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FOS-ACTIVATION OF FOXP2 AND LMX1B NEURONS IN THE PARABRACHIAL NUCLEUS EVOKED BY HYPOTENSION AND HYPERTENSION IN CONSCIOUS RATS

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Abstract—The parabrachial nucleus (PB) is a brainstem cell group that receives a strong input from the nucleus tractus solitarius regarding the physiological status of the internal organs and sends efferent projections throughout the forebrain. Since the neuroanatomical organization of the PB remains unclear, our first step was to use specific antibodies against two neural lineage transcription factors: Forkhead box protein2 (FoxP2) and LIM homeodomain transcription factor 1 beta (Lmx1b) to define the PB in adult rats. This allowed us to construct a cytoarchitectonic PB map based on the distribution of neurons that constitutively express these two transcription factors. Second, the *in situ* hybridization method combined with immunohistochemistry demonstrated that mRNA for glutamate vesicular transporter Vglut2 (Slc17a6) was present in most of the Lmx1b+ and FoxP2+ parabrachial neurons, indicating these neurons use glutamate as a transmitter. Third, conscious rats were maintained in a hypotensive or hypertensive state for 2 h, and then, their brainstems were prepared by the standard c-Fos method which is a measure of neuronal activity. Both hypotension and hypertension resulted in c-Fos activation of Lmx1b+ neurons in the external lateral-outer subdivision of the PB (PBel-outer). Hypotension, but not hypertension, caused c-Fos activity in the FoxP2+ neurons of the central lateral PB (PBcl) subnucleus. The Kölliker–Fuse nucleus as well as the lateral crescent PB and rostral-most part of the PBcl contain neurons that co-express FoxP2+ and Lmx1b+, but none of these were activated after blood pressure changes. Salt-sensitive FoxP2 neurons in the pre-locus coeruleus and PBel-inner were not c-Fos activated following blood pressure changes. In summary, the present study shows that the PBel-outer and PBcl subnuclei originate from two different neural progenitors, contain glutamatergic neurons, and are affected by blood pressure changes, with the PBel-outer reacting to both hypo- and hypertension, and the PBcl signaling only hypotensive changes.

Key words: parabrachial nucleus, blood pressure, transcription factors, FoxP2, Lmx1b.

INTRODUCTION

The brainstem neurons that regulate the cardiovascular system have been well-defined, although there are still gaps in our knowledge (Dampney, 1994; Blessing, 1997; Pilowsky and Goodchild, 2002; Guyenet, 2006; Goodchild and Moon, 2009). High pressure receptors (viz., baroreceptors) originating from the aortic arch and carotid sinus as well as low pressure receptors (viz., volume receptors) distributed in the walls of the atria and entrance sites of the great veins (viz., superior and inferior venae cavae) send afferent information via the vagus nerve into the nucleus tractus solitarius (NTS). The NTS is the first-order relay site in the brain for cardiovascular as well as other visceral information, and it functions like a clearing house by processing this incoming information and then, sending outputs to the numerous brainstem and forebrain targets for reflex adjustments and general integrative alterations of the autonomic and neuroendocrine systems (Loewy, 1990).

The NTS sends one of its strongest outputs to the parabrachial nucleus (PB) – a cell group that lies in the dorsolateral pons surrounding the brachium conjunctivum. This fiber bundle, also termed the superior cerebellar peduncle, divides the PB region into two main divisions: medial and lateral, but subsequent research divided the PB into ten subnuclei (Fulwiler and Saper, 1984). The latter nomenclature will be used here.

The NTS projection to the PB targets selective PB subnuclei in a viscerotopic fashion (Herbert et al., 1990), although some PB subnuclei receive converging inputs from multiple NTS regions. The medial and commissural NTS regions, which receive primary afferent inputs from

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Abbreviations: Atoh 1, atonal homolog 1; BP, blood pressure; CGRP, calcitonin gene-related peptide; DIG, digoxigenin; EDTA, ethylenediaminetetraacetic acid; FoxP2, Forkhead box protein 2; Lmx1b, LIM homeodomain transcription factor 1 beta; NTS, nucleus tractus solitarius; PB, parabrachial nucleus; PBcl, central lateral parabrachial nucleus; PBdl, dorsal lateral parabrachial nucleus; PBdm, dorsal medial parabrachial nucleus; PBel, external lateral parabrachial nucleus; PBem, external medial parabrachial nucleus; PBil, internal lateral parabrachial nucleus; PBlc, lateral crescent parabrachial nucleus; PBm, medial parabrachial nucleus; PBsl, superior lateral parabrachial nucleus; PBvl, ventral lateral parabrachial nucleus; PBwa, waist area of parabrachial nucleus; PCR, polymerase chain reaction; PHAL, Phaseolus vulgaris leucoagglutinin; RunX1, Runt-related transcription factor 1; TF, transcription factor; Vglut2, vesicular glutamate transporter 2; VMc, caudal ventral medial thalamic nucleus.

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the cardiovascular, gastrointestinal, and respiratory systems, project to five different PB sites: central lateral PB (PBcl), dorsal lateral PB (PBdl), PB external lateral (PBel), PB ventral lateral (PBvl), and medial PB (PBm) subnuclei (Herbert et al., 1990; Karimnamazi et al., 2002). The rostral NTS, a taste recipient region, is equally complex since it projects to four PB sites: medial PB (PBm), external medial PB (PBem), waist area of PB (PBwa), and PBvl (Herbert et al., 1990; Karimnamazi et al., 2002). The ventrolateral NTS, which subserves respiratory functions, targets three PB subnuclei: Kölliker–Fuse nucleus, PBem, and PBcl (Herbert et al., 1990). For the most part, with the exception for the PBvl, these projections are non-overlapping and suggest a pattern of viscerotopic organization within the PB, but its exact organization remains unknown.

Several c-Fos studies have described the location of PB neurons that become activated by blood pressure changes (Li and Dampney, 1994; Graham et al., 1995; Rocha and Herbert, 1996; Potts et al., 1997, 2000). For example, blood pressure sensitive PB neurons have been localized in three PB subnuclei: PBcl, PBdl, and PBel (Rocha and Herbert, 1996), but Dampney and colleagues found that only the PBel becomes activated after prolonged hypotension or hypertension in conscious rabbits (Potts et al., 1997). Some doubt, therefore, remains regarding which PB subnuclei are activated during blood pressure changes.

A detailed electrophysiological map of the PB in the rat reported that the firing rate of PBcl, PBel, and Kölliker–Fuse neurons increased during hemorrhage and remained elevated during restoration of blood volume by blood transfusion (Ward, 1989). By using multivariate regression analysis, Ward (1989) suggested that neurons found in these three sites were more likely to be responding to arterial blood pressure changes than blood volume changes. Neurons in the dorsal cap of the PBcl and caudal medial PB were selectively responsive to blood volume changes. Even though these results correlate well with the findings of Rocha and Herbert (1996), we felt it was reasonable to re-examine the issue because the Fos-activation pattern has not yet been related to the chemical nature of the individual neurons that form the PB subnuclei (see below).

A further unresolved issue is little is known about the neurotransmitters expressed in the PB neurons, although several papers have detailed the presence of neuropeptide-containing PB neurons (Uhl et al., 1979; Block and Hoffman, 1987; Herbert and Saper, 1990; Guo et al., 2005; D'Hanis et al., 2007; Westberg et al., 2009). With the exception of one report (Guo et al., 2005), nothing is known about the PB neuronal phenotypes that are involved in processing cardiovascular information. This latter study found that stimulation of the cardiac sympathetic afferents on the surface of the heart, which is similar to the sensory events that occur during myocardial infarction, resulted in Fos-activation of PBel neurons that express both glutamate and neuronal nitric oxide synthase (Guo et al., 2005).

An alternative way to examine the PB is to determine which transcription factors (TFs) that are present at embryonic and early postnatal developmental stages (Gray et al., 2004; Gray, 2008) are also constitutively expressed in the adult rat brain. The combinational pattern of TF expression within specific neurons has been proposed to define essential components of neural identity such as neurotransmitter, axonal projection pattern, and dendritic morphology. As such, differences in TF expression can be causal to functional differences between adjacent neuronal populations. Because of their relative specificity, TFs have been remarkably useful tools for defining unique subpopulations of neurons, especially within highly heterogeneous neural complexes. Here, we used the TFs as a means to identify the PB subgroups that are responsive to blood pressure changes. Earlier, we reported the localization and efferent projections of the Forkhead box protein2 (FoxP2) neurons in the PB of adult rats that are sensitive to sodium deprivation (Stein and Loewy, 2010; Geerling et al., 2011; Shin et al., 2011).

The PB complex contains neurons derived from at least two developmental progenitor populations, one expressing the proneural TF Atoh 1 (atonal homolog 1) and a second expressing the LIM homeodomain transcription factor 1 beta (Lmx1b). FoxP2 is expressed in several brainstem populations including a subset of Atoh-derived PB neurons. To differentiate these populations, here, we used another antibody to localize PB neurons that express Lmx1b. In this report, we utilized specific antibodies directed against FoxP2 and Lmx1b to define specific PB subnuclei, and then, subsequently, used these reagents in combination with the standard c-Fos method (a marker of neuronal activity) to describe the PB areas that become excited during sustained blood pressure changes.

EXPERIMENTAL PROCEDURES

General background

The surgical and experimental procedures were approved by the St. Louis University and Washington University School of Medicine Institutional Animal Care and Use Committees and followed NIH guidelines. At the end of each experiment, the rats were anesthetized with 3.5% chloral hydrate, and perfused through the heart with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4), and their brains were removed immediately and placed in fixative for 1–2 weeks.

Immunohistochemistry

Transverse sections of the pons were cut on freezing microtome at 50 μm thick, collected, and stored as a one-in-five series in 0.1 M sodium phosphate buffer (pH 7.4) solution containing 1% sodium azide. Tissues were processed with the following combinations of primary antibodies: FoxP2 & Lmx1b, c-Fos & FoxP2, c-Fos & Lmx1b, cFos & FoxP2 & Lmx1b, c-Fos & tyrosine hydroxylase & FoxP2, c-Fos & tyrosine hydroxylase & Lmx1b, Lmx1b & calcitonin gene-related peptide, and FoxP2 & dynorphin A.

The primary antibodies used in this study were as follows: rabbit anti-calcitonin gene-related peptide (1:8K; Cat. #AB 1971; Millipore, Temecula, CA, USA), rabbit anti-dynorphin A (1:6K; Cat. #H021-03; Phoenix, Burlingame, CA, USA), rabbit anti-Fos (1:10K; Cat. #PC38; Calbiochem, San Diego, CA, USA), sheep anti-FoxP2 (1:5K; Cat. #AF5647; R&D Systems, Minneapolis, MN, USA), guinea-pig anti-Lmx1b (1:2K; C. Birchmeier and T. Jessell), rabbit anti-Lmx1b (1:250 or 1:500; Y.-Q. Ding), and mouse anti-tyrosine hydroxylase (1:1K; Cat. #MAB318; Millipore, Temecula, CA, USA).