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Further characterization of the juxtaglomerular neurons in the mouse main olfactory bulb by transcription factors, Sp8 and Tbx21

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ABSTRACT

Juxtaglomerular neurons in the mouse main olfactory bulb consist of various types of neurons, especially classified by their chemical properties such as transmitter-related molecules and calcium binding proteins. In addition several transcription factors have been revealed to characterize neuronal subpopulations. In this study we examined the immunoreactivities of two transcription factors, Sp8 and Tbx21, in the juxtaglomerular neuronal subpopulations containing calretinin, calbindin, secretagogin, tyrosine hydroxylase (TH) and nitric oxide synthase (NOS). Both Sp8 and Tbx21 immunoreactivities were so diverse in their staining intensities. Almost all calretinin and secretagogin positive neurons were relatively strongly Sp8 positive, whereas none of calbindin positive neurons were Sp8 positive. TH positive neurons were also usually Sp8 positive, although some were faintly positive. These four types of interneurons were Tbx21 negative. On the other hand large faintly NOS positive external tufted cells were occasionally Tbx21 positive but always Sp8 negative, whereas small NOS positive periglomerular cells without distinctly stained dendrites were usually Sp8 positive and Tbx21 negative. Strangely, most of strongly NOS positive periglomerular cells with distinctly stained dendritic processes were Sp8 negative and Tbx21 negative. Thus Sp8 and Tbx21 immunoreactivities further characterized juxtaglomerular neurons and, especially confirmed the heterogeneity of NOS positive juxtaglomerular neurons.

1. Introduction

The main olfactory bulb (MOB), the first relay station for transmission of olfactory information, has a rather concentric arrangement of seven layers and richly varied in chemical substances. Previously we reported the chemically defined neuronal subpopulations in the juxtaglomerular region of the mouse MOB using the immunocytochemistry for transmitter- or neuroactive substance-related molecules and calcium binding proteins (Kosaka and Kosaka, 2007a). Other group also reported chemically defined subpopulations of juxtaglomerular neurons of the mouse MOB using the immunocytochemistry in combination with transgenic mice (Parrish-Aungst et al., 2007). In addition to those chemical properties recent studies have indicated that several transcription factors are important for the development and differentiation of those neuronal subpopulations and that the expression of some transcription factors persist toward adulthood, thus characterizing subpopulations (Yoshihara et al., 2005; Waclaw et al., 2006; Allen II et al., 2007; Li et al., 2011); those transcription factors characterizing OB interneurons include Sp8 (Waclaw et al., 2006; Allen II et al., 2007; Li et al., 2011), Arx (Yoshihara et al., 2005), ER81 (Stenman et al., 2003; Allen II et al., 2007; Saino-Saito et al., 2007), Pax6 (Dellovade et al., 1998; Kohwi et al., 2005; Hack et al., 2005; Allen II et al., 2007; Brill et al., 2008), Meis2 (Allen II et al., 2007) and those characterizing mitral/tufted cells include Tbr1, Tbr2 and Tbx21 (Faedo et al., 2002; Yoshihara et al., 2005; Winpenny et al., 2011). Furthermore Waclaw et al. (2006) and Li et al. (2011) showed that in the Sp8 conditional knock-out mice "the most severe reductions are seen in the populations which continue to express Sp8 as mature neurons (Waclaw et al., 2006)", and thus suggested that Sp8 is required for their normal production.

In the present study we extended our analyses on the juxtaglomerular neurons of the mouse MOB by examining the immunoreactivities of two well-known transcription factors, Sp8 and Tbx21, of the chemically defined subpopulations we reported previously (Kosaka and Kosaka, 2007a,b, 2009a), that is, calretinin (CR), calbindin (CB), tyrosine hydroxylase (TH) and nitric oxide synthase (NOS) positive groups, as well as a newly reported secretagogin positive group (Mulder et al., 2009). Special attention was

Abbreviations: CB, calbindin D28k; CLSM, a confocal laser scanning microscope; CR, calretinin; DAPI, 4′,6-diamidino-2-phenylindole; EPL, external plexiform layer; GCL, granule cell layer; GL, glomerular layer; IPL, internal plexiform layer; ML, mitral cell layer; MOB, main olfactory bulb; NOS, nitric oxide synthase; ONL, olfactory nerve layer; SE, subependymal/ependymal layer; TH, tyrosine hydroxylase.

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paid to the expression of these transcription factors in the TH and NOS positive juxtaglomerular neurons, for, as we reported previously, both TH and NOS positive juxtaglomerular neurons in the mouse MOB are heterogeneous (Kosaka and Kosaka, 2007b, 2008, 2009b).

2. Materials and methods

2.1. Tissue preparations

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, 1996) and the institutional guidance for animal welfare (the Guidelines for Animal Experiment in Graduate School of Medical Sciences, Kyushu University) and have been approved by the Committee of the Ethics on Animal Experiment in Graduate School of Medical Sciences, Kyushu University. All efforts were made to minimize the number of animals used and their suffering. Nine adult male C57BL/6J mice (22–25 g body weight, 7–11 weeks old, specific pathogen free; Japan SLC, Inc., Hamamatsu, Japan) were used in this study. For the fixation animals were deeply anesthetized with sodium pentobarbital (100 mg/kg body weight) and perfused transcardially with phosphate buffered saline (PBS, pH 7.4) followed by three kinds of fixatives: (1) fixative PFA1 (2 mice); 1% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4), (2) fixative PFA2 (4 mice); 2% paraformaldehyde in the same buffer, and (3) fixative PFA4; 4% paraformaldehyde in the same buffer (3 mice). The brains were left in situ for 1–2 h at room temperature and then were removed from the skull. The OBs were dissected out, encapsulated in 5% agar in PBS and cut horizontally into 50 μm-thick (for PFA2 and PFA4 fixed brains) or 80 μm-thick (for PFA1 fixed brains) serial sections on a vibratome (VT1000; Leica, Heidelberg, Germany or Vibratome 3000, Technical Products International Inc., St. Louis, MI).

2.2. Immunofluorescent multiple labeling

The sections were incubated overnight with 1% BSA in PBS containing 0.3% Triton X-100 and 0.05% sodium azide at room temperature. Then, they were incubated for 7–14 days at 20 °C in mixtures of following primary antibodies raised in different species (Table 1): goat anti-Sp8 antibody (1:500, Santa Cruz Biotechnology), rabbit anti-Sp8 antibody (1:10,000; Millipore), guinea-pig anti-Tbx21 (1:10,000; gift from Dr. Yoshihara; Yoshihara et al., 2005), rabbit anti-TH antibody (1:10,000; gift from Dr. Nagatsu; Nagatsu, 1983), rabbit anti-secretagogin antibody (1:20,000; gift from Dr. Wagner; Wagner et al., 2000), mouse monoclonal anti-CR antibody (1:5000; Swant, Bellinzona, Switzerland), goat polyclonal anti-CR antibody (1:5000; Swant, Bellinzona, Switzerland), mouse monoclonal anti-CB antibody (1:20,000; gift from Dr. Heizmann; Pinol et al., 1990), and sheep anti-NOS antibody (1:5000: gift from Dr. Emson; Herbison et al., 1996). Then, after incubation in biotinylated donkey anti-guinea-pig IgG (1:200; Jackson Immunoresearch, West Grove, PA, USA) overnight, the sections were rinsed several times in PBS, and incubated overnight in a mixture of pacific blue-conjugated streptavidin (stPaB) (1:200, Molecular Probe, Eugene, OR, USA), fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (1:200; Jackson Immunoresearch), cyanine 3 (Cy3)-conjugated donkey anti-goat IgG (1:200; Jackson Immunoresearch), and indodicarbocyanine (Cy5)-conjugated donkey anti-mouse IgG (1:100; Jackson Immunoresearch). Other combinations of fluoroprobes were also applied and there appeared to be no appreciable differences among them. Furthermore, some of those sections, double- or triple-stained with FITC-, Cy3- and Cy5-conjugated fluoroprobes, were incubated with a 4′,6-diamidino-2-phenylindole (DAPI) solution (10 μg/ml; Sigma, St. Louis, MO) in PBS for 30 min. After rinsing several times in PBS, the sections were mounted in the Vectashield (Vector, Burlingame, CA).

For the combination of goat anti-Sp8 and sheep anti-NOS antibodies, we applied the tyramide signal amplification (TSA) method to the Sp8 immunostaining, using the PerkinElmer TSA plus palette system (NEL 760). Sections were incubated overnight with 1% BSA in PBS containing 0.3% Triton X-100 and 0.05% sodium azide at room temperature. Then, they were incubated for 7–14 days at 20 °C in highly diluted goat anti-Sp8 antibody (1:5000) and then processed for the TSA system according to the supplier's manual. Then sections were rinsed in PBS, incubated in the 1% BSA in PBS and processed for the conventional immunofluorescent staining. We used Cy3 tyramide for the TSA system and other fluorochromes for conventional immunofluorescent stainings.

2.3. Image acquisition and processing

The sections were observed under a confocal laser-scanning microscope (CLSM; TCS-SP2; Leica Microsystems, Vienna, Austria). Single laser beams, 405, 488, 564 and 647 nm in wavelength, were alternately used to collect images (1024 × 1024 pixels and 256 grey levels) for different fluorescent signals. Most images were acquired using an oil immersion objective lens (X63, NA 1.35) and their x & y pixel sizes were 232.51 nm and z step was 407 nm. At the acquisition of the Sp8 channel images the gain was adjusted at which few profiles were saturated. Confocal image stacks were transferred to a personal computer, analyzed using the image analysis software Image J (Version 1.3) and subsequently processed by Adobe Photoshop 7.0J (Adobe Systems, Mountain View, CA).

The Sp8 immunostaining was so various in its intensity that it was sometimes difficult to determine whether a profile was negative or faintly positive. Thus the grey level of the Sp8 immunoreactivity of individual chemically defined neurons was measured not in the raw images but in the images processed by Image J. The Sp8 channel stacks were processed first by the "Background subtract (Rolling Ball radius = 50 pixels)" and "Despeckle" and, in some cases, "Median filter (2.0 pixels)" in the "Process" menu

Table 1
Primary antibodies used in this study.

Antigen	Animal	Dilution	Source/Reference
Sp8	Goat	1:500	sc-104661; Santa Cruz Biotechnology
Sp8	Goat	1:5000 (TSA)	sc-104661; Santa Cruz Biotechnology
Sp8	Rabbit	1:10,000	AB15260; Millipore
Tbx21	Guinea-pig	1:10,000	Gift from Dr. Yoshihara; Yoshihara et al. (2005)
Nitric oxide synthase (NOS)	Sheep	1:5000	Gift from Dr. Emson; Herbison et al. (1996)
Tyrosine hydroxylase (TH)	Rabbit	1:10,000	Gift from Dr. Nagatsu; Nagatsu (1983)
Secretagogin	Rabbit	1:20,000	Gift from Dr. Wagner; Wagner et al. (2000)
Calretinin (CR)	Goat	1:5,000	Swant, Bellinzona, Switzerland
Calretinin (CR)	Rabbit	1:5,000	Swant, Bellinzona, Switzerland
Calretinin (CR)	Mouse	1:5,000	Swant, Bellinzona, Switzerland
Calbindin 28k (CB)	Mouse	1:20,000	Gift from Dr. Heizmann; Pinol et al. (1990)