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ANATOMICAL AND FUNCTIONAL PATHWAYS OF RHYTHMOGENIC INSPIRATORY PREMOTOR INFORMATION FLOW ORIGINATING IN THE PRE-BÖTZINGER COMPLEX IN THE RAT MEDULLA

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Abstract—The pre-Bötzinger complex (preBötC) of the ventrolateral medulla is the kernel for inspiratory rhythm generation. However, it is not fully understood how inspiratory neural activity is generated in the preBötC and propagates to other medullary regions. We analyzed the detailed anatomical connectivity to and from the preBötC and functional aspects of the inspiratory information propagation from the preBötC on the transverse plane of the medulla oblongata. Tract-tracing with immunohistochemistry in young adult rats demonstrated that neurokinin-1 receptor- and somatostatin-immunoreactive neurons in the preBötC, which could be involved in respiratory rhythmogenesis, are embedded in the plexus of axons originating in the contralateral preBötC. By voltage-imaging in rhythmically active slices of neonatal rats, we analyzed origination and propagation of inspiratory neural activity as depolarizing wave dynamics on the entire transverse plane as well as within the preBötC. Novel combination of pharmacological blockade of glutamatergic transmission and mathematical subtraction of the video images under blockade from the control images enabled to extract glutamatergic signal propagations. By ultra-high-speed voltage-imaging we first demonstrated the inter-preBötC conduction process of inspiratory action potentials. Intra-preBötC imaging with high spatiotemporal resolution during a single spontaneous inspiratory cycle unveiled deterministic nonlinearities, i.e., chaos, in the population recruitment. Collectively, we comprehensively elucidated the anatomical pathways to and from the preBötC and dynamics of inspiratory neural information propagation: (1) From the preBötC in one side to the contralateral preBötC, which would synchronize the bilateral rhythmogenic kernels, (2) from the preBötC directly to the bilateral hypoglossal premotor and motor areas as well as to the nuclei tractus solitarius, and (3) from the hypoglossal premotor areas toward the hypoglossal motor nuclei. The coincidence of identified anatomical and functional connectivity between the preBötC and other regions in adult and neonatal rats, respectively, indicates that this fundamental connectivity is already well developed at the time of birth.

Key words: voltage-sensitive dye, tract tracing, medulla oblongata, pre-Bötzinger complex, rhythmically active slice, respiratory rhythm.

INTRODUCTION

In mammals, respiratory motor drive originates in the brainstem. In the rhythmically active slice preparation of the medulla oblongata, inspiratory neural activity is generated in the pre-Bötzinger complex (preBötC) and is thought to propagate to other respiratory-related medullary regions (Smith et al., 1991; Koshiya and Smith, 1999; Koizumi et al., 2008; Tarras-Wahlberg and Rekling, 2009). There have been a number of analyses of preBötC neurons regarding their anatomical features (e.g., Gray et al., 2001; Stornetta et al., 2003; Thoby-Brisson and Greer, 2007) and electrophysiological properties (e.g., Del Negro et al., 2002a; Thoby-Brisson et al., 2005; Kuwana et al., 2006; Pace et al., 2007; Koizumi and Smith, 2008; Zavala-Tecuapetla et al., 2008; Koizumi et al., 2010). However, anatomical pathways and functional connections from the preBötC to other respiratory-related regions have not been comprehensively understood, although there have been a few, mostly fragmental, reports of anatomical (Stornetta et al., 2003; Bouvier et al., 2010; Tan et al., 2010) and physiological studies by calcium-imaging

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Abbreviations: ABC, avidin–biotin peroxidase complex; BDA, biotinylated dextran amine; DAB, diaminobenzidine; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; *F*, fluorescence intensity; Δ*F*, change in fluorescence intensity relative to the initial intensity of fluorescence; FG, Fluoro-gold; nXII, hypoglossal motor nucleus; NK1R, neurokinin-1 receptor; nTS, nucleus tractus solitarius; PB, phosphate buffer; PBS, phosphate-buffered saline; preBötC, pre-Bötzinger complex; SST, somatostatin.

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(e.g., Koshiya and Smith, 1999; Thoby-Brisson et al., 2005; Koizumi et al., 2008) showing connections between bilateral preBötC. In order to better understand the mechanism of respiratory rhythm and pattern generation in the medulla oblongata, we aimed to thoroughly elucidate the anatomical pathways and propagating excitation wave dynamics of inspiratory neural information from the preBötC to other respiratory-related medullary regions. To analyze the distribution of neurons projecting to the preBötC region, retrograde tracing was conducted together with immunohistochemistry for neurokinin-1 receptor (NK1R) that indicates putative rhythmogenic neurons in the contralateral preBötC (Gray et al., 2001). Also, to anatomically elucidate the axonal projections from the preBötC region, anterograde tracing was conducted with immunohistochemistry for NK1R and in some rats simultaneously for somatostatin (SST) that is also a marker of putative rhythmogenic neurons in the preBötC (Tan et al., 2010; Wei et al., 2012).

Although calcium-imaging, which has been widely applied, is advantageous in simultaneous recording of multiple cell activities at a single cellular resolution, it is not suitable for detecting the dynamics of neural information flow such as action potential conductions (Berger et al., 2007). Therefore, we applied a high-speed voltage-sensitive dye imaging (voltage-imaging), which is suitable for the analysis of fast neural information propagation (Tokumasu et al., 2001; Onimaru and Homma, 2003, 2005; Berger et al., 2007; Okada et al., 2007; Oku et al., 2007; Ruangkittisakul et al., 2009; Aoyama et al., 2011). We used the rhythmically active slice preparation for voltage-imaging, because components essential to rhythm generation and representative motor output formation are exposed on the slice surface, providing an ideal configuration for imaging analysis. Here we report the comprehensively analyzed results of the anatomical and functional pathways of inspiratory premotor information flow originating in the preBötC on the transverse plane of the medulla oblongata.

EXPERIMENTAL PROCEDURES

All experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 and with the Guiding Principles for the Care and Use of Animals of the Physiological Society of Japan. Experiments for anatomical analyses and for functional imaging were approved by the animal experiment ethics committees of Shimane University (Permit Nos.: 03-34, H17-7, H19-53, H20-32 and IZ25-14) and of Keio University (Permit No.: 020062), respectively. We attest that all efforts were made to minimize the number of animals used and their suffering.

Combined retrograde tracing and immunohistochemistry

Young adult male Wistar rats (8–10 weeks old, $n = 12$) were anesthetized by intraperitoneal injection of chloral hydrate (350 mg kg^{-1}). The dorsal surface of the medulla oblongata was exposed, and a micropipette filled with a 5% solution of Fluoro-gold (FG) (Fluorochrome, Denver, CO, USA) dissolved in saline was inserted into the medulla oblongata. The micropipette tip was stereotaxically positioned in the preBötC region at coordinates 12.3–13.0 mm caudal, 2.0 mm lateral and 10.0 mm ventral to the bregma, and the accuracy of the micropipette tip positioning was confirmed in subsequent histological analyses. In each rat, a single injection of FG was made by iontophoresis. The driving current (3 μA, 400 ms, 1 Hz) was delivered for 15–20 min. After 7–10 days survival, the rats were anesthetized with a lethal dose of chloral hydrate (700 mg kg^{-1}) and perfused transcardially with 150 ml of saline followed with 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.3). After perfusion, the brains were removed and postfixed in 4% paraformaldehyde in PB for 6–16 h at 4 °C, and then saturated with a cold solution of 20% sucrose in the same buffer. Subsequently, the brains were cut serially into frontal sections of 30 μm thickness on a freezing microtome, and collected in 0.1 M phosphate buffered saline (PBS; pH7.3). The sections containing the preBötC region were washed in PBS, and incubated overnight in PBS containing 3% normal goat serum, 0.2% Triton-X, guinea pig anti-NK1R antibody (1:1000; EMD Millipore, Billerica, MA, USA) and rabbit anti-FG antibody (1:3000; EMD Millipore). Subsequently, the sections were washed in PBS, incubated in PBS containing Alexa488-conjugated anti-guinea pig IgG antibody (1:500; Invitrogen, Carlsbad, CA, USA) and Alexa633-conjugated anti-rabbit IgG (1:500; Invitrogen) for 3 h, and then mounted on gelatinized slides. Finally, the sections were observed under a fluorescent microscope (ECLIPSE, Nikon, Tokyo, Japan) as well as under a confocal laser scanning microscope (FV300, Olympus, Tokyo, Japan). Injection site of FG in the preBötC region and the distributions of FG-labeled neurons, NK1R-immunoreactive neurons and double-labeled neurons in the contralateral preBötC region were analyzed from confocal images, and these neurons were counted by a previously reported method (Wang et al., 2001) in every sixth section of a series of serial frontal sections.

Combined anterograde tracing and immunohistochemistry

Anterograde tracing from the preBötC region with immunohistochemistry for NK1R was conducted in young adult rats ($n = 14$). For anterograde tracing, biotinylated dextran amines (BDAs) (Invitrogen) were injected into the preBötC region. In each rat a single BDA injection was made by iontophoresis through a glass micropipette filled with a 10% solution of BDA dissolved in 0.01 M PB. The micropipette tip was positioned in the preBötC region as in the retrograde tracing. The driving current (5 μA, 400 ms, 1 Hz) was delivered for a period of 40–60 min. After 7–10 days of survival, the rats were anesthetized and perfused transcardially with 150 ml of saline, followed by 500 ml