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Calcium-binding protein, secretagogin, specifies the microcellular tegmental nucleus and intermediate and ventral parts of the cuneiform nucleus of the mouse and rat

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ABSTRACT

Secretagogin (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. SCGN characterizes some particular neuron groups in various regions of the nervous system and is considered as one of the useful neuron subpopulation markers. In the present study we reported that SCGN specifically labelled a particular neuronal cluster in the brainstem of the mice and rats. The comparison of the SCGN immunostaining with the choline acetyltransferase immunostaining and acetylcholinesterase staining clearly indicated that the particular cluster of SCGN positive neurons corresponded to the microcellular tegmental nucleus (MiTg) and the ventral portion of the cuneiform nucleus (CnF), both of which are components of the isthmus. The analyses in mice indicated that SCGN positive neurons in the MiTg and CnF were homogeneous in size and shape, appearing to compose a single complex: their somata were small comparing with the adjacent cholinergic neurons in the pedunculotegmantal nucleus, 10.5 vs 16.0 μm in diameter, and extended 2–3 slender smooth processes. SCGN might be one of significant markers to reconsider the delineations of the structures of the mouse, and presumably rat, brainstem.

1. Introduction

Secretagogin (SCGN) is a newly discovered calcium binding protein (Wagner et al., 2000) which characterizes some particular neuron groups in various regions of the nervous system (Mulder et al., 2009, 2010; Puthussery et al., 2009; Alpár et al., 2012; Maj et al., 2012; Shi et al., 2012; Gyengesi et al., 2013; Kosaka and Kosaka, 2013; Weltzien et al., 2014; Garas et al., 2016; Kosaka et al., 2017). Mulder et al. (2009) analyzed mouse and primate brains and reported the interspecies differences between them in the SCGN distribution. We also noticed SCGN as one of useful neuronal markers in addition to other calcium binding proteins, especially, parvalbumin (PV), calbindin (CB) and calretinin (CR), for some particular neuronal populations in various regions of the nervous system. Previously we reported SCGN positive neurons in the mouse olfactory bulb (OB) and rat striatum (Kosaka and Kosaka, 2013; Kosaka et al., 2017): in both regions SCGN positive neurons included novel types of neurons in these brain regions. Among the brain regions containing SCGN positive neurons we noticed a particular region in the brainstem of mice and rats, in which small–medium sized SCGN positive neurons are compactly clustered. The region containing SCGN neuron clusters appeared to be

Abbreviations: 3N, oculomotor nucleus; 4N, trochlear nucleus; 5N, motor trigeminal nucleus; 7n, facial nerve; 7N, facial nucleus; AChE, acetylcholinesterase; APT, anterior pretectal nucleus; Aq, aqueduct; AV, anteroventral thalamic nucleus; BSA, bovine serum albumin; CA, hippocampus; CB, calbindin; ChAT, choline acetyltransferase; CnF, cuneiform nucleus; CnFD, dorsal part of the cuneiform nucleus; CnFI, intermediate part of the; CnFV, ventral part of the cuneiform nucleus; CPu, caudate putamen; Cr, cerebellum; CR, calretinin; Cy3, indocarbocyanine; Cy5, indodicarbocyanine; DAPI, 4′, 6-diamidino-2-phenylindole; DLG, dorsal lateral geniculate nucleus; DLL, dorsal nucleus of the lateral lemniscus; FITC, fluorescein isothiocyanate; fr, fasciculus retroflexus; GFP, green fluorescent protein; HDB, nucleus of the horizontal limb of the diagonal band; IC, inferior colliculus; IG, indusium griseum; isRt, isthmic reticular formation; LDTg, laterodorsal tegmental nucleus; LDTgV, laterodorsal tegmental nucleus, ventral part; lfp, longitudinal fasciculus of the pons; ll, lateral lemniscus; LPB, lateral parabrachial nucleus; LV, lateral ventricle; m5, motor root of the trigeminal nerve; mcp, middle cerebellar peduncle; MiTg, microcellular tegmental nucleus; ml, medial lemniscus; mlf, medial longitudinal fasciculus; MT, medial terminal nucleus of the accessory optic tract; OB, olfactory bulb; PB, phosphate buffer; PBG, parabigeminal nucleus; PBS, phosphate-buffered saline; PTg, pedunculotegmental nucleus; PV, parvalbumin; s5, sensory root of the trigeminal nucleus; SC, superior colliculus; SCGN, secretagogin; scp, superior cerebellar peduncle; VLPAG, ventrolateral part of periaqueductal grey; xscp, decussation of the superior cerebellar peduncle.

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located at the midbrain-hindbrain border, that is, isthmus (Paxinos and Franklin, 2013; Watson et al., 2017). In addition the in situ hybridization data (Allen Brain Atlas Scgn experiment 583549, specimen 04-0545) show the intensive expression of SCGN around the midbrain-hindbrain border. In the present study we examined the spatial relationship of these SCGN positive neurons with the acetylcholinesterase (AChE) stained regions as well as the choline acetyltransferase (ChAT)-immunostained regions including adjacent cholinergic neuron groups (Mesulam et al., 1983; Wainer et al., 1984; Mufson et al., 1986), that is, pedunculotegmental nucleus (PTg; Ch5), laterodorsal tegmental nucleus (LDTg; Ch6) and parabigeminal nucleus (PBG; Ch8). We identified this particular region containing clustered SCGN positive neurons at the isthmus as the microcellular tegmental nucleus (MiTg) and ventral portion of the cuneiform nucleus (CnF). In the present study we report the distribution of SCGN positive neurons in this particular region at the isthmus and their structural features revealed with the SCGN immunostaining.

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 "Guide for the Care and Use of Laboratory Animals 8th edition (2011)änd the institutional guidance for animal welfare (the Guidelines for Animal Experiment in International University of Health and Welfare). Every experimental procedure was approved by the Committee of the Ethics on Animal Experiment in International University of Health and Welfare. All efforts were made to minimize the number of animals used and their suffering.

In this study we used 8 male Wistar rats, 5–8weeks old, 110–180 g (Japan SLC, Inc., Hamamatsu, Japan), and 12 C57BL/6J mice (8 male and 4 female mice), 6–8weeks old 16–25 g (Japan SLC, Inc.). Animals were deeply anesthetized with 2.5–3.5% isoflurane or with sodium pentobarbital (100 mg/kg body weight) and perfused transcardially with phosphate-buffered saline (PBS, pH7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH7.2–7.4). The brains were left in situ for 1–2 h at room temperature, then removed from the skull and stocked in the same fixative at 7 °C for 1–10 days. The brains were dissected out, encapsulated in 4% agar in PBS and cut transversely or horizontally or parasagittally into 50 μm-thick serial sections on a vibratome (Leica VT1000S).

2.2. Immunohistochemistry

The sections were incubated overnight with 10% horse serum + 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, that is, rabbit anti-SCGN (gift from Dr. Wagner, 1:20,000; Wagner et al., 2000), goat anti-ChAT (Chemicon, AB144P, 1:500 or 1000), guinea-pig anti-PV (Frontier Institute, AB2571615, 1: 5000), mouse anti-NeuN (Chemicon, MAB 377, 1:2000), mouse anti-CR (Swant 6B3, 1:5000) and mouse anti-CB (gift from Dr. Heizmann, 1:20,000; Pinol et al., 1990). We tried double-, triple- and quadruple-immunostaining of various combinations of primary antibodies. The sections were rinsed 3 times in PBS, and incubated overnight in a mixture of fluorochrome-conjugated donkey secondary antibodies (Jackson Immunoresearch) such as Alexa Fluor 488-conjugated anti-goat IgG (1:250), fluorescein isothiocyanate (FITC) or Alexa Fluor 488-conjugated anti-guinea pig IgG (1:250), indocarbocyanine (Cy3)-conjugated anti-rabbit IgG (1:1000), and indodicarbocyanine (Cy5)-conjugated anti-mouse IgG (1:250). Fluorescently labelled sections were usually incubated in PBS containing 4′, 6-diamidino-2-phenylindole (DAPI, 1 μg/ml, Dojin) for 30–60 min. After rinsing several times in PBS, the sections were mounted in the Vectashield (Vector, Burlingame, CA) and covered with a cover glass.

The fluorescent images of individual sections were examined and photographed with a fluorescence stereoscopic microscope (Leica MZ FL III) equipped with a color CCD digital camera (Olympus DP70). Fluorescent filter sets of the Leica stereoscopic microscope used were as follows; GFP Plus (excitation 480/40 nm, barrier 510 nm) for Alexa 488 and FITC, UV fluorescence (excitation 360/40 nm, barrier 420 nm) for DAPI and Green fluorescence (excitation 546/10 nm, barrier 590 nm) for cy3. The sections were also examined with a fluorescence microscope (Nikon Eclipse 80i) equipped with a monochrome CCD digital camera (Retiga Exi; QImaging), motorized digital imaging head (Nikon DIH-E) and motor-driven stage and focus drive (Ludl Electronic Products Ltd.) controlled by the Neurolucida image analysis system (MBF Bioscience). Fluorescent filter sets of the Nikon microscope used were as follows; Semrock GFP-3035D for FITC and Alexa Fluor 488, Semrock TRIC-B for cy3, Semrock DAPI–1160 B for DAPI and Semrock cy5-4040C for cy5. Image stacks of 3 or 4 channels were obtained using planapochromat objective lenses ×4 or ×10 or ×20. Projection images were made from these image stacks using the plugin module 'stack focuser' of ImageJ.

The soma sizes of SCGN positive neurons in the MiTg and CnF and ChAT positive neurons in the PTg were measured on the light microscopic image stacks using the image analysis software imageJ. Briefly, in the image stacks we applied the best fitting ellipse to each selected somatic profile and obtained the areas of these ellipses. Then the area equivalent diameters (mean diameter = 2 (soma area/π) $^{1/2}$) of individual somata were calculated and the normalities of their distributions were tested with the Shapiro-Wilk test using Origin 8 (Origin Lab.).

2.3. Acetylcholinesterase histochemistry

For the Aacetylcholinesterase (AChE) histochemistry, a modified Karnovsky-Roots method (Butcher, 1983) was used. The sections adjacent to those for immunofluorescent multiple labeling were selected and incubated in the solution (10 ml) consisting of acetylthiocholine iodide 5 mg, 0.2 M Tris maleate buffer (pH 5.7) 6.5 ml, 0.1 M sodium citrate 0.5 ml, 0.03 M cupric sulfate 1.0 ml, 0.005 M potassium ferricyanide 1.0 ml and MilliQ pure water 1.0 ml for 6 h at 25 °C or 2 h at 35 °C. The sections were rinsed in PBS and then with 0.1 M PB, air-dried on the glass slides, mounted in the M.X (Matsunami) or Aquatex (Merk) and covered with a cover glass. The AChE stained sections were examined and photographed with a stereoscopic microscope (Leica MZ FL III) equipped with a color CCD digital camera (Olympus DP70).

3. Results

In the supporting information Fig. S3A of their paper, Mulder et al. (2009) showed the distributions of SCGN positive neurons in the mouse brain. We encountered SCGN positive neurons in those regions previously reported (Mulder et al., 2009) such as olfactory bulb (OB), indusium griseum (IG), superior colliculus (SC), dorsal lateral geniculate nucleus (DLG), medial terminal nucleus of the accessory optic tract (MT) and hypothalamic region. In the same figure Mulder et al. (2009) also mapped SCGN somata at the cuneiform nucleus (CnF)-lateral parabrachial nucleus (LPB) border at the level interaural P1.22, that is, Fig. 73 of the stereotaxic atlas

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