

A part of the cholinergic fibers in mouse superior cervical ganglia contain GABA or glutamate

Tetsufumi Ito*, Satoshi Iino, and Yoshiaki Nojyo.

Department of Anatomy, Faculty of Medical Sciences,
University of Fukui, Fukui, Japan

* Corresponding author. Tel.: +81 (776) 61 8302; Fax: +81 (776) 61 8132; e-mail: itot@fmsrsa.fukui-med.ac.jp

Abstract

The localizations and functions of glutamate and GABA, the major amino acid neurotransmitters in the central nervous system, are still unclear in the peripheral nervous system. We immunohistochemically double-stained mouse superior cervical ganglia with antibodies for the vesicular acetylcholine transporter (VAChT), GAD65, the vesicular glutamate transporters 1-3 (VGluTs1-3), the marker of the sympathetic preganglionic neuron (SPN), GABAergic, and glutamatergic terminals, respectively. All GAD65-positive terminals showed VAChT-immunoreactivity, indicating that GABAergic fibers originate from SPNs. VGluT2-immunoreactive terminals showing colocalization with VAChT were observed, but VGluT1 and 3 immunoreactive terminals were not. Colocalization of GAD65 and VGluT2 was rarely found. All VGluT2-immunopositive terminals were also immunopositive for neuronal nitric oxide synthase (nNOS), a marker for the subpopulation of the SPNs, while about half of the GABA-immunopositive fibers were immunopositive for nNOS. The origin of these fibers was discussed.

Theme: Neurotransmitters, modulators, transporters, and receptors

Topic: Excitatory amino acids: anatomy and physiology

Keywords: sympathetic preganglionic neuron, superior cervical ganglion, mouse, GABA, acetylcholine, vesicular acetylcholine transporter, colocalization

Abbreviations: SPN, sympathetic preganglionic neuron; Ach, acetylcholine; VGluT, vesicular glutamate transporter; VAChT, vesicular acetylcholine transporter; nNOS,

neuronal nitric oxide synthase; SCG, superior cervical ganglion

The other abbreviations used in this manuscript:

oC: Degrees celsius

grmu: Greek character "mu"

Sympathetic preganglionic neurons (SPNs) contain not only acetylcholine (Ach), but also other neurotransmitters (and/or neuromodulators) including neuropeptides (cf: VIP, enkephalin) and bases(cf: ATP). Preganglionic fibers innervate postganglionic neurons according to certain rules, known as "chemical coding."

Glutamate is a major excitatory neurotransmitter in the central nervous system. It has been reported that there is immunoreactivity for AMPA type glutamate receptor in the superior cervical ganglion (SCG) of the rat [13]. Furthermore, glutamate synthesising enzyme glutaminase expression has been reported in the autonomic preganglionic neurons [17]. However, glutaminase-immunoreactivity was also reported in GABAergic interneurons, which might not release glutamate [1]. Therefore, another, more reliable marker for glutamatergic neurons was needed to elucidate whether a glutamatergic projection to the sympathetic ganglia exists or not.

GABA is a major inhibitory neurotransmitter in the central nervous system, and a GABAergic projection to the SCG has been reported [4]-[6][21]. Some authors insisted there were other neurons innervating postganglionic neurons and that they contained GABA [5][21]. For example, Dobo and colleagues [5] argued that SIF-like neurons in the sympathetic trunk projected to the SCG, because there were GABA-like immunoreactivities observed in fibers of the SCG, SIF-like cells in the sympathetic trunk showed a GABA-like immunoreactivity, and there were few GABA-like neurons in the spinal cord. Because their argument was based upon indirect evidence, further direct evidence is needed to elucidate the source of the GABAergic projection to the SCG.

The vesicular acetylcholine transporter (VAChT) is a reliable marker for the preganglionic terminal because the antibody for VAChT shows very specific staining. GAD65 is a better GABAergic terminal marker than GABA itself because the intensity of the GABA-like immunoreactivity was the highest when the fixative contained 5% glutaraldehyde [4], and this fixative is inadequate of the immunostaining for other markers. We used antibodies for vesicular glutamate transporters (VGluTs) because these proteins are directly concerned with glutamate exocytosis [18]-[20]. We immunohistochemically double-stained the mouse SCGs with antibodies for the VAChT,

GAD65, and the VGluTs to clarify whether SPNs contain glutamate and/or GABA.

Twelve male ddY mice were used. All the animals were maintained and treated according to the guidelines established in Fukui Medical University's Guidelines for Animal Experiments. Animals were anesthetized with an overdose of chloral hydrate, perfused transcardially with 10 ml of saline and then with 40 ml of fixative solution containing 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). For GABA immunohistochemistry, we used a fixative containing 4% paraformaldehyde and 0.05% glutaraldehyde in 0.1M phosphate buffer instead. Immediately after perfusion, the right SCGs were dissected out and immersed in the same fixative for 1 h at 4°C. SCGs were then immersed in 20% sucrose in 0.1M phosphate buffer (pH 7.4) for 24 h. Tissues were embedded in the OCT compound (Tissue Tek, USA) and frozen at -80°C. Longitudinal sections 14 μ m thick were made with a cryostat and mounted on glass slides. Sections were incubated overnight with goat anti-VAChT (Chemicon, 1:1000), rabbit anti-GAD65 (Sigma, USA, 1:1000), rabbit anti-GABA (Sigma, USA, 1:3000), guinea-pig anti-VGluT1 (1:500), rabbit anti-VGluT2 (1:50), guinea-pig anti-VGluT2(1:200), guinea-pig anti-VGluT3 (1:500), rabbit anti-NOS1 (Santa Cruz, USA, 1:500), and sheep anti nNOS (1:2000) diluted in 0.3% Triton-X, 1% normal donkey serum in 0.05M PBS, followed by Cy3 donkey anti-goat IgG (Jackson ImmunoResearch, USA, 1:400), FITC donkey anti-rabbit IgG (Jackson ImmunoResearch, USA, 1:200), Cy3 donkey anti-rabbit IgG (Rockland, 1:400, USA), Cy3 donkey anti-sheep IgG (Rockland, USA, 1:400), and Cy3 donkey anti-guinea-pig IgG (Rockland, USA, 1:400). Sections were observed with a confocal laser scanning microscope (TCS-SP2-AOBS, Leica, Germany) and photographed with a x63 objective lens (N.A.=1.4). Antibodies for VGluT1-3 [8][10] were a gift from Dr. Kaneko (Kyoto University, Japan). Antibody for nNOS raised from sheep [9] was a gift from Dr. Emson (The Babraham Institute, UK). The specificity of antibodies used in this study were already examined by other authors with Western blot(VGluT1,2[8], VGluT3[10], nNOS[9], NOS1[3], and VAChT[12]) or dot blot(GABA[16]). The specificity of anti-GAD65 was examined with Western blot, and we observed single band at 65kD(not shown).

GAD65-immunoreactivity in the SCG had a bouton-like expression. This bouton-like expression is the same as in the central nervous system. GAD65-immunopositive boutons were scattered throughout the SCG, and sometimes they had a basket-like appearance with relatively large postganglionic neurons. We used the term "basket" for

the condition in which boutons richly surround the cellbody. The number of GAD65-immunopositive baskets per one section (12 grmu m thick) were 48.6 ± 9.8 (n=5). All GAD65-immunopositive boutons exhibited VAChT immunoreactivity (Fig. 1). GAD65-immunopositive boutons and nNOS-immunopositive fibers often encircled the same postganglionic neurons and were very close to each other (not shown), so that it was difficult to judge whether co-localization existed. To overcome this problem, we used the anti-GABA antibody instead. Judging the co-localization of GABA and nNOS is easier compared to GAD65 and nNOS because the anti-GABA antibody can visualize the GABAergic fibers and boutons while the anti-GAD65 antibody visualizes the GABAergic boutons only. We found that about half of the GABA-immunopositive fibers were also immunopositive for nNOS (Fig. 2). Although the majority of the GAD65-immunopositive boutons encircled large NPY-immunonegative postganglionic neurons (Fig. 3a), as previously reported in a rat study using the anti-GABA antibody [6], about ten-per cent of the NPY-immunopositive neurons were encircled by GAD65-immunopositive boutons (Fig. 3b).

VGluT1 and 3-immunoreactivities were not found in the SCG, while the control sections (cerebral cortex, cerebellum, spinal cord) on the same slide showed marked stainings, as reported elsewhere [7][11]. Some VGluT2-immunopositive boutons were found in the SCG. In contrast to the GAD65-immunopositive boutons, they were not scattered, but gathered around relatively small numbers of postganglionic neurons. The number of VGluT2-immunopositive baskets per one section (12 grmu m thick) were 31.6 ± 9.6 (n=5). All VGluT2-immunopositive boutons exhibited VAChT-immunoreactivity (Fig. 4). All VGluT2-immunopositive boutons also showed nNOS-immunoreactivity (Fig. 5). VGluT2-immunopositive boutons encircled relatively large NPY-negative postganglionic neurons (Fig. 6).

Although almost all the GAD65-immunopositive boutons were VGluT2-negative (Fig. 7), GAD65- and VGluT2-immunopositive boutons often encircled the same postganglionic neurons and were very close each other, so that even high magnification pictures could not disprove the possibility that some GAD65-immunopositive boutons were also immunopositive for VGluT2. However, in serial confocal sections analysis (pixel size: 162x162 nm, 0.23 grmu m-thick, 23 optical sections), we found that areas of GAD65-immunoreactivity were slightly removed from areas of VGluT2-immunoreactivity. Double immunoelectron microscopic observations will help whether the colocalization of GAD65 and VGluT2-immunoreactivities exists or not.

The present study is the first report on VGluT2-immunoreactivity, and shows that

GAD65- and VGluT2-immunopositive terminals are cholinergic in mouse SCGs. In the SCG, ionotropic non-NMDA type glutamate receptor (GluR1 and 2/3) expressions have been reported [13], while NR1 subunit (an NMDA receptor component) expression has not (unpublished data). The metabotropic glutamate receptor, mGluR7, was shown to be expressed [15]. Our findings provide further evidence of the existence of glutamatergic transmission in the SCG. VGluT2-immunopositive fibers may originate from SPNs because VGluT2-immunopositive terminals also expressed VAChT. Furthermore, glutaminase-immunoreactivity was found in SPNs [17].

We also found that GABAergic terminals showed cholinergic properties. In previous studies, the authors presumed that GABAergic fibers originated from SIF-like cells in the cervical sympathetic trunk and cervical sympathetic ganglia [5][21]. However, their speculations came from indirect evidence; this being that GABAergic SIF-like cells were found in the sympathetic trunk and GABAergic fibers were more abundant in the rostral sympathetic trunk. Although they stated that GABA-immunopositive neurons were not found in the intermediolateral cell column of the spinal cord, the article they referred to was inadequate [14] because it dealt with the cervical spinal cord, which does not include intermediolateral cell column. Indeed, GABA-immunoreactivity is hardly found in the intermediolateral cell column of the normal specimen, but GABA-immunopositive SPNs were found in colchicine-treatment animals in our preliminary data. Furthermore, it has not been reported that SIF-like cells have cholinergic properties. We also did not observe VAChT-immunopositive SIF-like cells at all (data not shown).

Nitric oxide-containing neurons are the major subpopulation of SPNs. For example, half of the SPNs projecting to the adrenal medulla were immunopositive for nNOS [2]. Interestingly, almost all the VGluT2-immunopositive boutons were also immunopositive for nNOS, while about half GABA-immunopositive fibers were nNOS-negative. It is likely that VGluT2 and GAD65 are chemically distinct populations in the SCG (Fig. 8). Although a small number of GAD65-immunopositive boutons encircled NPY-positive neurons, the majority of GAD65-immunopositive boutons and almost all VGluT2-immunopositive boutons encircled large NPY-negative neurons, indicating that these boutons had a weak association with vasoconstriction. Future studies will clarify the function of these chemically coded pathways.

The authors are grateful to Dr. Takeshi Kaneko for the VGluT antibodies, and Dr. Emson for the sheep anti-nNOS antibody. This work was supported by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan (Y. Nojyo, No. 14580726).

References

- [1] H. Akiyama, T. Kaneko, N. Mizuno, and P.L. McGeer, Distribution of phosphate-activated glutaminase in the human cerebral cortex, *J. Comp. Neurol.*, 297(1990) 239-52.
- [2] D. Blottner and H.G. Baumgarten, Nitric oxide synthetase (NOS)-containing sympathoadrenal cholinergic neurons of the rat IML-cell column: evidence from histochemistry, immunohistochemistry, and retrograde labeling, *J. Comp. Neurol.*, 316(1992) 45-55.
- [3] J.Y. Chan, L.L. Wang, K.L. Wu, and S.H. Chan, Reduced functional expression and molecular synthesis of inducible nitric oxide synthase in rostral ventrolateral medulla of spontaneously hypertensive rats. *Circulation*, 104(2001) 1676-81.
- [4] E. Dobo, P. Kasa, R.J. Wenthold, and J.R. Wolff, Pronase treatment increases the staining intensity of GABA-immunoreactive structures in the paravertebral sympathetic ganglia. *Histochemistry*, 93(1989) 13-8.
- [5] E. Dobo, P. Kasa, F. Joo, R.J. Wenthold, and J.R. Wolff, Structures with GABA-like and GAD-like immunoreactivity in the cervical sympathetic ganglion complex of adult rats, *Cell Tissue Res.*, 262(1990) 351-61.
- [6] E. Dobo, F. Joo, and J.R. Wolff, Distinct subsets of neuropeptide Y-negative principal neurons receive basket-like innervation from enkephalinergic and gabaergic axons in the superior cervical ganglion of adult rats, *Neuroscience*, 57(1993) 833-44.
- [7] R.T. Fremeau, J. Burman, T. Qureshi, C.H. Tran, J. Proctor, J. Johnson, H. Zhang, D. Sulzer, D.R. Copenhagen, J. Storm-Mathisen, R.J. Reimer, F.A. Chaudhry, and R.H. Edwards, The identification of vesicular glutamate transporter 3 suggests novel modes of signaling by glutamate, *Proc. Natl. Acad. Sci. U S A.*, 99(2002) 14488-93.
- [8] F. Fujiyama, T. Furuta, and T. Kaneko, Immunocytochemical localization of candidates for vesicular glutamate transporters in the rat cerebral cortex, *J. Comp. Neurol.*, 435(2001) 379-87.

- [9] A.E. Herbison, S.X. Simonian, P.J. Norris, and P.C. Emson, Relationship of neuronal nitric oxide synthase immunoreactivity to GnRH neurons in the ovariectomized and intact female rat, *J. Neuroendocrinol.* 8(1996) 73-82.
- [10] H. Hioki, F. Fujiyama, K. Nakamura, S.X. Wu, W. Matsuda, and T. Kaneko, Chemically specific circuit composed of vesicular glutamate transporter 3- and preprotachykinin B-producing interneurons in the rat neocortex, *Cereb. Cortex.* 14(2004) 1266-75.
- [11] T. Kaneko T, F. Fujiyama, and H. Hioki, Immunohistochemical localization of candidates for vesicular glutamate transporters in the rat brain, *J. Comp. Neurol.*, 444(2002) 39-62.
- [12] M.H. Kim, M. Lu, M. Kelly, and L.B. Hersh, Mutational analysis of basic residues in the rat vesicular acetylcholine transporter. Identification of a transmembrane ion pair and evidence that histidine is not involved in proton translocation, *J. Biol. Chem.*, 275(2000) 6175-80.
- [13] H. Kiyama, K. Sato, T. Kuba, and M. Tohyama, Sympathetic and parasympathetic ganglia express non-NMDA type glutamate receptors: distinct receptor subunit composition in the principle and SIF cells, *Brain Res. Mol. Brain Res.*, 19(1993) 345-8.
- [14] T. Kosaka, M. Tauchi, and J.L. Dahl, Cholinergic neurons containing GABA-like and/or glutamic acid decarboxylase-like immunoreactivities in various brain regions of the rat, *Exp. Brain Res.*, 70(1988) 605-17.
- [15] J. Li, H. Ohishi, T. Kaneko, R. Shigemoto, A. Neki, S. Nakanishi, and N. Mizuno, Immunohistochemical localization of a metabotropic glutamate receptor, mGluR7, in ganglion neurons of the rat: with special reference to the presence in glutamatergic ganglion neurons, *Neurosci. Lett.*, 204(1996) 9-12.
- [16] M.A. Ligorio, W. Akmentin, F. Gallery, and J.B. Cabot, Ultrastructural localization of the binding fragment of tetanus toxin in putative gamma-aminobutylic acidergic terminals in the intermediolateral cell column: A potential basis for sympathetic dysfunction in generalized tetanus, *J. Comp. Neurol.*, 419(2000) 471-484.

- [17] E. Senba, T. Kaneko, N. Mizuno, and M. Tohyama, Somato-, branchio- and visceromotor neurons contain glutaminase-like immunoreactivity, *Brain Res. Bull.*, 26(1991) 85-97.
- [18] S. Takamori, J.S. Rhee, C. Rosenmund, and R. Jahn, Identification of a vesicular glutamate transporter that defines a glutamatergic phenotype in neurons, *Nature*, 407(2000) 189-94.
- [19] S. Takamori, J.S. Rhee, C. Rosenmund, and R. Jahn, Identification of differentiation-associated brain-specific phosphate transporter as a second vesicular glutamate transporter (VGLUT2), *J. Neurosci.*, 21(2001) RC182.
- [20] S. Takamori, P. Malherbe, C. Broger, and R. Jahn, Molecular cloning and functional characterization of human vesicular glutamate transporter 3, *EMBO Rep.*, 3(2002) 798-803.
- [21] J.R. Wolff, P. Kasa, E. Dobo, H.J. Romgens, A. Parducz, F. Joo, and A. Wolff, Distribution of GABA-immunoreactive nerve fibers and cells in the cervical and thoracic paravertebral sympathetic trunk of adult rat: evidence for an ascending feed-forward inhibition system, *J. Comp. Neurol.*, 334(2)(1993) 281-93.

Figure legends:

Figure 1-7: Double immunofluorescent micrographs of mouse SCGs.

Figure 1

All GAD65-immunopositive boutons (red) were also immunopositive for VAChT (green). Arrowheads: boutons immunopositive for both GAD65 and VAChT. Bar: 20 grmu m.

Figure 2

Some GABA-immunopositive fibers were also immunopositive for nNOS (arrowheads), while the other GABA-immunopositive fibers were not (arrows). Reconstructed from serial 45 images. Green: GABA-immunoreactivity. Red: nNOS-immunoreactivity. Bar: 10 grmu m.

Figure 3

a: GAD65-immunopositive boutons (green) were closely appositioned to a large NPY-negative postganglionic neuron (asterisk). Reconstructed from serial 7 images. b: Two NPY-immunopositive postganglionic neurons (+) made close appositions with GAD65-immunopositive boutons (green). Red: NPY-immunoreactivity. Bar: 50 grmu m.

Figure 4

All VGluT2-immunopositive boutons (green) were also immunopositive for VAChT (red). Arrowheads: boutons immunopositive for both VGluT2 and VAChT. Bar: 20 grmu m.

Figure 5

The localization of VGluT2-immunopositivity (green) was consistent with that of nNOS-immunoreactive varicosity (red). Arrowheads: varicosities immunopositive for both VGluT2 and nNOS. Bar: 10 grmu m.

Figure 6

VGluT2-immunoreactive boutons (red) encircled a NPY-negative postganglionic neuron (asterisk). Green: NPY-immunoreactivity. Bar: 50 grmu m.

Figure 7

Colocalizations of GAD65 (green) and VGluT2 (red) were not observed except one bouton (arrowhead). Reconstructed from serial 14 images. Bar: 20 grmu m.

Figure 8

Schematic diagram of the chemical classification of cholinergic fibers in mouse SCGs assumed in this study.

