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Secretagogin-containing neurons in the mouse main olfactory bulb☆

Katsuko Kosaka[a,1], Toshio Kosaka[b,*]

[a] Department of Health Sciences, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan
[b] Department of Anatomy and Neurobiology, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan

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ABSTRACT

Secretagogin (SCGN) is a recently discovered calcium binding protein of the EF hand family. We studied the structural features of SCGN-positive neurons in the mouse main olfactory bulb (MOB). SCGN-positive neurons were localized throughout layers but clustered in the glomerular layer (GL), mitral cell layer (MCL) and granule cell layer (GCL). They were heterogeneous, including numerous juxtaglomerular neurons, granule cells, small to medium-sized neurons in the external plexiform layer (EPL), and a few small cells in the ependymal/subependymal layer. Calretinin and/or tyrosine hydroxylase occasionally colocalized in SCGN-positive juxtaglomerular neurons. Calretinin also frequently colocalized in SCGN-positive EPL and GCL neurons. Morphologically some of juxtaglomerular SCGN-positive neurons were classical periglomerular cells, whereas others were apparently different from those periglomerular cells, although they were further heterogeneous. Some extended one slender process into a glomerulus which passed the glomerulus and further penetrated into another nearby glomeruli, and thus their dendritic processes spanned two or three or more glomeruli. We named this type of juxtaglomerular neurons "transglomerular cells." With the stereological analysis we estimated total number of juxtaglomerular SCGN-positive neurons at about 80,000/single MOB. The present study revealed the diversity of SCGN-positive neurons in the mouse MOB and their particular structural properties hitherto unknown.

1. Introduction

The olfactory bulb (OB) is known to richly vary in chemical substances (Kosaka and Kosaka, 2009a). Among chemical substances and their related enzymes which characterize various types of neurons in the OB, calcium binding proteins such as parvalbumin (PV), calretinin (CR), calbindin D28k (CB), neurocalcin and secretagogin (SCGN) are considered to be valuable chemical markers to reveal the neuronal organization of this part of the cortex in various species; rodents (Kosaka et al., 1987, 1994, 1998, 2008; Celio, 1990; Rogers and Résibois, 1992; Alonso et al., 1993; Toida et al., 1994, 1996, 1998; Briñón et al., 1992, 1997; Kosaka and Kosaka, 2004, 2005, 2007a, 2008a, 2010, 2011; Mulder et al., 2009), hedgehog (Briñón et al., 2001; Kosaka and Kosaka, 2004), musk shrew (Kakuta et al., 1998, 2001; Kosaka and Kosaka, 2001, 2004), mole (Kosaka and Kosaka, 2004), bat (Kosaka and Kosaka, 2004), opossum (Jia and Halpern, 2004), monotremes (Ashwell, 2006), monkey (Alonso et al., 2001; Mulder et al., 2009) and human (Ohm et al., 1991). In this study, we analyzed the neurons containing SCGN in the main olfactory bulb (MOB) of the C57BL/6J mouse strain. SCGN is a recently discovered calcium binding protein of the EF hand family, cloned from β cells of pancreatic island of Langerhans and endocrine cells of the gastrointestinal gland (Wagner et al., 2000). CGN expression has been also shown in developing and adult neurons (Alpár et al., 2012). In adult brain SCGN expression is restricted to some regions (Mulder et al., 2009; Alpár et al., 2012; Maj et al., 2012; Allen Brain Atlas Scgn-RP_040831_02_G06-sagittal). CGN's cellular roles are still inconclusive but SCGN is proposed to be a functionally multifaceted calcium binding protein and to be implicated in neuronal turnover, differentiation,

Abbreviations: ABC, avidin–biotin-peroxidase complex; AOB, accessory olfactory bulb; BDA, biotinylated dextran amine; BrdU, 5-bromo-2′-deoxyuridine; BSA, bovine serum albumin; CB, calbindin D28k; CLSM, confocal laser scanning microscope; CR, calretinin; Cy3, indocarbocyanine; Cy5, indodicarbocyanine; DAB, diaminobenzidine tetrahydrochloride; DAPI, 4′,6-diamidino-2-phenylindole; EdU, 5-ethynyl-2′-deoxyuridine; EM, electron microscope; EPL, external plexiform layer; FITC, fluorescein isothiocyanate; GCL, granule cell layer; GL, glomerular layer; IPL, internal plexiform layer; MCL, mitral cell layer; MOB, main olfactory bulb; MNL, myelinated nerve layer of the accessory olfactory bulb; MTL, mitral and tufted cell layer of the accessory olfactory bulb; NOS, nitric oxide synthase; OB, olfactory bulb; ON, olfactory nerve/olfactory nerve terminal; ONL, olfactory nerve layer; PaB, pacific blue; PB, phosphate buffer; PBS, phosphate buffered saline; PV, parvalbumin; RMS, rostral migratory stream; SCGN, secretagogin; SEL, ependymal/subependymal layer; stPaB, pacific blue-conjugated streptavidin; TH, tyrosine hydroxylase; VN, vomeronasal nerve; VNL, vomeronasal nerve layer.

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* Corresponding author at: Department of Anatomy and Neurobiology, Graduate School of Medical Sciences, Kyushu University, Higashi-ku, Fukuoka 812-8582, Japan. Tel.: +81 92 642 6053; fax: +81 92 642 6059.
E-mail address: kosaka@med.kyushu-u.ac.jp (T. Kosaka).
[1] Present address: Department of Medical Science Technology, Faculty of Health and Welfare Sciences in Fukuoka, International University of Health and Welfare, 137-1 Enokizu, Okawa City, Fukuoka 831-8501, Japan.

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proliferation, vesicular exocytosis of neuroactive substances and neuroprotection (Alpár et al., 2012; Maj et al., 2012). Regarding with the SCGN-positive neurons in the MOB, Mulder et al. (2009) reported the SCGN containing elements in some strains of mice and one species of primate, gray mouse lemur, including the species differences between rodent and primate. However, the details of their structural features remain to be elucidated. In the present study we analyzed the structural features of the SCGN-positive neurons in the mouse MOB. We confirmed numerous SCGN-positive juxtaglomerular neurons in the glomerular layer (GL) and granule cells in the granule cell layer (GCL), as reported by Mulder et al. (2009). However, we also encountered some types of SCGN-positive neurons located in the juxtaglomerular region, which we could not necessarily classify into previously known distinctive neuron groups such as periglomerular cells and superficial short-axon cells clearly. In the present study we first describe the general distribution pattern of SCGN-positive neurons, and describe the chemical properties of SCGN-positive neurons throughout layers. Then we focus on the structural features of some types of SCGN-positive neurons in the juxtaglomerular region. We also describe a stereological estimation of the total number of juxtaglomerular SCGN-positive neurons, electron microscopic analysis of synaptic contacts of the SCGN-positive neurons and also touch the adult neurogenesis of SCGN-positive neurons, although some results are preliminary and will be firmed by future studies.

2. Experimental procedures

2.1. Tissue preparations

All experiments were carried out in accordance with "the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions" of the Ministry of Education, Culture, Sports, Science and Technology of Japan, the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, 1996) and the Regulations for Animal Experiment at Kyushu University and have been approved by the Institutional Animal Care and Use Committee in Kyushu University. All efforts were made to minimize the number of animals used and their suffering. Forty-five adult male C57BL/6J mice (22–25 g body weight, 4–16 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) 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4), (2) 2% paraformaldehyde in 0.1 M PB and (3) a mixture of 4% paraformaldehyde and 1% glutaraldehyde in the same buffer. 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 transversely or horizontally or parasagittally into 50 μm-thick serial sections on a vibratome (VT1000; Leica, Heidelberg, Germany or Vibratome 3000, Technical Products International Inc., St. Louis, MI).

2.2. Immunocytochemistry

2.2.1. Immunostaining for SCGN: ABC method

Sections from 4% and 2% paraformaldehyde-fixed OBs were used. The sections were incubated in 3% H_2O_2 in 80% methanol for 30 min at room temperature to reduce the endogenous peroxidase activities and then were rinsed briefly in PBS. After incubation overnight in 1% bovine serum albumin (BSA) in PBS containing 0.3% Triton X-100 and 0.05% sodium azide, the sections were incubated in rabbit polyclonal anti-SCGN antibody (dilution 1:20,000; gift from Dr. Wagner; Wagner et al., 2000) for 10 days at 20 °C. Then the sections were incubated in biotinylated donkey anti-rabbit IgG (1:200; Jackson Immunoresearch, West Grove, PA, USA) overnight, rinsed several times in PBS, and were processed according to the avidin–biotin-peroxidase complex (ABC) method using Vector kits (Vector Laboratories, Burlingame, CA, USA). After rinses in PBS, they were incubated in diaminobenzidine tetrahydrochloride (DAB) solution (Vector DAB substrate kit for peroxidase SK 4100) diluted with PBS for 5–15 min at room temperature. The sections were rinsed in PBS and then in PB, treated with 0.4% OsO_4 in 0.1 M PB for 30 min at room temperature to enhance and stabilize the reaction products, and then dehydrated in graded series of ethanol, infiltrated in propylene oxide, and flat-embedded in Epon-Araldite.

Some SCGN-positive cells were examined and drawn with light microscopes equipped with camera lucida apparatus at high magnifications using oil-immersion lens (Olympus BX50 Plan Apo 100; NA, 1.40; Olympus, Tokyo, Japan and Leica DM2500 Plan Apo 100; NA 1.40; Leica Microsystems, Tokyo, Japan).

2.2.2. 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 10 days at 20 °C in mixtures of primary antibodies raised in different species. The primary antibodies used are listed in Table 1. Then, after incubation in biotinylated donkey secondary antibodies (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), indocarbocyanine (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).

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 for different fluorescent signals.

2.3. Combined CLSM and EM

For combined CLSM and EM examinations we used the sections fixed with a mixture of 4% paraformaldehyde and 1% glutaraldehyde. After cryoprotection in 0.1 M PB containing 30% sucrose and freeze-thaw procedure using liquid N_2, the sections were treated with 1% sodium borohydride in PBS for 30 min before immunocytochemical procedures (Kosaka et al., 1986). The sections were preincubated overnight with 1% BSA in PBS containing 0.05% sodium azide at 4 °C, and then incubated in a mixture of rabbit anti-SCGN antibody (1:20,000), mouse anti-CR antibody (1:5000) and sheep anti-tyrosine hydroxylase (TH; 1:5000) overnight, rinsed several times in PBS. Then sections were incubated in biotinylated donkey anti-rabbit IgG (1:200; Jackson Immunoresearch, West Grove, PA, USA) overnight, and, after several rinses in PBS, incubated overnight in a mixture of stFITC (1:200, Molecular Probe, Eugene, OR, USA), Cy3-conjugated donkey anti-goat IgG (1:200; Jackson Immunoresearch) and Cy5-conjugated donkey anti-mouse