 7.4; Electron Microscopy Sciences, Fort Washington, PA, USA). The brain was removed and stored in the perfusion fixative for 24–48 h at 4 °C. A series of coronal sections (40 μm) from the brain were cut using a vibrating microtome and stored in cryoprotectant solution at −20 °C for up to 2 weeks (20% glycerol plus 30% ethylene glycol in 50 mM phosphate buffer, pH 7.4) until histological processing. All of the histochemical procedures were performed using free-floating sections according to previously described protocols (Rosin et al., 2006; Barna et al., 2014). BDA was detected using either streptavidin-Cy3 (1:200; 60 min; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or avidin-Alexa 488 (1:200; 60 min; Molecular Probes, Eugene, OR, USA; Takakura et al., 2006). Vesicular glutamate transporter 2 (VGLUT2, *Slc17a6*) and glutamic acid decarboxylase 65/67 (GAD65/67) were detected by immunofluorescence using a guinea-