Respiratory Physiology & Neurobiology 250 (2018) 14–18

Contents lists available at ScienceDirect

Respiratory Physiology & Neurobiology

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

Short communication

Expression of the transcription factor FOXP2 in brainstem respiratory circuits of adult rat is restricted to upper-airway pre-motor areas

Davor Stanić*, Rishi R. Dhingra, Mathias Dutschmann*

Systems Neurophysiology Division, Florey Institute of Neuroscience and Mental Health, University of Melbourne, Parkville, Victoria 3010, Australia

ARTICLE INFO

Keywords:
Brainstem vocalization
Voice
Postinspiration
Motor control
Voice
Brainstem
Laryngeal adductor
Immunohistochemistry
Parabrachial

ABSTRACT

Expression of the transcription factor FOXP2 is linked to brain circuits that control motor function and speech. Investigation of FOXP2 protein expression in respiratory areas of the ponto-medullary brainstem of adult rat revealed distinct rostro-caudal expression gradients. A high density of FOXP2 immunoreactive nuclei was observed within the rostral pontine Kölliker-Fuse nucleus, compared to low densities in caudal pontine and rostral medullary respiratory nuclei, including the: (i) noradrenergic A5 and parafacial respiratory groups; (ii) Bötzinger and pre-Bötzinger complex and; (iii) rostral ventral respiratory group. Moderate densities of FOXP2 immunoreactive nuclei were observed in the caudal ventral respiratory group and the nucleus retroambiguus, with significant density levels found in the caudal half of the dorsal respiratory group and the hypoglossal pre-motor area lateral around calamus scriptorius. FOXP2 immunoreactivity was absent in all cranial nerve motor nuclei. We conclude that FOXP2 expression in respiratory brainstem areas selectively delineates laryngeal and hypoglossal pre-motor neuron populations essential for the generation of sound and voice.

1. Introduction

Forkhead box protein P2 (FOXP2) is a transcription factor required for brain and lung development and is closely associated with the generation of speech in humans and bird song, and ultrasonic echolocation in bats (Enard, 2011). FOXP2 in the rodent forebrain (Campbell et al., 2009) is enriched within areas involved in vocalization (e.g. cortex, amygdala, nucleus accumbens) and fine-tuning of voluntary motor action (e.g. striatum, substantia nigra, olivo-cerebellar system). In addition, selective autonomic brainstem areas contain FOXP2 immunoreactivity, particularly the parabrachial complex (Miller et al., 2015). FOXP2-expressing neurons in the lateral parabrachial nuclei are associated with salt and fluid homeostasis (Miller et al., 2015) and also with thermoregulation (Geerling et al., 2016). Ventral to the lateral parabrachial nuclei, FOXP2 is marker for the neonatal pontine Kölliker-Fuse nucleus (Gray, 2008) and remains densely expressed in the KF of adult rats and mice (Geerling et al., 2017). Moreover, these FOXP2-expressing KF neurons are predominantly glutamatergic and project to nuclei of the lateral respiratory column located in the ponto-medullary brainstem (Geerling et al., 2017).

The KF is an integral part of the brainstem respiratory central pattern generator (rCPG) and serves the gating of the postinspiratory phase of the respiratory motorcycle (Dutschmann and Herbert, 2006). The respiratory motor output for the postinspiratory phase is expressed at the level of the laryngeal adductors to valve and modulate expiratory airflow (see Dutschmann and Dick, 2012) and importantly, laryngeal adductor function is critical for voice production (see Dutschmann et al., 2014).

We quantitatively investigated the profile of FOXP2 protein expression across the lateral respiratory column to elucidate respiratory FOPX2 circuitry within the rCPG. We observed that the density of FOXP2 immunoreactivity in the lateral respiratory column is highest in the pontine KF, but significant expression was also found in the most caudal aspect of the rCPG, such as the nucleus retro-ambiguus, the caudal part of dorsal respiratory group and hypoglossal pre-motor area lateral to the hypoglossal motor nucleus. Low or absent FOXP2 immunoreactive cells were found in other parts of the rCPG, including primary motor nuclei (e.g. trigeminal, facial and hypoglossal) and respiratory modulatory areas such the Raphé nuclei. Thus, FOXP2 protein expression appears to be largely restricted to laryngeal and hypoglossal pre-motor areas that are an essential component for the generation of vocalizations.

2. Material and methods

2.1. Immunohistochemistry

2.1.1. Tissue preparation

All animals (n = 4) were deeply anaesthetized using pentobarbitone sodium (Ilium Pentobarbitone, Troy Laboratories, Smithfield, NSW,

* Corresponding authors at: Florey Institute of Neuroscience and Mental Health, Gate 11 Royal Parade, University of Melbourne, VIC 3010, Australia.
E-mail addresses: davor.stanic@florey.edu.au (D. Stanić), mathias.dutschmann@florey.edu.au (M. Dutschmann).

https://doi.org/10.1016/j.resp.2018.01.014
Received 23 January 2018; Accepted 24 January 2018

Australia, 200 mg/kg i.p.) and perfused through the heart via the ascending aorta with 50 ml Ca2 + -free Tyrode's buffer (37 °C), followed by 50 ml of a mixture of 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) and 0.2% picric acid (Sigma) diluted in 0.16 M phosphate buffer (pH 6.9, 37 °C) and 250 ml of the same fixative at 4 °C, the latter for approximately 5 min. The brains were dissected out and postfixed in the same fixative for 90 min at 4 °C, and finally immersed for 72 h at 4 °C in 10% sucrose dissolved in 0.1 M phosphate buffer (PBS, pH 7.4) containing 0.01% sodium azide (Sigma) and 0.02% bacitracin (Sigma), before rapid freezing by CO2. Sections were cut using a cryostat (Leica CM1850, Wetzlar, Germany) at a thickness of 40 µm, and stored in a cyroprotectant solution [30% v/v ethyleneglycol (Merck); 15% w/v sucrose; 35% v/v 0.1 M phosphate buffer; 35% v/v distilled H2O], at −20 °C.

2.1.2. Incubation protocol

Sections were dipped in 0.01 M PBS, washed with 0.03% H_2O_2 in PBS for 30 min followed by PBS alone (3× in 20 min), and incubated for 24 h at room temperature (RT) with a sheep anti-FoxP2 antibody (1 ug/10 ml; R&D Systems Inc, MN, USA) diluted in PBS containing 0.3% Triton X-100 and 0.5% BSA. To visualize the immunoreactivity, sections were processed using a commercial kit (Metal Enhanced DAB Substrate Kit, Thermo Scientific, IL, USA, Product # 34065). Briefly, sections were washed in PBS (3× in 20 min), incubated with PBS containing 5% normal donkey serum (Merck Millipore, MA, USA) for 1 h, and incubated for 1 h with a biotinylated donkey anti-sheep conjugate (Jackson ImmunoResearch Laboratories, PA, USA) diluted 1:500 in PBS and 1% normal donkey serum. Sections were then washed in PBS and incubated in a streptavidin-horseradish peroxidase conjugate (Jackson) diluted 1:500 in PBS, followed by PBS washes and incubation in DAB metal concentrate (Thermo Scientific, Product # 1856090) diluted in Peroxide Substrate Buffer (1:10; Thermo Scientific, Product # 1855910) for 30 min. Sections were then washed in PBS, mounted onto slides coated with 0.5% gelatin- (Sigma) and 0.05% chromium(III) potassium sulphate dodecahydrate (Merck), and coverslipped using DPX (Merck).

2.1.3. Primary antisera used

Sheep anti-FoxP2 polyclonal antibody was raised against purified *E. coli*-derived recombinant human FoxP2 isoform 1 (rhFoxP2; aa 640–715). Western blot analysis of lysates from a A172 human glioblastoma cell line and human lung tissue revealed a band of approximately 90 kDa (R&D Systems Inc). The staining patterns of this antibody were consistent with those reported previously in rat (Geerling et al., 2017; Miller et al., 2015).

2.2. Image processing

Sections were examined using a Leica DM LB2 microscope (Leica, Wetzlar, Germany), equipped with objective lenses of ×10 (N.A. 0.45) and ×60 oil (N.A. 1.40). Photographs were taken using a Hamamatsu ORCA-R2 digital camera attached to the microscope, using Hamamatsu HCImage-Live software (Hamamatsu Photonics K.K., Hamamatsu City, Japan). Digital images from the microscopy were slightly modified to optimise for image resolution, brightness and contrast using Adobe Photoshop CS6, version 13, software (Adobe Systems Inc., San Jose, CA), to best represent the immunohistochemistry observed at the microscope.

2.3. Data analysis

Quantitative analysis (n = 4 animals) of the expression profile of FOXP2-immunoreactive (IR) cell nuclei was performed on the ponto-medullary lateral respiratory column (LRC) and the hypoglossal premotor area later the XII motor nuclei in the caudal medulla oblongata. The number of FoxP2-IR cell nuclei within the boundaries of the LRC were obtained from every fourth 40 µm-thick section, each 160 µm apart, starting from the transition of cervical spinal cord and medulla oblongata (Bregma −16 mm, Paxinos and Watson, 2007) until the commencement of the superior colliculi at the brainstem-midbrain transition (Bregma −8 mm). In addition, cell counts for the caudal dorsal respiratory group (Jones et al., 2016) and hypoglossal pre-motor area were plotted across the caudal medulla. Initial analysis revealed no laterality of FOXP2 expression and, thus, cell counts were restricted to the unilateral LRC, caudal dorsal respiratory group and hypoglossal pre-motor area. Counts were made using a fractionator sampling design (Stanic et al., 2003), with regular predetermined intervals (x = 150 µm, y = 150 µm) and counting frame dimensions (150 × 150 = 22,500 μm^2) derived by means of a grid program (Stereoinvestigator v.7.0, MicroBrightField, Williston, VT, viewed through a microscope and × 60 oil objective, Leica). The number of FoxP2-ir cell nuclei per section was obtained by multiplying the number of cell bodies counted within the sampling regions with reciprocals of the fraction of sectional area sampled (in this case 1). Values are expressed as the mean ± SEM (standard error) (Fig. 1).

The density of FOXP2-IR nuclei in additional (i) respiratory-related brain areas such as the nucleus of the solitary tract, raphe nuclei or respiratory motor nuclei, and (ii) non-respiratory brainstem and cerebellar areas, was assessed using semi-quantitative comparative analysis (Table 1) on a five point scale: (0) absence of labelled terminals; (±) very low density; (+) low density; (++) moderate density; and (+++) high density.

3. Results

Unilateral cell counts revealed a high density of FOXP2-IR nuclei within the rostral pontine KF of the LRC, and comparatively low densities in other regions of the LRC, except for the moderate density levels observed in the caudal ventral respiratory group. FOXP2 immunoreactivity was restricted to cell nuclei throughout all brainstem areas analysed in the present study.

3.1. FOXP2 immunoreactivity in the LRC

The highest number of FOXP2-IR nuclei within the ponto-medullary LRC of the adult rat was found in the KF, where intermediate regions of the KF (Bregma −8.8 mm) contained 324 ± 64 FOXP2-IR neurons per section (Fig. 1A and D). The number of FOXP2-IR neurons was reduced in caudal pontine regions of the LRC, such as the intertrigeminal area (Bregma −9.5 mm) and A5 (Bregma −10.2 mm), in which 46 ± 11 and 25 ± 2 neurons per section were quantified, respectively. A gradual decrease in the number of FOXP2-IR neurons was observed more caudally in critical rostral medullary respiratory nuclei such as the Bötzinger complex (Bregma −12.2 mm) and pre-Bötzinger complex (Bregma −12.6 mm), in which 13 ± 1 and 26 ± 2 neurons per section were quantified, respectively (Fig. 1A and C). Towards the caudal end of the LRC, the number of FOXP2-IR neurons increased slightly in the caudal ventral respiratory group (48 ± 3 neurons per section, Bregma −13.8 mm), and remained low in the nucleus retroambiguus (11 ± 2 neurons per section, Bregma −15.5 mm).

3.2. FOXP2 immunoreactivity in dorsal respiratory areas of the medulla oblongata

Outside the LRC an abundant level of FOXP2-IR neurons was found in the dorsolateral medulla oblongata overlapping with hypoglossal premotor area (see Gestreau et al., 2005; and Fig. 1A and B). The most pronounced levels of FOXP2-IR neurons in the dorsal medullary region was observed just caudal to the level of calamus scriptorius (Bregma −14.7 mm), where 66 ± 16 neurons per section were quantified (Fig. 1A and B). Numbers of FOXP2-IR neurons declined (7 ± 1 neurons per section) in the rostral dorsolateral medulla rostral to area postrema (Bregma −13 mm), but remained robust (44 ± 6 neurons