GABA-containing sympathetic preganglionic neurons in rat thoracic spinal cord send their axons to the superior cervical ganglion.

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Title

GABA-containing sympathetic preganglionic neurons in rat thoracic spinal cord send their axons to the superior cervical ganglion.

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Abstract

GABA-containing fibers have been observed in the rat superior cervical ganglion (SCG) and, to a lesser extent, in the stellate ganglion (STG). The aim of present study is to clarify the source of these fibers. No cell body showed mRNAs for glutamic acid decarboxylases (GADs) or immunoreactivity for GAD of 67 kDa (GAD67) in the cervical sympathetic chain. Thus, GABA-containing fibers in the ganglia are suggested to be of extraganglionic origin. Since GAD67-immunoreactive fibers were found not in the dorsal roots or ganglia, but in the ventral roots, GABA-containing fibers in the sympathetic ganglia were considered to originate from the spinal cord. Furthermore, almost all GAD67-immunoreactive fibers in the sympathetic ganglia showed immunoreactivity for vesicular acetylcholine transporter, suggesting that GABA was utilized by some cholinergic preganglionic neurons. This was confirmed by the following results: 1) after injection of Sindbis/palGFP virus into the intermediolateral nucleus, some anterogradely labeled fibers in the SCG were immunopositive for GAD67, and 2) after injection of fluorogold into the SCG, some retrogradely labeled neurons in the thoracic spinal cord were positive for GAD67 mRNA. Finally, when the ventral roots of the eighth cervical to the fourth thoracic segments were cut, almost all GAD67- and GABA-immunoreactive fibers disappeared from the ipsilateral SCG and STG, suggesting that the vast majority of GABA-containing fibers in those ganglia were of spinal origin. Thus, the present findings strongly indicate that some sympathetic preganglionic

neurons are not only cholinergic, but also GABAegic.

Keywords: nerve transection, co-localization, anterograde tracing, retrograde tracing, immunohistochemistry, in situ hybridization

Abbreviations: SPN, sympathetic preganglionic neuron; VAchT, vesicular acetylcholine transporter; IML, nucleus intermediolateralis; SCG, superior cervical ganglion; STG, stellate ganglion; DRG, dorsal root ganglion; VR, ventral root; DR, dorsal root; LF, lateral funiculus; PF, posterior funiculus; cc, central canal; DH, dorsal horn; FG, fluorogold; GAD, glutamic acid decarboxylase; GFP, enhanced green fluorescent protein; SIF, small intensely fluorescent; PB, 0.1M phosphate buffer (pH 7.4); PBS, 0.05M phosphate-buffered saline (pH 7.4); PBS-X, PBS containing 0.3% Triton X-100; PBS-XD, PBS containing 0.3% Triton X-100, 1% normal donkey serum; RT, room temperature; NLS, N-lauroylsarcosine; DAB, diaminobenzidine; FITC, fluorescein isothiocyanate; TSA, tyramide signal amplification; GST, glutathione-S-transferase.

1. Introduction

Sympathetic postganglionic neurons receive many kinds of chemical inputs in the sympathetic ganglia. The chemical inputs are classified into two major groups: 1) cholinergic excitatory or facilitatory afferents, and 2) inhibitory or suppressive afferents (e.g. GABA, enkephalin, and dopamine). These inputs are derived not only from sympathetic preganglionic neurons (SPNs) in the spinal cord, but also from primary sensory neurons in the dorsal root ganglia (DRGs), enteric neurons in the gastro-intestinal tract, and small intensely fluorescent (SIF) cells within the sympathetic ganglia. It is very important for understanding the function of sympathetic ganglia (i.e. neural computation, integration of inputs, etc) to investigate both the chemical characteristics and origin of afferent fibers projecting to the ganglia.

The superior cervical ganglion (SCG) is located in the most rostral part of the sympathetic chain, and is the only sympathetic ganglion which projects to various organs in the head and face (e.g. pineal body, iris, salivary glands, cochlea, lacrimal gland, etc). Because these organs have quite unique roles in autonomic functions, the neural circuit of the ganglia is likely to be also specialized. For understanding the function of the SCG, it is very important to identify specific features (e.g. chemical properties of preganglionic fibers and postganglionic neurons, physiological properties, morphology of postganglionic neurons, etc) in the ganglion.

The usage of GABA, an inhibitory neurotransmitter in the central nervous system, in the sympathetic nervous system, including the SCG (Amenta et al., 1992; Dobo et al., 1990, 1993; Guillermo et al., 1994; Liu and Burt, 1999; Wolff et al., 1989, 1993a, 1993b) has been reported. Interestingly, GABA-immunopositive fibers are distributed in the rostral sympathetic trunk with a rostrally deviated gradient, and the number of GABA-immunopositive fibers reaches a maximum in the cervical sympathetic trunk (Wolff et al., 1993a). These GABA-containing fibers make numerous axon baskets with varicosities in the SCG (Wolff et al., 1989). These observations imply that GABA has an unique role in SCG-specific neural functions.

Wolff and colleagues argued that, in the cervical sympathetic chain, which is composed of the SCG, STG, and cervical sympathetic trunk, SIF-like cells extended their axons and projected to the SCG (Dobo et al., 1990; Wolff et al., 1993a). We recently reported that, in the mouse SCG, almost all GABA-containing varicosities were immunopositive for vesicular acetylcholine transporter (VAchT), suggesting a spinal origin of the GABA-containing cholinergic fibers (Ito et al. 2005). The DRG neurons may be another candidate for the origin of GABA-containing fibers because a few axons of the DRG fibers pass through the cervical sympathetic trunk (Murata et al., 1982), and GABA-immunopositive DRG neurons have been reported (Szabat et al., 1992). Therefore, there are three candidates for the source (SIF-like cells, SPNs, and the DRG neurons) of GABA-containing fibers in the SCG.

To reveal the source of GABA-containing fibers in the SCG, we first investigated the distribution of mRNAs for GADs of 65 and 67kDa (GAD65 and GAD67). Second, we studied possible sources by double immunohistochemistry, nerve transection, and anterograde and retrograde tracer labelings.

2. Materials and methods

2. 1. Animals

Twenty-two adult male Sprague-Dawley rats (body weight: 200-250g, Japan SLC, Shizuoka Japan) were used in this study. All animals were maintained and treated according to the Guidelines for Animal Experiments of the University of Fukui and Kyoto University. All efforts were made to minimize animal suffering and the number of animals used.

2. 2. Surgery

2. 2. 1. Anterograde tracing

Three rats were deeply anesthetized with chloral hydrate (400 mg/kg body weight, i.p.) and fixed in a stereotaxic apparatus in the prone position. A laminectomy of vertebra T1 was carried out to expose the dorsal surface of the spinal cord. About 0.6 μl of viral solution containing Sindbis/palGFP virus (Furuta et al., 2001) was injected by pressure (0.7 kg/cm^2) of N_2 gas through a glass micropipette. The injection coordinates were at 0.5 mm right of the posterior median

sulcus and 0.6 mm below the dorsal surface of the spinal cord. Overflowing liquid was carefully absorbed with kimwipe (Crecia, Tokyo, Japan). After the surgery, animals were allowed to survive for 18 hours. Throughout the survival period, a large amount of palmitoylated GFP was produced in the infected neurons, and outlined the complete neuronal morphology (i.e. Golgi-like staining). Within this survival period, the death of infected neurons was minimal (Furuta et al., 2001).

2. 2. 2. Retrograde tracing

Three rats were deeply anesthetized with chloral hydrate (400 mg/kg body weight, i.p.), supinely fixed in a stereotaxic apparatus, and injected with 1 μl of 4 % (w/v) FG in the right SCG by pressure (0.5 kg/cm^2) of N_2 gas through a glass micropipette. After the injection, animals were allowed to survive for 2 days.

2. 2. 3. Nerve transection

Seven rats were deeply anesthetized with chloral hydrate (400 mg/kg body weight, i.p.). The inferior articular processes of vertebrae C8-T4 were removed to expose the dorsal surface of the right DRGs, and dorsal and ventral roots (DRs and VRs) of C8-T4 were cut with a spring scissors.

After the surgery, animals were allowed to survive for 4 days. We dissected these animals after the perfusion and confirmed that the transection was complete.

2. 3. Immunohistochemistry

2. 3. 1. Antibodies

In this study, we used a mouse monoclonal antibody for GAD67 (MAB5406, Chemicon, Temecula, CA, USA), a goat polyclonal antibody for VAchT (AB1578, Chemicon), and rabbit polyclonal antibodies for FG (AB153, Chemicon), GFP (Tamamaki et al., 2000), and GABA (A2052, Sigma-Aldrich, St Louis, MO, USA) as primary antibodies (Table 1).

Although the specificity of the mouse monoclonal antibody for GAD67 had already been checked by immunoblotting (Fong et al., 2005), an absorption test had not been conducted. In the present study, we carried out an absorption test with the glutathione-S-transferase (GST)-GAD67 fusion protein as an antigen. First, the entire coding sequence of the rat GAD67 cDNA (GenBank accession number M76177) was subcloned into the SmaI site of pGEX-4T2 (GE healthcare bioscience, Fairfield, CT). Then, expression of GST-GAD67 was induced in *E. coli* by adding isopropyl-l-thio-beta-D-galactopyranoside to the medium. The fusion protein was extracted from

the medium with CelLytic B (Sigma), and purified on a GST-column (GE Healthcare Bio-Sciences Piscataway, NJ, USA). In immunoblot, the fusion protein was recognized by the anti-GAD67 antibody, giving a single band around 100 kDa. This molecular weight was in good agreement with the estimated molecular weight of GST-GAD67 (approximately 97 kDa).

We preincubated the anti-GAD67 antibody (1:3000) with this fusion protein (180 μg/ml) diluted in 0.3% (w/v) Triton X-100, 1% (v/v) normal donkey serum in 0.05M phosphate-buffered saline (PBS-XD; pH 7.4) for 1 hour at room temperature (RT). The sections immunostained with the anti-GAD67 antibody preabsorbed with GST-GAD67 showed no immunopositive signal in the SCG (Fig. $1e_2$), STG (Fig. $1f_2$), or brain (Fig. $1h_2$). Furthermore, GAD67 immunoreactivity in the brain (Fig. $1h_1$) was very similar to that in a previous study (Mugnaini and Oertel, 1985), and the distribution of GAD67-immunopositive cell bodies was well consistent with that of GAD67-expressing cell bodies (Fig. $2f_1$). These observations strongly indicated that the antibody was specific for GAD67.

To check the specificity of the goat polyclonal antibody for VAchT, we again performed an absorption test. We preincubated the anti-VAchT antibody (1:4000) with VAchT control peptide (1:50; AG260; Chemicon) diluted in PBS-XD for 1 hour at RT, and confirmed the absence of immunostaining (not shown). Likewise, to check the specificity of the rabbit polyclonal antibody for GABA, we preincubated the antibody (1:10000) with GABA (0.5M) diluted in PBS-XD for 1

hour at RT. The sections of the SCG incubated with the anti-GABA antibody preabsorbed with GABA showed no immunoreactivity in any region examined (Fig. 1d). The specificity of antibodies for GFP and FG was confirmed by a lack of stainings in the sections of animals not injected with Sindbis/palGFP or FG.

2. 3. 2. Tissue preparation

Animals were deeply anesthetized with an overdose of chloral hydrate (800 mg/kg body weight, i.p.) and perfused transcardially with saline, followed by a fixative containing 4% (w/v) paraformaldehyde diluted with 0.1M phosphate buffer (PB; pH 7.4). In the immunohistochemistry for GABA, we instead used 4% (w/v) paraformaldehyde, 0.05% (v/v) glutaraldehyde diluted with 0.1M PB as a fixative. The SCGs and STGs of both sides, the spinal cord, DRs, VRs, and DRGs from C8 to T4 levels were dissected out and postfixed with the same fixative for 2 hours at RT. The specimens were immersed in 30% (w/v) sucrose in 0.1M PB overnight at 4°C. After embedding in OCT compound (Pelco, Redding, CA, USA), frozen sections of the spinal cord were made transversely at a thickness of 30 μm by cryostat, and collected in 0.05M phosphate-buffered saline (PBS; pH 7.4). Sections of the DRs, VRs, DRG, STG, and SCG were made pallarel to the longitudinal axis at a thickness of 14 μm by cryostat, mounted on APS-coated glass slides, and

air-dried. Sections for the immunoperoxidase reaction were immersed in PBS containing 0.3% (v/v) H_2O_2 for 15 minutes to remove the endogenous peroxidase reactivity.

2. 3. 3. Immunohistochemistry for confocal microscopy

Sections were incubated overnight with goat anti-VAchT (1:1000), rabbit anti-FG (1:2000), rabbit anti-GFP (1:100), mouse anti-GAD67 (1:1000), or rabbit anti-GABA (1:3000) diluted in PBS-XD, followed by fluorescein isothiocyanate (FITC) donkey anti-goat IgG (1:200; Rockland Immunochemicals, Gilbertsville, PA, USA) to visualize VAchT-immunoreactivity, Cy3 donkey anti-rabbit IgG (1:400; Chemicon) for FG, Cy3 donkey anti-mouse IgG (1:400; Rockland) for GAD67, and FITC donkey anti-rabbit IgG (1:200; Chemicon) for GFP and GABA diluted in PBS-XD. As GAD-immunoreactivity in neuronal somata increases when the sections are incubated without Triton X-100 (Mugnaini and Oertel, 1985), we incubated some sections without Triton X-100 to enhance the GAD67-immunoreactivity of neuronal somata. To amplify immunopositive signals, some sections incubated with anti-GAD67 or anti-GABA were then incubated with HRP-conjugated donkey antibodies for mouse or rabbit IgG (1:100; Chemicon), respectively, and reacted with a tyramide signal amplification (TSA) fluorescein system (Perkin Elmer, Wellesley, MA, USA). Then, sections were mounted on glass slides with

VECTORSHIELD (Vector Laboratories, Burlingame, CA, USA).

2. 3. 4. Immunohistochemistry for bright field microscopy

Some sections were pre-incubated with an Avidin/Biotin Blocking Kit (Vector) to block endogenous biotin. The sections were then incubated with goat anti-VAchT (1:4000), rabbit anti-GFP (1:400), mouse anti-GAD67 (1:3000), and rabbit anti-GABA (1:3000) diluted in PBS-XD, followed by biotinylated donkey secondary antibodies (biotinylated anti-mouse IgG, biotinylated anti-rabbit IgG or biotinylated anti-goat IgG; 1:200; Rockland) diluted in PBS-XD, and further incubated with avidin-biotinylated peroxidase complex (1:50; ABC-Elite, Vector) in PBS containing 0.3% (w/v) Triton X-100 (PBS-X). In some sections of the SCG, we omitted primary antibodies, and confirmed an absence of staining. To reveal GAD67-immunoreactivity in the neuronal somata, we processed some sections without Triton X-100. These sections were used for a diaminobenzidine (DAB) or Nickel-DAB reaction. The sections were dehydrated with graded alcohols, cleared with xylene, and mounted on glass slides with Entellan (Merck, Germany).

2. 4. In situ hybridization histochemistry

2. 4. 1. Single in situ hybridization

Complementary DNA fragments corresponding to a region of the GAD67 (nucleotides 227-845, GenBank accession number BC_027059) and GAD65 (69-693, D42051) cDNAs were cloned into pBluescript II SK (+) (Stratagene, La Jolla, CA, USA). Using this plasmid as a template, sense and antisense single-strand RNA probes were synthesized with a digoxigenin labeling kit (Roche Diagnostics, Mannheim, Germany). The procedure for non-radioactive in situ hybridization was described elsewhere (Liang et al., 2000). Briefly, three rats were anesthetized with an overdose of chloral hydrate (800 mg/kg body weight, i.p.) and perfused transcardially with saline, followed by 4% paraformaldehyde diluted in PB. The whole brain, spinal cord from C8 to T5, right DRGs from C8 to T5, right SCG, and right STG with cervical sympathetic trunk were dissected out and postfixed with the same fixative for 4 hours at RT. The specimens were immersed in DEPC-treated cryoprotectant (0.1M PB containing 30% (w/v) sucrose) overnight at 4°C and cut parallel to the longitudinal axis at a thickness of 45 μm by cryostat. The free-floating sections were washed in 0.1M PB for 5 minutes twice, immersed in 0.1M PB containing 0.3% (w/v) Triton X-100, and washed in 0.1M PB. The sections were treated with acetylation solution (0.003% (v/v) acetic acid anhydrate/ 1.3% (v/v) triethanolamine/ 6.5% (w/v) HCl in DEPC-treated water) for 10 minutes at RT. After two washes in PB, they were incubated in a prehybridization solution

containing 50% (v/v) formamide (Nacalai Tesque, Kyoto, Japan)/ 5 x SSC/ 2% (w/v) blocking reagents (Roche)/ 0.1% (w/v) N-lauroylsarcosine (NLS)/ 0.1% (w/v) SDS for 1 hour at 60°C. The sections were hybridized with 1 μg/ml digoxigenin-labeled sense or antisense RNA probe for GAD67 and GAD65 in the prehybridization solution for 20 hours at 60°C and 72°C, respectively. After two washes in 2xSSC/ 50% (v/v) formamide/ 0.1% (w/v) NLS for 20 minutes at 60°C or 72°C, the sections were incubated with 20 μg/ml RNase A (Nacalai) for 30 minutes at 37°C, and washed in 2xSSC/ 0.1% (w/v) NLS for 20 minutes twice at 37°C, followed by 0.2xSSC/ 0.1% (w/v) NLS for 20 minutes twice at 37°C. The sections were blocked with 1% (w/v) blocking reagent (Roche) diluted in Tris-HCl (pH7.5), 0.15M NaCl (TS7.5) for 1 hour at RT, and incubated with an alkaline phosphatase-conjugated anti-digoxigenin antibody Fab fragment (1:2000; Roche) in 1% (w/v) blocking reagent (Roche) diluted in TS7.5 at RT overnight. The bound phosphatase was visualized by a reaction with NBT/BCIP for 4 hours at 37°C in Tris-HCl (pH9.5), 0.15M NaCl. Sections were mounted on glass slides, dehydrated, cleared with xylene, and coverslipped.

2. 4. 2. In situ hybridization combined with immunohistochemistry

Three rats which received FG in the right SCG as described above were anesthetized with an overdose of chloral hydrate (800 mg/kg body weight, i.p.) and perfused transcardially with saline,

followed by 4% paraformaldehyde diluted with 0.1M phosphate buffer. The spinal cord from C8 to T5 was dissected out and postfixed with the same fixative for 4 hours at RT. The specimens were immersed in DEPC-treated cryoprotectant (PB containing 30% (w/v) sucrose) overnight at 4°C and cut parallel to the longitudinal axis at a thickness of 45 μm by cryostat. Every second section was processed for a combination of immunoperoxidase and alkaline phosphatase reactions, and the rest were processed for a combination of immunofluorescent and fluorescent in situ hybridization histochemistries.

For double labeling bright field microscopy, the sections were washed in DEPC-treated PBS twice, and incubated with rabbit anti-FG (1:3000) diluted in 1% (w/v) blocking reagent (Roche) in DEPC-treated PBS-X at RT overnight. After three washes in DEPC-treated PBS, the sections were incubated for 1 hour in 1% (w/v) blocking reagent (Roche) in DEPC-treated PBS-X with biotinylated anti-rabbit IgG donkey antibody (1:200; Rockland), and further incubated with ABC-Elite diluted in DEPC-treated PBS-X. FG immunoreactivity was revealed as the DAB reaction product. Then these sections were processed with bright field in situ hybridization histochemistry as described above.

For double fluorescent labeling, sections were washed in DEPC-treated PBS twice, and incubated with rabbit anti-FG (1:500) diluted in 1% (w/v) blocking reagent (Roche) in DEPC-treated PBS-X at RT overnight. After three washes in DEPC-treated PBS, sections were

incubated for 1 hour in 1% (w/v) blocking reagent (Roche) in DEPC-treated PBS-X with FITC-labeled anti-rabbit IgG donkey antibody (1:200; Chemicon), and fixed with 4% (w/v) paraformaldehyde in 0.1M PB for 15 minutes. These sections were processed with in situ hybridization. To visualize alkaline phosphatase by fluorescent microscopy, sections were developed with 0.005% (w/v) FastRed (Roche), 1% (v/v) HNPP (Roche) diluted in Tris-HCl (pH8.0), 0.15M NaCl for 2 hours at RT. The sections were mounted on glass slides with Permafluor (Beckman Coulter, Fullerton, CA).

2. 5. Micrographs

All micrographs were collected digitally. Bright field micrographs were taken by CCD-camera (HC2500, Fujifilm, Japan). Fluorescent micrographs were taken by confocal laser scanning microscope (TCS-SP2-AOBS, Leica Microsystems, Germany). FITC was excited with a 488-nm laser beam and observed through a 510-560 nm AOBS emission filter. FastRed and Cy3 were excited with a 543-nm laser beam, and observed through ≥ 570 nm AOBS emission filters. We used a x63 oil-immersive objective (N.A. = 1.4; Leica) for the determination of axonal co-localization in double immunostained sections. Digital images were stored in TIFF format and edited for optimal contrast and brightness in Photoshop CS2 (Adobe Systems, San Jose, CA, USA)

for both bright field and confocal micrographs.

2.6. Counting procedures

When we counted the number of varicosities, we used a laser confocal microscope, observed the specimen with a x63 oil-immersive lens through a x4 zoom, and took digital images with a resolution of 512x512. Under the conditions, pixel size (0.12 μm) was smaller than the resolution of the lens (approximately 200 nm). We counted the number of varicosities from randomly chosen areas of 3 ganglia. When we counted the number of axon baskets, we only chose cells which were completely surrounded by varicose fibers (inset of Fig. $1e_1$). Concerning cell counting, only those cells whose nuclei were observed were chosen. Because we chose every second section for counting FG-positive cells, and the thickness of these sections (45 μm) was much greater than the mean diameter of FG-positive neuronal nuclei (13.7 ± 3.0 μm; mean ± S.D., obtained from 30 neurons of 3 animals), the occurrence of double counting error should have been avoided.

3. Results

3. 1. GABA and GAD67 immunoreactivities in the SCG and STG

In the SCG and STG, many GABA-immunopositive varicose fibers were observed, which often made basket-like structures (Fig. 1a). These basket-like structures were fewer in the STG (not shown) than in the SCG. As reported previously (Dobo et al., 1990, Wolff et al., 1993a), a few SIF-like cells showed GABA immunoreactivity (arrows in Fig. 1b, c). Most of them had GABA-immunopositive processes, and appeared to be the type I cells described by Dobo et al. (1990). GABA-immunopositive SIF-like cells were often in close proximity to GABA-immunopositive varicosities and fibers (arrowheads in Fig. 1b, c).

In the SCG, almost all GAD67-immunopositive structures were varicose fibers which frequently made basket-like structures, as observed with GABA immunostaining (Fig. 1e$_1$). We counted the number of neurons which were surrounded by GAD67-positive varicose fibers (inset of Fig. 1e$_1$). One SCG contained 387.7 ± 58.4 neurons (mean ± S.D.; n=7) surrounded densely by GAD67-immunopositive axon baskets. In the STG, only a few GAD67-positive axon baskets were observed (Fig. 1f$_1$), and 35.3 ± 26.4 neurons (mean ± S.D.; n=4) were surrounded by GAD67-immunopositive baskets. In contrast to the GABA immunoreactivity, no GAD67-immunopositive cell body was found in the ganglia (Fig. 1e$_1$, f$_1$, g). Even when sections

were incubated in the primary antibody solution without Triton X-100, and processed with the TSA method to enhance GAD67 immunoreactivity in neuronal somata, no immunoreactive cell body was observed (Fig. 1g). These findings imply that, although some SIF-like cells contain GABA, they do not produce enough GAD67 to be detected in their cell bodies.

3. 2. GAD expression in the SCG and STG

Since SIF-like cells showed GABA immunoreactivity, we examined whether these small cells express GAD mRNA by in situ hybridization histochemistry, but detected no GAD67 or GAD65 mRNA signal in the SCG, STG (Fig. 2a-d), or cervical sympathetic trunk (not shown). With the same antisense probes and histochemistry, strong signals for GAD65 (Fig. $2e_1$) and GAD67 (Fig. $2f_1$) mRNAs were observed in many brain regions as reported previously (Esclapez et al., 1993). These results indicate that the paravertebral ganglionic cells express little, if any, mRNA for the GADs.

3. 3. GABAergic neuronal somata in the thoracic spinal cord and GABAergic fibers in the ventral roots.

In the gray matter of the thoracic spinal cord, including the intermediolateral nucleus (IML),

many neurons expressed mRNAs for GAD65 and GAD67 (Fig. 3a_1, b_1). However, no mRNA signal for GAD65 or GAD67 was observed in the DRGs (Fig. 3c, d), indicating that no DRG cell has synthetic mechanisms for GABA. Moreover, although the presence of GABA-immunopositive DRG neurons was reported previously (Szabat et al., 1992), we did not detect any GABA- (Fig. 3e) or GAD67-immunopositive structures (Fig. 3h) in the DRGs. Nor were observed GABA- or GAD67-immunopositive fibers in the DRs of the proximal or distal side of the DRGs (Fig. 3f, i). In contrast, many GABA- and GAD67-immunopositive fibers were found in the VRs (Fig. 3g, j), suggesting that some GABAergic efferents ran from the spinal cord to the periphery.

3. 4. Origin of GABAergic fibers in the SCG and STG

The presence of GABA- and GAD67-immunopositive fibers in the VRs implies that GABAergic fibers in the SCG originate in the spinal cord. Because all spinal cord neurons which send axons to the periphery are known to be cholinergic, GAD67-immunopositive fibers in the SCG and STG were expected to display immunoreactivity for VAchT. Actually, almost all GAD67-immunopositive varicosities showed immunoreactivity for VAchT in the ganglia (97.1%; 268/276 randomly chosen GAD67-immunopositive varicosities; n=3 ; Fig. 4a-c'').

In an attempt to confirm the spinal origin of GAD67-immunopositive fibers in the SCG and STG, GAD67 immunoreactivity was studied in the anterogradely labeled preganglionic fibers. After a small injection of Sindbis/palGFP virus into the lateral horn of the T1 segment, many neurons were infected with the virus (Fig. 4d), and some of them were located in the IML (box in Fig. 4d; Fig. 4e). Some GFP-labeled axons were observed in the ipsilateral STG and SCG, and had extensive varicose branches and basket-like structures (Fig. 4f). In all 3 cases injected with the virus, GAD67 immunoreactivity was observed in some GFP-positive varicose structures (arrowheads in Fig. 4g-g").

We next examined whether retrogradely labeled SPNs showed GAD67 mRNA signals or not. After the injection of FG into the SCG, many neurons in the IML of C8-T5 spinal segments and a few neurons around the central canal were retrogradely labeled, being well consistent with previous reports (Rando et al., 1981; Strack et al., 1988). With the combination of FG immunofluorescence and fluorescent in situ hybridization for GAD67, some retrogradely labeled neurons showed mRNA signals for GAD67 (arrow in Fig. 5a-a''). To further clarify the distribution of GAD67-expressing SPNs, we performed combined staining with an immunoperoxidase reaction for FG and an alkaline phosphatase reaction for GAD67 mRNA (Fig. 5b-d) from every other section collected. In all, 540 ± 42 (mean ± S.D.; n=3) neurons showed FG immunoreactivity, and 15 ± 3 neurons (2.78 ± 0.5 %) were positive for both FG and GAD67. They were located mainly in the rostral part of the IML

and, to a much lesser extent, in other parts of the IML and around the central canal (Fig. 5d-f). Actually, the number of these neurons was significantly larger in the most rostral part of the IML (bar 0-2.3mm in Fig. 5e) than any other part of the IML and around the central canal (Tukey's multiple analysis, $p<0.05$). GAD67-expressing SPNs tended to be located in the central and lateral part of the gray matter (Fig. 5f), although no significance was detected (ANOVA, $p=0.073$). Although the morphological reconstruction was limited by insufficient labeling of FG, GAD67-expressing (A-6, B-4-18, C-10 in Fig. 5c) and non-expressing SPNs (A-N1, B-N1, 2, C-N1 in Fig. 5c) displayed no obvious differences in the shape of cell bodies and proximal dendrites. Thus, at least some GABA- and GAD67-immunopositive fibers in the SCG and STG were considered to be of spinal origin.

3. 5. The effect of VR transection.

The ventral roots of C8 to T4 were transected to examine whether or not the SCG and STG had other sources of GAD67-immunopositive fibers than the spinal cord. Almost all GAD67-immunopositive structures disappeared from the SCG and STG on the operated side (Fig. 6a, c), whereas GAD67-immunopositive structures remained almost unchanged in the contralateral ganglia (Fig. 6b, d). Furthermore, when the sections were immunostained for GABA, not only

GABA-immunopositive fibers but also GABA-immunopositive SIF-like cells disappeared from the sympathetic ganglia of the operated side (Fig. 6e). On the contralateral side, however, GABA-immunopositive structures, including varicose fibers (Fig. 6f) and SIF-like cells (inset in Fig. 6f), were observed as in the normal ganglia. These results indicate that almost all GABAergic fibers in the SCG come from the spinal cord through the ipsilateral VRs.

4. Discussion

In this study, we demonstrated that 1) GABAergic neurons were present in the IML, but not in the sympathetic chain or DRGs, 2) GABAergic fibers were present in the VRs but not in the DRs, 3) almost all GAD67-positive varicosities showed immunoreactivity for VAchT in the sympathetic ganglia, 4) GAD67 was expressed in the terminals and somata of SPNs, and 5) GABAergic fibers in the sympathetic ganglia disappeared after the VR transection. These observations clearly indicate that almost all GABAergic fibers in the cervical sympathetic chain originate from SPNs. The fact that some SPNs express GAD67 implies that these neurons co-release both acetylcholine and GABA.

4. 1. Technical consideration

Because there is a limitation to the sensitivity of in situ hybridization histochemistry, especially in the case of GAD65, the number of GAD-expressing cells might be underestimated. Therefore, we cannot exclude the possibility that SIF cells and DRG neurons express a small amount of GAD65, but not GAD67. However, this is unlikely because GAD65 and GAD67 are known to be expressed in the same neuron (Esclapez et al., 1993). Even if SIF cells and DRG neurons contain GAD65, these cells seem to have less importance in the GABAergic fibers in the SCG, because 1)

GABAergic fibers were found in the VRs, but not in the DRs, 2) no GABAergic SIF-like cell was found in the VRs, and 3) GABAergic fibers disappeared after the transection of the ventral roots.

Since the immunoperoxidase reaction product interfered with the alkaline phosphatase reaction, it is possible that the number of GAD67-expressing SPNs was underestimated in Figure 5b-f. We estimated the number of GAD67-expressing SPNs projecting to the unilateral SCG at about 30 because we observed about 15 neurons in half of all serially collected sections. Since a single preganglionic axon branches into 11-14 terminal baskets in the SCG (Asamoto et al., 1997), the number of GAD67-positive basket may be about 330-420, being in good agreement with our observation (387.7 ± 58.4 baskets within an SCG). Thus, the underestimation, if any, may not greatly affect our conclusion.

4.2. GABA immunoreactivity in SIF-like cells and DRG neurons

Wolff and colleagues emphasized the existence of GABAergic SIF-like cells in the cervical sympathetic chain (Dobo et al., 1990; Wolff et al., 1993a). Although we found GABA-immunopositive SIF-like cells in the SCG and STG (Fig. 1b, c), we did not observe SIF-like cells expressing GAD65 or GAD67. Similarly, although GABA-immunopositive DRG neurons were reportedly detected using monoclonal antibody for GABA (Szabat et al., 1992), we did not

detect a mRNA signal for GAD65 or GAD67 in the DRG or GAD67 immunoreactivity in the DRs. Thus, neither DRG neurons nor SIF-like cells showed evidence of GABA production. It is suggested that GABA-immunopositive SIF-like cells do not produce GABA, but take up and accumulate GABA released from the GABAergic terminals. Actually, the disappearance of GABA-immunopositive SIF-like cells after the transection of VRs is consistent with this suggestion.

4. 3. Unique properties of SPNs located in the most rostral part of the IML

One of the most interesting results from this study is that most GABAergic SPNs are located in the most rostral part, about C8-T2, of the IML. The rostral IML is known to have some unique properties: SPNs in this area receive strong orexin input (Llewellyn-Smith et al., 2003) and much less serotonin input than SPNs in more caudal segments (Jensen et al., 1995). In addition, neurokinin-1 receptor-immunopositive SPNs are fewer in T1 and T2 than in the more caudal segments (Llewellyn-Smith et al., 1997). These findings suggest that rostral SPNs are chemically coded, and may have a specific function. These characteristics of rostral SPNs, which mainly project to the SCG, are likely to be associated with the functions of head- and face-specific organs (e.g. the pineal body, iris, salivary glands, cochlea, lacrimal gland, etc).

4. 4. Functional considerations

Wolff and colleagues proposed the "feed-forward inhibition" mechanism (Wolff et al., 1993a), in which SPNs excited SIF-like cells in the cervical sympathetic chain, and GABA-immunopositive SIF-like cells sent the axons to the SCG and inhibited the postganglionic neurons. However, in the present study, we showed that 1) no signal for GAD mRNAs was detected in the cervical sympathetic chain, and 2) all GAD67-immunopositive varicosities were cholinergic and of spinal origin. Thus, the "feed-forward inhibition" mechanism seems unlikely in the sympathetic ganglia.

Because both acetylcholine receptors and $GABA_A$ receptors are expressed in the SCG (Amenta et al., 1992; Liu and Burt, 1999), the present findings imply the co-release of excitatory (i.e. acetylcholine with nicotinic receptor) and “inhibitory” (i.e. GABA with $GABA_A$ receptor) neurotransmitters from some preganglionic fibers in the SCG. Dobo and colleagues (1993) reported that GABA-immunopositive fibers mainly innervated NPY-negative large postganglionic neurons. Since about half of all postganglionic neurons are NPY-positive vasoconstrictors (Gibbins, 1995), it can be postulated that half of all GAD-positive baskets would encircle NPY-positive postganglionic neurons if GABAergic fibers randomly choose the postganglionic target. However, only 10% of GAD-immunopositive baskets actually encircled NPY-positive postganglionic neurons (Ito et al., 2005), suggesting that GABAergic baskets do not prefer

NPY-positive vasoconstrictor neurons. Because the majority of NPY-negative large postganglionic neurons are involved in the secretomotor pathway (Gibbins, 1995), and postganglionic neurons which are encircled by GABA-positive baskets exit the SCG through the internal carotid nerve (Wolff et al., 1989), postganglionic neurons encircled by GABA-positive preganglionic fibers may regulate the activity of glands located in the cranium.

Since the resting membrane potential is −45~−90 mV (Adams and Harper, 1995) and the reversal potential for chloride ion is −40~−50 mV in the SCG neurons (Sacchi et al., 1999), GABA may depolarize these neurons. On the other hand, because the threshold for action potential production is 12.2 mV in the SCG neurons (Adams and Harper, 1995), activation of $GABA_A$ receptors may inhibit action potential production in postganglionic neurons. It seems strange that fibers from a single SPN co-release neurotransmitters which have opposite effects on action potential production.

Nevertheless, the co-expression of excitatory and inhibitory neurotransmitters has been reported in other regions of the brain such as granule cells in the hippocampus (Gutierrez, 2003), interneurons in the neocortex (Hioki et al., 2004), and immature lateral superior olivary neurons (Gillespie et al., 2005), although the function remained unclear.

It has also been reported that GABA strongly inhibits long-term potentiation via $GABA_A$ receptors in the rat SCG (Guillermo et al., 1994). As noted above, GABAergic preganglionic

fibers preferentially innervate NPY-negative secretomotor neurons, and associate with only a small number of NPY-positive postganglionic neurons. Therefore, postganglionic neurons innervated by GABAergic fibers, which may be associated with secretomotor functions, may be inhibited from producing long-term potentiation.

However, there is no direct evidence of targets which receive sympathetic postganglionic innervation related to the spinal preganglionic GABAergic input, or that the LTP blockade phenomenon caused by stimulating GABAergic fibers exists. There also arises the question of whether the chloride equilibrium potential for cells surrounded by GABAergic baskets differs from that those for the other postganglionic neurons or not. The identification of postganglionic neurons richly innervated by GABAergic fibers, and morphological, electrophysiological, and pharmacological studies of these neurons are needed.

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Figure legends:

Figure 1 (monochrome)

Immunostaining for GABA and GAD67 in the SCG and STG. Many GABA-immunopositive varicose fibers (a) and a few small GABA-immunopositive cells (arrows in b, c) were observed in the ganglia after staining with the TSA method. These GABA-immunopositive cells were often in close proximity to GABA-immunopositive varicose fibers (arrowheads in b, c). No immunoreactivity was observed when sections were incubated with anti-GABA antibody preabsorbed with GABA (d). GAD67-immunoreactive varicose fibers were observed frequently in the SCG (e_1) and less frequently in the STG (f_1), and often made basket-like structures (inset in e_1), but no immunoreactive cell body was found in the ganglia even when the sections were incubated in the primary antibody solution without Triton X-100 and processed with the sensitive TSA method (g). No immunoreactivity was observed in the ganglia (e_2, f_2) or brain (h_2) when the sections were incubated with anti-GAD67 antibody preabsorbed with GAD67-GST fusion protein. Scale bars: 25 μm in a, d-g, 10 μm in b, c, and 2 mm in h.

Figure 2 (monochrome)

GAD-expression in the SCG and STG. No cell body which expresses GAD65 mRNA was found in the SCG (a) or STG (b). Nor was found GAD67 mRNA expression in the SCG (c) and STG (d).

Brain sections reacted with GAD65 (e_1) and GAD67 (f_1) antisense riboprobes showed strong signals in many regions as reported previously. No signal was observed in the brain sections reacted with the sense probe for GAD65 (e_2) or GAD67 (f_2). Note: the olfactory bulb and inferior colliculus are not included in the section of e_1. Scale bars: 50 μm in a-d, and 2 mm in e, f.

Figure 3 (monochrome)

GAD-expression in the thoracic spinal cord and DRGs. Many neurons expressed mRNAs for GAD65 (a_1) and GAD67 (b_1) in the gray matter of thethoracic spinal cord, including the intermediolateral nucleus (IML) and region around the central canal (cc). On the other hand, no GAD65 (c) or GAD67 (d) mRNA-expressing cell was found in the DRGs of C8-T5 spinal segments. Moreover, no cell body or fiber immunopositive for GABA was observed in the DRGs (e) or DRs (f) of C8-T5 spinal segments. Nor was observed GAD67 immunoreactivity in the DRGs (h) and DRs (i). In contrast, GABA- (g) and GAD67- (arrows in j) immunopositive fibers were observed in the VRs. No staining was observed when the sections were reacted with the sense riboprobe (a_2, b_2). In the immunohistochemistry, sections were incubated in the primary antibody solution without Triton X-100. In the immunohistochemistry for GABA (e, g), the TSA method was used for signal enhancement. Scale bars: 50 μm in a, b, e, f, h, i, 400 μm in c, d, and 20 μm in g, j.

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Figure 5 (color figure)

GABA-producing SPNs in the thoracic spinal cord. In sections stained with fluorescent dyes (a; FG immunoreactivity is represented by green in a, and GAD67 mRNA signal is represented by magenta in a''), some retrogradely labeled SPNs showed signal for GAD67 mRNA (arrows in a-a'') after the injection of FG into the right SCG. Combined immunoperoxidase staining for FG (visualized with DAB; brown structures in b) and alkaline phosphatase in situ hybridization for

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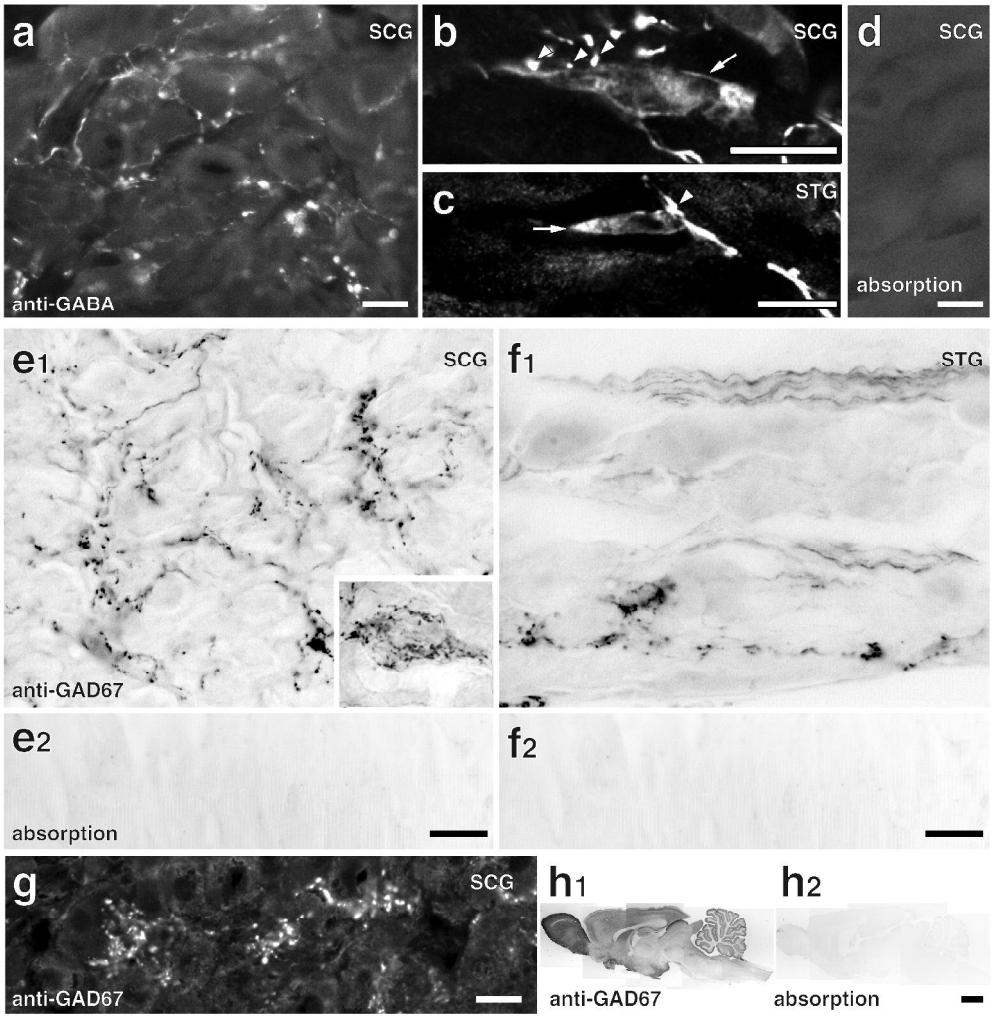

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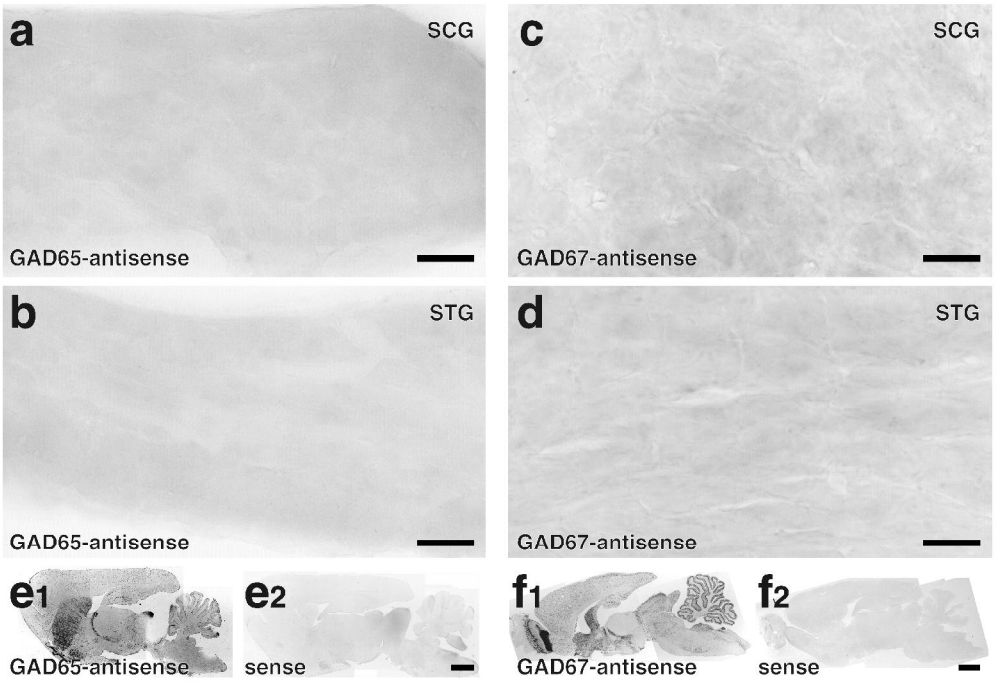

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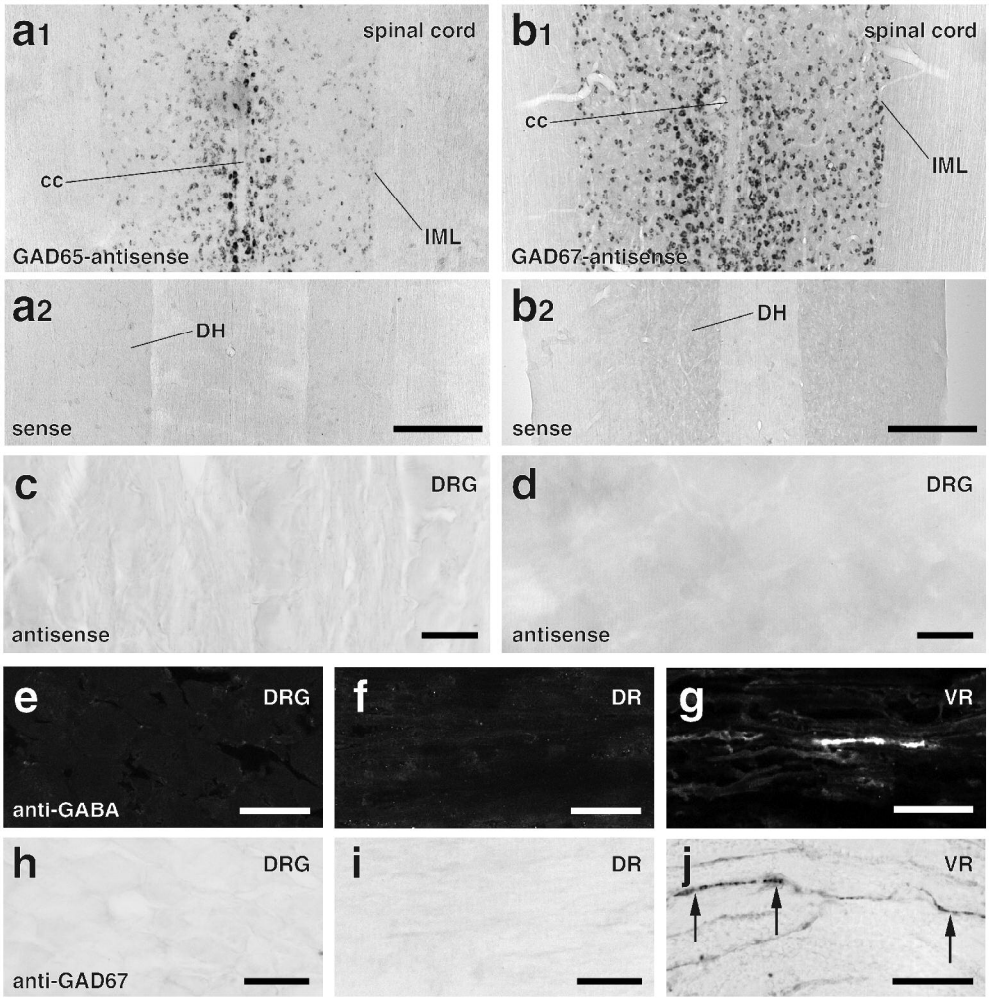

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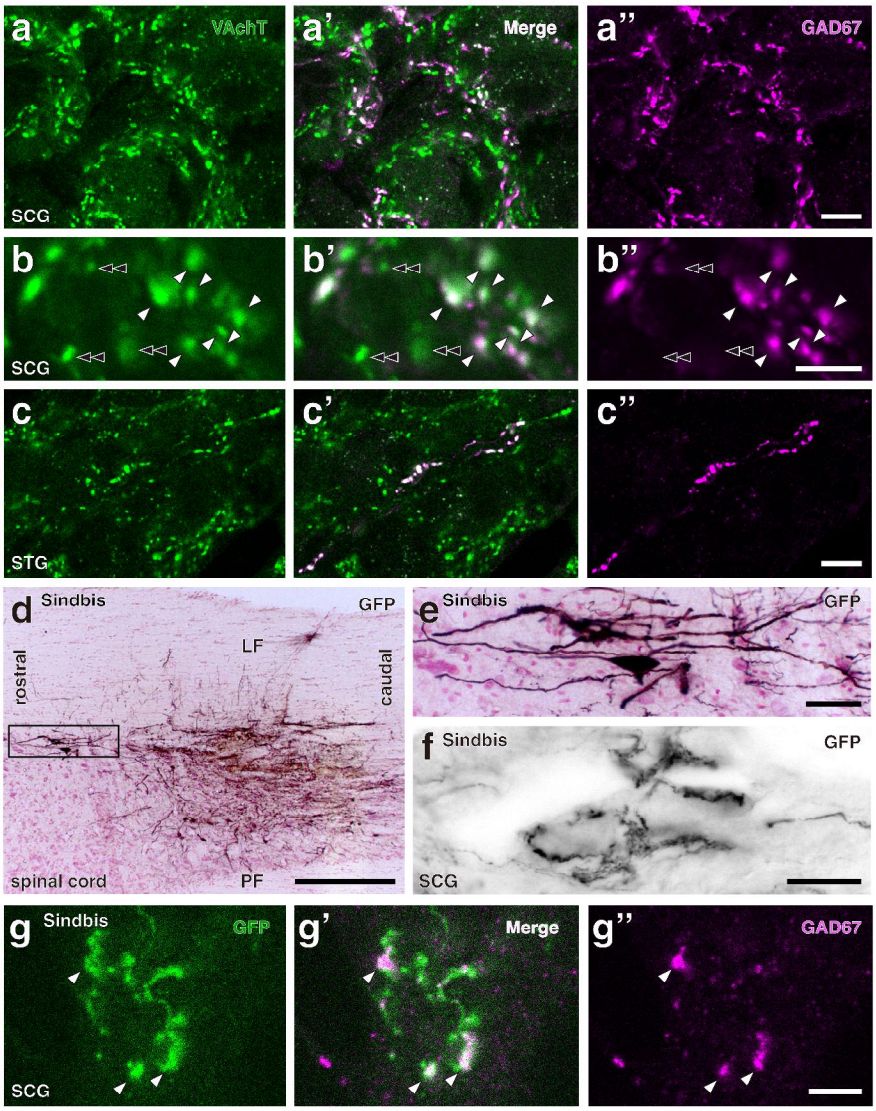

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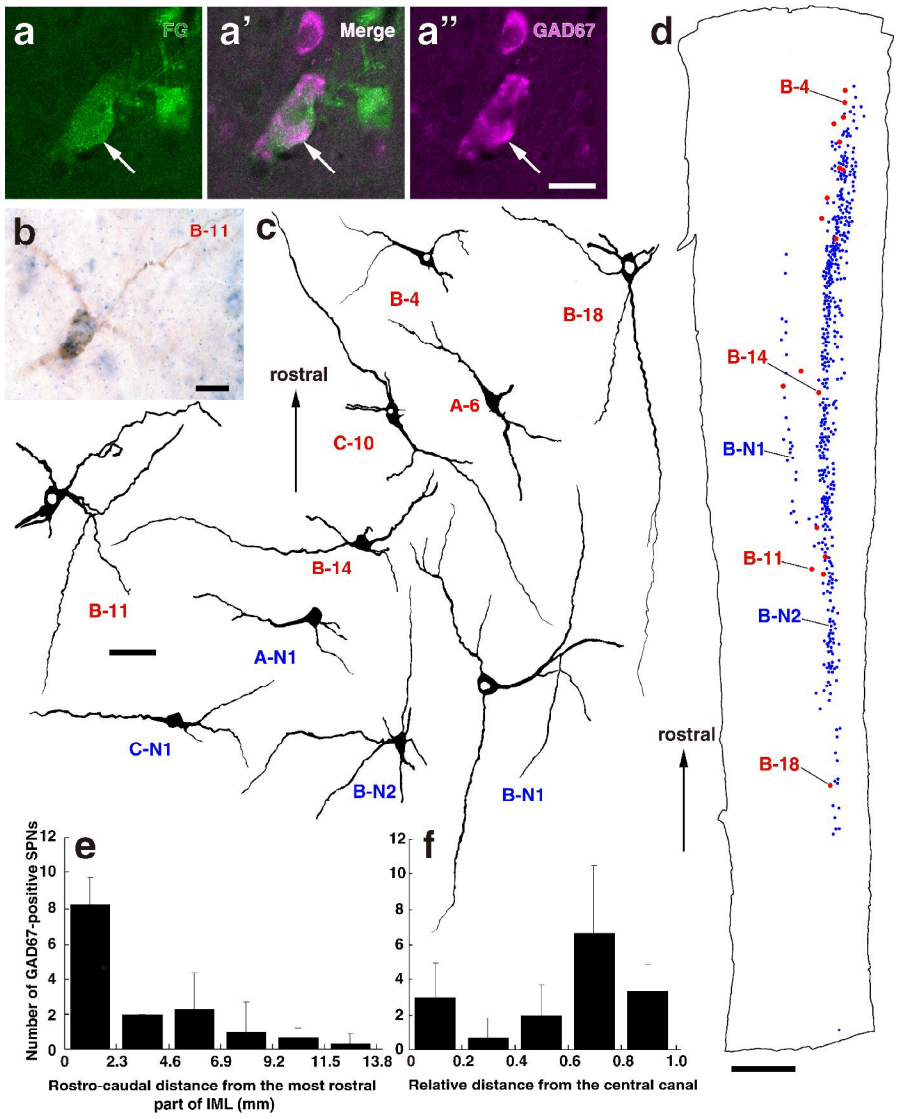

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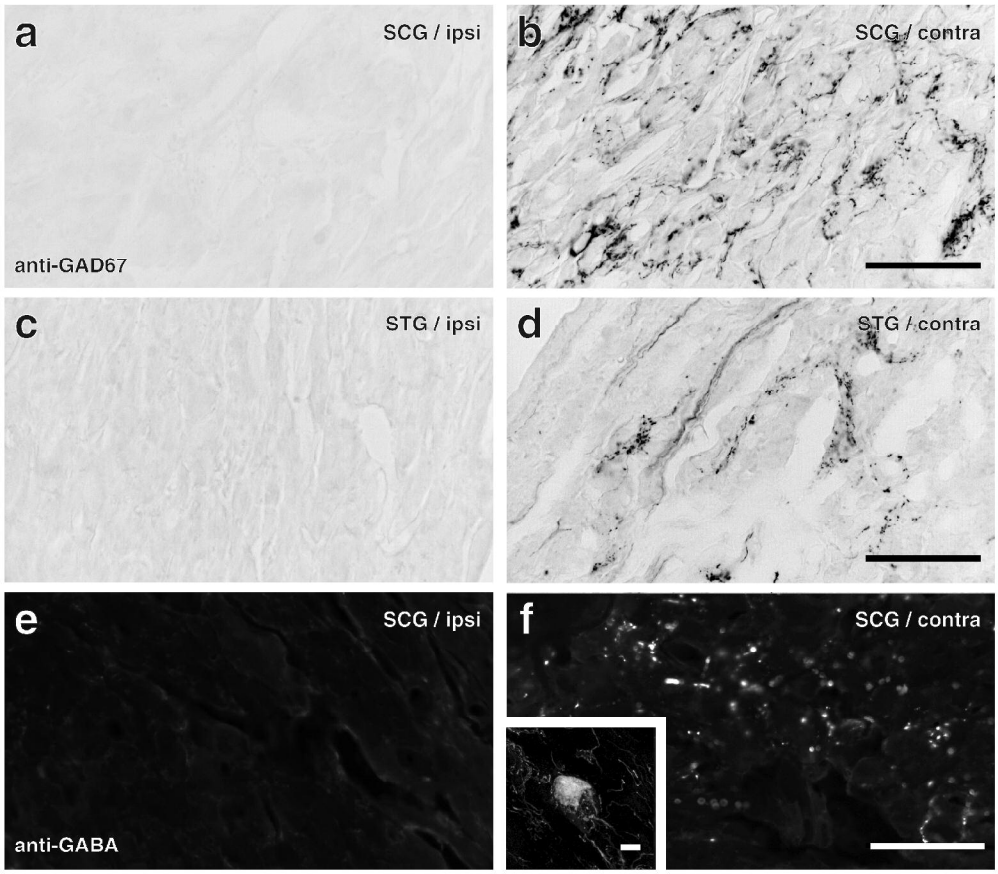

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Antibody	Host	Mono or polyclonal	Source	Catalog no.	Lot no.	Antigen	Specificity
Anti-vesicular acetylcholine transporter (VAchT)	Goat	Poly	Chemicon	AB1578	18112610	Synthetic peptide corresponding to C-terminus of cloned rat VAchT (CSPPGPFDGCEDDYNYYSRS; suplied by Chemicon on request)	Absorbed with control peptide (AG260, Chemicon); single band around 65-70kDa on immunoblot (http://www.chemicon.com/browse/productdetail.asp?ProductID=ab1578).
Anti-gamma aminobutyric acid (GABA)	Rabbit	Poly	Sigma-Aldrich	A2052	101K4837	GABA conjugated with bovine serum albumin (GABA-BSA)	Absorbed with GABA; only GABA-BSA, but not glycine-BSA, was detected on dot blot (Ligorio et al.,2000).
Anti-glutamic acid decarboxylase of 67kDa (GAD67)	Mouse	Mono	Chemicon	MAB5406	25080061	Recombinant GAD67 protein	Absorbed with GST-GAD67 fusion protein; single band around 67kDa on the immunoblot (Fong et al., 2005); immunoreactivity was in very good agreement with a previous study (Mugnaini and Oeltel, 1985).
Anti-fluorogold	Rabbit	Poly	Chemicon	AB153	0509010863	Fluorogold	Lack of immunoreactivity in the sections of animals which were not injected with fluorogold.
Anti-enhanced green fluorescent protein (GFP)	Rabbit	Poly	Prof. T. Kaneko (Kyoto Univ)	N/A	N/A	Recombinant GFP protein	Lack of immunoreactivity in the sections of animals which do not express GFP; affinity purified (Tamamaki et al., 2000).