Neuroscience Research 119 (2017) 53–60

Contents lists available at ScienceDirect

Neuroscience Research

journal homepage: www.elsevier.com/locate/neures

Short communication

Calcium-binding protein, secretagogin, characterizes novel groups of interneurons in the rat striatum

Toshio Kosaka*, Seiko Yasuda, Katsuko Kosaka

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

ARTICLE INFO

Article history:
Received 13 October 2016
Received in revised form
28 December 2016
Accepted 18 January 2017
Available online 11 February 2017

Keywords:
Calretinin
Choline acetyl transferase
Nitric oxide synthase
Parvalbumin
GABA
Heterogeneity

ABSTRACT

In the rat striatum numerous secretagogin (SCGN) positive neurons were scattered. They were heterogeneous in their morphological and chemical properties. We examined the colocalization of SCGN with known four interneuron markers, parvalbumin (PV), calretinin (CR), nitric oxide synthase (NOS) and choline acetyl transferase (ChAT). 60–70% of SCGN positive striatal neurons contained either PV or CR or ChAT, but none contained NOS. On the other hand the remaining 30–40% expressed none of these markers, most of which were GAD positive. The present study indicates that there are hitherto unknown groups of striatal interneurons in the rat striatum.

Telencephalic interneurons are heterogeneous and major groups are generally distinguished based on the expression of histochemical markers such as calcium binding proteins, neuroactive substances and transmitter receptor subtypes. Those chemically defined groups of interneurons were further characterized and/or correlated with morphological and physiological features. In the striatum four major classes of interneurons have been identified, cholinergic neurons and GABAergic neurons containing parvalbumin (PV), somatostatin/nitric oxide synthase (NOS) and calretinin (CR) (Kawaguchi et al., 1995; Tepper and Bolam, 2004; Tepper et al., 2010; Dudman and Gerfen, 2015). Recently, mainly based on the analyses of several transgenic mice expressing a fluorescent marker specifically and selectively in some neurons, additional GABAergic striatal interneurons were reported such as tyrosine hydroxylase-expressing interneurons (Tepper et al., 2010; Silberberg and Bolam, 2015) and serotonin receptor 3a (5HT3a)-expressing interneurons (Silberberg and Bolam, 2015). Muñoz-Manchado et al. (2016) analyzed the striatum of the transgenic mouse 5HT3a^{EGFP} and reported 5HT3a^{EGFP} -positive (+) cells are novel major groups of GABAergic interneurons; according to Table 1 in Muñoz-Manchado et al. (2016), 5HT3a^{EGFP} -positive (+) cells were about one-third of all striatal GABAergic interneurons, although about one-fourth of striatal 5HT3a^{EGFP} -positive (+) cells were overlapped with previously characterized interneurons. At any rate some new chemical markers could reveal novel groups of neurons in various brain regions.

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., 2010; Alpár et al., 2012; Maj et al., 2012; Shi et al., 2012; Gyengesi et al., 2013; Kosaka and Kosaka, 2013; Weltzien et al., 2014). Mulder et al. (2009) analyzed mouse and primate brains and reported the interspecies differences between them in the SCGN distribution. The striatum is one of the regions showing such interspecies differences. In the primate putamen approximately 50% of cholinergic interneurons were SCGN+, whereas in the mouse striatum SCGN+ neurons were rare and GABAergic with elaborate, spiny dendritic tufts (Mulder et al., 2009). Among rodents mice and rats are major targets in neuroscience researches. These two rodent species are generally similar in their distribution of various chemical markers, but in some cases show vast differences. Recently, Garas et al. (2016) reported the species differences in the striatal neurons expressing SCGN between mice and rats. They reported the colocalization of SCGN and PV in the striatum of rats and monkeys and the structural and functional details of PV+/SCGN+ and PV+/SCGN- neurons. Furthermore they showed that SCGN+ neurons in the rat striatum

* Corresponding author.
E-mail address: t-kosaka@iuhw.ac.jp (T. Kosaka).

http://dx.doi.org/10.1016/j.neures.2017.01.004
0168-0102/

Table 1
Primary antibodies used in this study.

Antigen	Host animal	Dilution	Source/references
calretinin CR	mouse	1: 5000	Transduction Lab., clone 34
calretinin CR	rabbit	1: 5000	Swant, 7699/4
calretinin CR	goat	1: 5000	Swant, CG1
choline acetyl transferase ChAT	goat	1: 500	Chemicon, AB144P
glutamic acid decarboxylase 67 GAD67	mouse	1: 5000	Chemicon, MAB5406, clone 1G10.2
nitric oxide synthase NOS	sheep	1: 10,000	gift from Dr. P.C. Emson (Herbison et al., 1996, J. Neuroendocrinol. 8, 211–216.)
parvalbumin PV	guinea pig	1: 5000	Frontier Institute Co. Ltd, AB2571615
parvalbumin PV	rabbit	1: 5000	gift from Dr. C. W. Heizmann (Kägi et al., 1987, J. Biol. Chem. 262, 7314–7320)
secretagogin SCGN	rabbit	1: 20,000	gift from Dr. L. Wagner (Wagner et al., 2000, J. Biol. Chem. 275, 24740-24751; Puthussery et al., 2010, J. Comp. Neurol. 518, 513-525.)
secretagogin SCGN	sheep	1: 5000	BioVendor, RD184120100

did not express the chemical marker of the striatal projection neurons, Ctip2, and thus indicated that striatal SCGN+ neurons were interneurons. However, in their study Garas et al. (2016) did not analyzed the colocalization of SCGN with other striatal interneuron markers such as CR, NOS and choline acetyl transferase (ChAT). In the present study we examined the colocalization relationship of SCGN with those markers in the rat striatum and noticed that SCGN+ neurons in the rat striatum showed some peculiar colocalization relationship with those markers. Furthermore the present results suggest that some SCGN+ neurons in the rat striatum are presumed novel groups of interneurons. The preliminary results of this study have been reported (Yasuda, 2016).

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)" and 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 5 male C57BL/6J mice, 8weeks old 22–25 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 (pH 7.2–7.4). The brains were left in situ for 1–2 h at room temperature and then removed from the skull. From each brain, coronal, parasagittal, or horizontal 50 μm thick sections were cut serially on a vibratome (Leica VT1000S or Lancer 1000). The sections were incubated overnight with 1% bovine serum albumin in PBS containing 0.3% Triton X-100 and 0.05% sodium azide at 20 °C. Then, they were incubated for 10 days at 20 °C in mixtures of primary antibodies raised in different species. The primary antibodies used are shown in Table 1. In the present study we used two anti-SCGN antibodies (Table 1), one rabbit and the other sheep polyclonal antibodies. The rabbit anti-SCGN antibody is raised against recombinant human SCGN (Wagner et al., 2000), which also recognizes rat SCGN (Wagner et al., 2000) and mouse SCGN (Puthussery et al., 2010). The sheep anti-SCGN antibody is also raised against recombinant human SCGN, that is, recombinant protein containing a 276 amino acid sequence of human SCGN and 10 extra amino acids, N-terminal His-tag, which was reported to show identical staining patterns to the rabbit anti-SCGN antibody described above in the macaque retina (Weltzien et al., 2014). We tried double-, triple- and quadruple-immunostaining of various combinations of primary antibodies as shown in Table 2. The sections were rinsed 3 times in PBS, and incubated overnight in a mixture of fluorochrome-conjugated donkey secondary antibodies (Jackson Immunoresearch) such as aminomethylcoumarin (AMCA)-conjugated anti-goat IgG, which cross-reacts with sheep IgG (1:250), fluorescein isothiocyanate (FITC)-conjugated anti-guinea pig IgG (1:250), indocarbocyanine (Cy3)-conjugated anti-rabbit IgG (1:1000), and indodicarbocyanine (Cy5)-conjugated anti-mouse IgG (1:250). Other combinations of fluoroprobes were also applied and there appeared to be no appreciable differences among them. To examine whether SCGN+ neurons different from 4 groups of known striatal interneurons were GABAergic or not, sections were processed in Triton X-100 free solutions; sections were incubated in a mixture of 5 primary antibodies, that is, mouse anti-GAD67, rabbit anti-SCGN, goat anti-ChAT, goat anti-CR, guinea pig anti-PV (No. 10 in Table 2), and then, after rinsing several times in PBS, incubated in a mixture of secondary antibodies composed with cy3-conjugated anti-mouse IgG, Alexa Fluor 488-conjugated anti-rabbit IgG, cy5-conjugated anti-goat IgG and cy5-conjugated anti-guinea pig IgG. After rinsing several times in PBS, the sections were mounted in the Vectashield (Vector).

Table 2
Combinations of primary antibodies and secondary antibodies. All secondary antibodies were fluorochrome-conjugated donkey affinity-purified antibodies against rabbit, goat, mouse or guinea-pig IgG and, according to the products specifications sheet, show minimally cross-reaction to serum proteins of several animals.

	host animals			
Combinations of primary antibodies	rabbit	goat/sheep	mouse	guinea pig
No. 1	SCGN	–	–	PV
No. 2	SCGN	CR	–	–
No. 3	SCGN	NOS	–	–
No. 4	SCGN	ChAT	–	–
No. 5	SCGN	–	CR	–
No. 6	PV	SCGN	CR	–
No. 7	CR	SCGN	–	PV
No. 8	SCGN	NOS	CR	PV
No. 9	SCGN	ChAT	CR	PV
No. 10	SCGN	ChAT + CR	GAD67	PV
No. 11	SCGN	SCGN		

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) and with a fluorescence microscope (Olympus BX53) equipped with a color CCD digital camera (Olympus DP73). The sections were also examined with a Neurolucida image analysis system (MBF Bioscience) composed of a fluorescence microscope (Nikon Eclipse 80i), a monochrome CCD digital camera (QIClick;