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ORIGINAL PAPER

Localization of substance P (SP)-immunoreactivity in the myenteric plexus of the rat esophagus

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Abstract

The present immunohistochemical study was performed to examine the number, distribution, and chemical coding of intrinsic substance P (SP) neurons and nerve fibers within the esophagus and discuss their functional roles. Many SP neurons and nerve fibers were found in the myenteric plexus, and the SP neurons gradually decreased from the oral side toward the aboral side of the esophagus. Double-immunolabeling showed that most SP neurons were cholinergic (positive for choline acetyltransferase), and few were nitrergic (positive for nitric oxide synthase). Some cholinergic SP nerve terminals surrounded cell bodies of several myenteric neurons. In the muscularis mucosa and lower esophageal sphincter, and around blood vessels, numerous SP nerve endings were present, and many of them were cholinergic. Also, SP nerve endings were found on only a few motor endplates of the striated muscles, and most of them were calcitonin gene-related peptide (CGRP)-positive. Retrograde tracing using Fast Blue (FB) showed that numerous sensory neurons in the dorsal root ganglia (DRGs) and nodose ganglion (NG) projected to the esophagus, and most FB-labeled SP neurons were CGRP-positive. These results suggest that the intrinsic SP neurons in the rat esophagus may play roles as, at least, motor neurons, interneurons, and vasomotor neurons, which are involved in local regulation of smooth muscle motility, neuronal transmission, and blood circulation, respectively. Moreover, SP nerve endings on only a minority of motor endplates may be extrinsic, derived from DRGs or NG, and possibly detect chemical circumstances within motor endplates to modulate esophageal motility.

Keywords Substance P · Intrinsic neurons · Motor endplates · Esophagus · Immunohistochemistry

Abbreviations

Ach	Acetylcholine
AMCA	Amino methyl coumarin
CGRP	Calcitonin gene-related peptide
ChAT	Choline acetyltransferase
DMV	Dorsal motor nucleus of the vagus
DMSO	Dimethyl sulfoxide
DRG	Dorsal root ganglion
FB	Fast Blue
GAL	Galanin
IR	Immunoreactivity
LES	Lower esophageal sphincter
M-ENK	Methionine-enkephalin
NA	Nucleus ambiguus
NG	Nodose ganglion
NK1R	Neurokinin 1 receptor
NOS	Neuronal nitric oxide synthase
NPY	Neuropeptide Y
PBS	Phosphate-buffered saline
SP	Substance P
TK	Tachykinin
TRPV1	Transient receptor potential ion channel of the vanilloid type 1
VIP	Vasoactive intestinal peptide

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Introduction

Substance P (SP), an undecapeptide, is a member of the tachykinin (TK) neuropeptide family, which includes neurokinin A and neurokinin B (Nakanishi 1987). It consists of 11 amino-acid residues and is largely distributed in numerous primary sensory neurons of the vagal (nodose and jugular) and spinal (dorsal root) ganglia (Dalsgaard et al. 1985; Ju et al. 1987; Helke and Hill 1988). A subset of their visceral

afferents containing SP and/or calcitonin gene-related peptide (CGRP) contributes to gut physiology by innervating the gastrointestinal tract (Holzer and Holzer-Petsche 1997a, b). In addition, intrinsic SP-containing neurons and nerve fibers located in the enteric nervous system have various putative physiological functions, including secretion, motility, and blood circulation in the gastrointestinal tract (Walling et al. 1977; Jensen and Gardner 1979; Costa et al. 1981; Domoto et al. 1983; Bartho and Holzer 1985; Daniel et al. 1985; Ekblad et al. 1987).

Myenteric SP-containing neurons and intrinsic and extrinsic SP nerve fibers are also present in the esophagus of some species, including cats, humans, pigs, opossums, and dogs (Leander et al. 1982; Aggestrup et al. 1986; Wattchow et al. 1987; Christensen et al. 1989; Sandler et al. 1993), but their number, distribution, and functions remain unclear. In the present immunohistochemical study, we examine the number and distribution of SP neurons, and intrinsic and extrinsic SP nerve fibers, throughout the rat esophagus and discuss their functions.

Materials and methods

Animals

Eighteen male Wistar/ST rats (200–230 g body weight; Shimizu Laboratory Supplies, Kyoto, Japan) were used in this study. Animals were housed in plastic cages lined with clean wooden chips, kept at 24 ± 2 ℃, and supplied laboratory chow and tap water ad libitum. All experiment procedures were carried out according to the Guide for the Care and Use of Laboratory Animals of the National Institute of Health in the USA and the guidelines of Kyoto Institute of Technology. We used the minimum possible number of animals, and took steps to minimize their suffering. The Animal Experiment Committee at Kyoto Institute of Technology has approved the animal care procedures and protocols of the experiment (authorization no. 100176 in 2021).

Administration of colchicine

To enhance SP-immunoreactivity in the neuronal cell bodies, colchicine (2 mg/kg; Nacalai Tesque, Kyoto, Japan) diluted with physiological saline was intraperitoneally administered to the rats, which were then maintained at 24 ± 2 ℃ for 18 h before they were killed.

Tissue preparation

Following mixed anesthesia with intraperitoneal administration of pentobarbital (50 mg/kg, i.p.) and inhalation of isoflurane, 14 rats were perfused from the heart with 150 mL of physiological saline, and then their entire esophagus was removed. The esophageal lumen was inflated by injection of Zamboni solution (2% paraformaldehyde + 15% saturated picric acid solution), with both ends of the esophagus ligated with a thread. The swollen esophagus was immersed for 18–24 h in the Zamboni solution at 4 ℃. It was then cut open longitudinally to prepare wholemounts, treated thrice with dimethylsulfoxide (DMSO) for 10 min, and washed thrice with 0.01 M phosphate-buffered saline (PBS; pH 7.3) for 10 min. The tissue was stored in 0.1% sodium-azide-containing PBS at 4 ℃ until it was processed for immunohistochemistry. After the mucosal layer and muscle coat had been separated, the inner muscle layer of the muscle coat was removed to prepare a wholemount of the outer muscle layer attached to the myenteric plexus.

Four rats anesthetized as described above were perfused from the heart with 150 mL of physiological saline, followed by 200 mL of Zamboni solution for fixation. The entire esophagus and lower esophageal sphincter (LES) were removed and immersed for 18–24 h in the same fixative at 4 ℃. The tissues were then treated thrice with DMSO for 10 min, washed thrice with PBS for 10 min, and stored in PBS containing 30% sucrose and 0.1% sodium azide at 4 ℃. The LES and the esophagus, which was divided into five segments (as described below), were embedded in OCT Compound (Sakura Finetek, Tokyo, Japan) and frozen at −40 ℃. Fifteen-micrometer transverse and longitudinal sections of the segments and the LES, respectively, were made using a cryostat (Sakura Seiki, Tokyo, Japan), mounted on poly-L-lysine-coated glass slides, and allowed to dry overnight.

Immunohistochemistry

Wholemounts and frozen sections of the esophagus were treated for 2–3 days and 1 h, respectively, with PBS containing 0.3% Triton X-100, exposed for 30 min to normal donkey serum (1:10; Chemicon International, Temecula, CA, USA) to decrease nonspecific immunoreactions, and washed thrice with PBS for 10 min. For double-immunolabeling, the wholemounts or frozen sections were incubated for 24–48 h with a mixture of two primary antibodies, washed thrice with PBS for 10 min, and incubated for 2 h with a mixture of two secondary antibodies. Triple-immunostaining was also performed to investigate relationships between nerve terminals with different chemical coding on motor endplates. After some wholemount preparations were double-immunolabeled with a mixture of SP and NOS or SP and CGRP antibodies, they were singly labeled with ChAT antibody for 12–18 h, followed by treatment with a biotin-labeled secondary antibody for 2 h and then with an AMCA-labeled streptavidin (42788, Jackson ImmunoResearch, West Grove, PA, USA) for 1 h.

All incubations were performed at room temperature. Table 1 gives details of the primary and secondary antibodies used. To determine the specificity of three SP antibodies, we immunostained wholemounts with rabbit anti-SP antibody (AB1566), rabbit anti-SP antibody (Y150), and guinea-pig SP antibody, which were preabsorbed with a full-length SP peptide (Peptide Institute, Osaka, Japan): 20 µg/mL, 20 µg/mL, and 40 µg/mL of diluted antibody, respectively. The specificities of NOS, ChAT, and CGRP antibodies have been described previously (Kuramoto et al. 1999; Kuramoto et al. 2004; Kuramoto et al. 2006).

Retrograde tracing

Three rats were anesthetized with pentobarbital sodium (25 mg/kg, i.p.) followed by inhaled isoflurane, and the abdominal esophagus and stomach were exposed after the midline of the abdomen was incised. Using a glass micropipette connected to a 10-µL microsyringe through polyethylene tube, 4–5 µL of 2% Fast Blue (FB; Polysciences, Warrington, PA, USA) dissolved in distilled water as a neural retrograding tracer was injected into four or five locations on the ventral side of the esophageal wall at 1.0–1.5 cm proximal to the lower esophageal sphincter. To avoid leakage or diffusion of the tracer, much care was taken. Then, the wound of the abdomen was sutured, enrofloxacin (0.5 mg/kg; Bayer, Tokyo, Japan) as an antibiotic was injected into the femoral muscle, and the rats were left in the recovery cage. Three or four days after surgical operation, the rats that were deeply anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and inhaled isoflurane were fixed by perfusion of Zamboni solution, and then the bilateral thoracic dorsal root ganglia (DRGs; T8 or T9), nodose ganglion (NG), and medulla oblongata were removed to process for frozen sections in the same way as described above.

Observation and data analysis

Each esophageal wholemount preparation was divided into five segments of equal length from the cervical to the abdominal end (Fig. 2d). In esophageal segments from eight rats, we counted the number of cell bodies of immunoreactive neurons in the myenteric plexus via a fluorescence microscope (Axioskop, Zeiss, Germany), and estimated the densities of positive neurons per 1 cm^2. In wholemount preparations from two animals, we selected 50 blood vessels to which varicose SP nerve fibers ran parallel to determine the extent of colocalization of SP and ChAT or NOS in the perivascular fibers.

Using the frozen sections, we examined the distribution of immunoreactive nerve fibers or endings throughout the esophagus (from the mucosa to the tunica muscularis) and the LES. We calculated the densities of immunoreactive nerve endings (number per 10^4 μm^2) in the muscularis mucosa in each segment and the LES. We imaged positive neurons and nerve fibers or endings using a charge-coupled device camera system (Visualix Pro2, Mitani Corporation, Tokyo, Japan) connected to the fluorescence microscope and a confocal laser microscope (FV10i, Keyence, Tokyo, Japan). The number of immunoreactive neurons in each segment was expressed as mean ± standard deviation (SD). The data were statistically analyzed using one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test, in R (R Core Team 2021) to examine whether the number of cells differed significantly among the segments.

In the retrograde tracing experiment, neurons showing moderate-to-bright labeling for FB in the DRGs and NG were selected, and the FB-labeled neurons and those showing SP-immunoreactivity (IR) or CGRP-IR occurring in six cryostat sections taken through the bilateral DRGs and NG were counted. Results are expressed as mean ± SD.

Table 1 Characteristics of primary and secondary antibodies

Antigen	Host	Dilution	Code and source
Primary			
SP	Rabbit (polyclonal)	1:5000	AB1566/Millipore
SP	Rabbit (polyclonal)	1:10,000	Y150/Yanaihara Institute
SP	Guinea pig (polyclonal)	1:10,000	GHC7451/Peninsula Laboratories
NOS	Sheep (polyclonal)	1:5000	H205/Dr. Emson
ChAT	Goat (polyclonal)	1:100	AB144P/Chemicon International
CGRP	Rabbit (polyclonal)	1:20,000	AB5920/Millipore
Secondary			
Rabbit IgG	Cy3-Donkey IgG	1:200	711-095-152/Jackson ImmunoResearch
Guinea-pig IgG	Cy3-Donkey IgG	1:200	705-0545-147/Jackson ImmunoResearch
Sheep IgG	Alexa 488 Donkey IgG	1:100	A-11015/Molecular Probes
Goat IgG	Alexa 488 Donkey IgG	1:100	A-11055/Molecular Probes

Results

Number and distribution of SP neurons in the esophagus

Numerous intrinsic neurons and nerve fibers showing SP-IR occurred in the myenteric plexus of the esophageal wholemount preparation (Fig. 1a). An average of 826 ± 147 (n = 8 rats) SP neurons were present in the esophagus, which accounted for approximately 12% of the total intrinsic PGP9.5-positive neurons (7207 ± 789, n = 10 rats, from data published by Kuramoto et al. 2019). In the five esophageal segments, the density of SP neurons per 1 cm^2 decreased gradually but significantly from the cervical to the abdominal end: 187.1 ± 28.2, 180.0 ± 35.5, 151.4 ± 34.3, 114.2 ± 24.8, and 79.9 ± 24.8 in segments 1–5, respectively (Fig. 2a).

Double-immunostaining revealed 708.3 ± 143.7 (n = 4 rats) SP neurons with ChAT-IR (Fig. 1b–b″) in the entire esophagus, and once again the number per 1 cm^2 decreased from the cervical to the abdominal end, 161.9 ± 12.9 in segment 1, followed by 156.1 ± 29.1, 130.2 ± 36.7, 94.5 ± 23.3, and 60.0 ± 13.0 in segments 2–5, respectively (Fig. 2b). The percentage of ChAT/SP neurons of the total SP neuron population in segments 1–5 was 93.9 ± 2.2%, 94.9 ± 1.4%, 94.0 ± 1.8%, 94.6 ± 1.7%, and 96.6 ± 1.5%, respectively (average for the whole esophagus: 94.6 ± 1.6%). On the

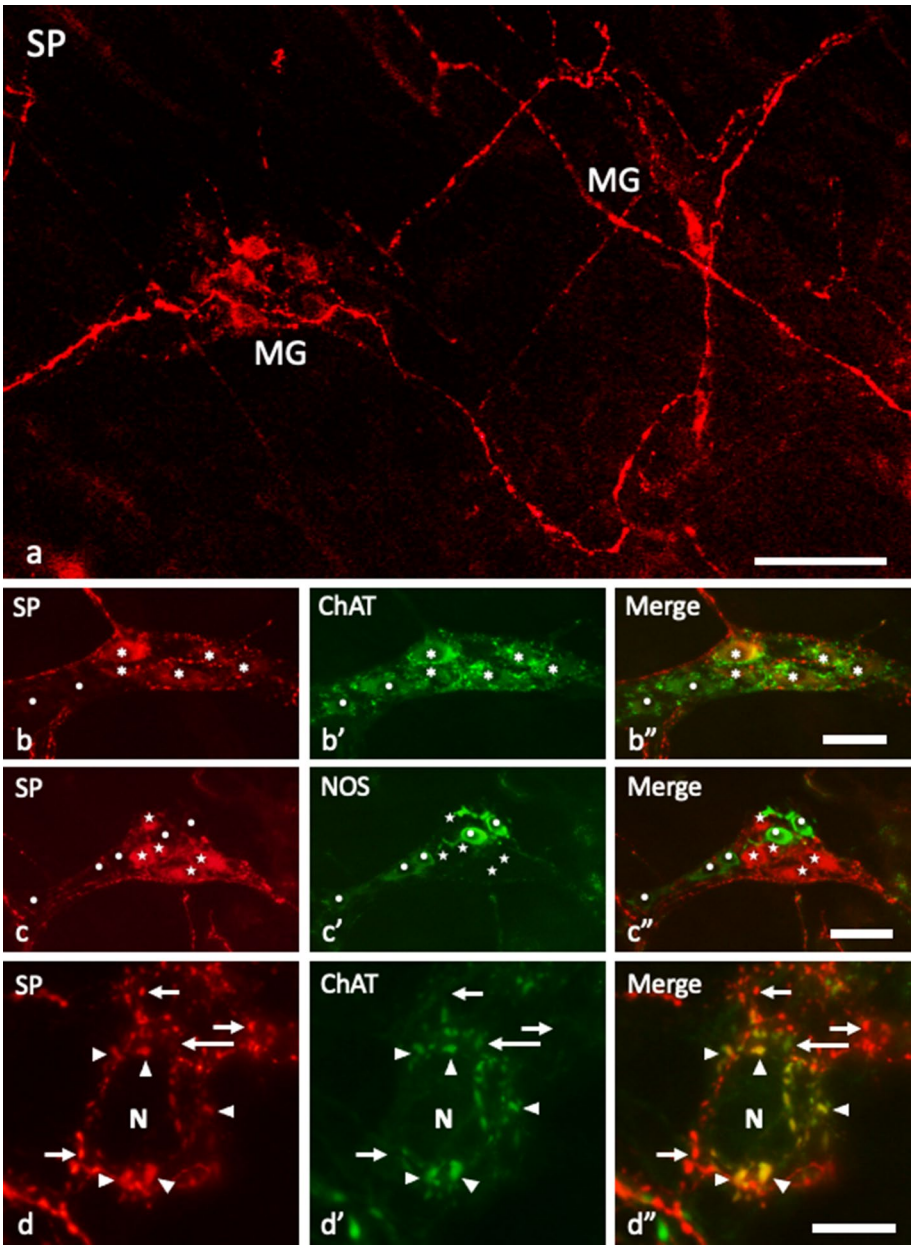

Fig. 1 Wholemount preparations of the myenteric plexus in the rat esophagus, which were immunostained with SP antibodies (AB1566 and Y150). **a** Some cell bodies of intrinsic neurons in the myenteric ganglia (MG) and nerve fibers are positive for SP. Scale bar, 100 µm. **b–b″** All SP neurons (✻) are ChAT-positive, and a few ChAT neurons are SP-negative (●) in a myenteric ganglion. Scale bar, 50 µm. **c–c″** All SP neurons are NOS-negative (★), and some NOS neurons are not SP-positive (●) in a myenteric ganglion. Scale bar, 50 µm. **d–d″** Many varicosities with both SP-IR and ChAT-IR (arrowheads) are seen around one neuronal cell body (N) in a myenteric ganglion. Several SP varicosities (short arrows) are ChAT-negative, and a ChAT varicosity (long arrows) is SP-negative. Scale bar, 20 µm

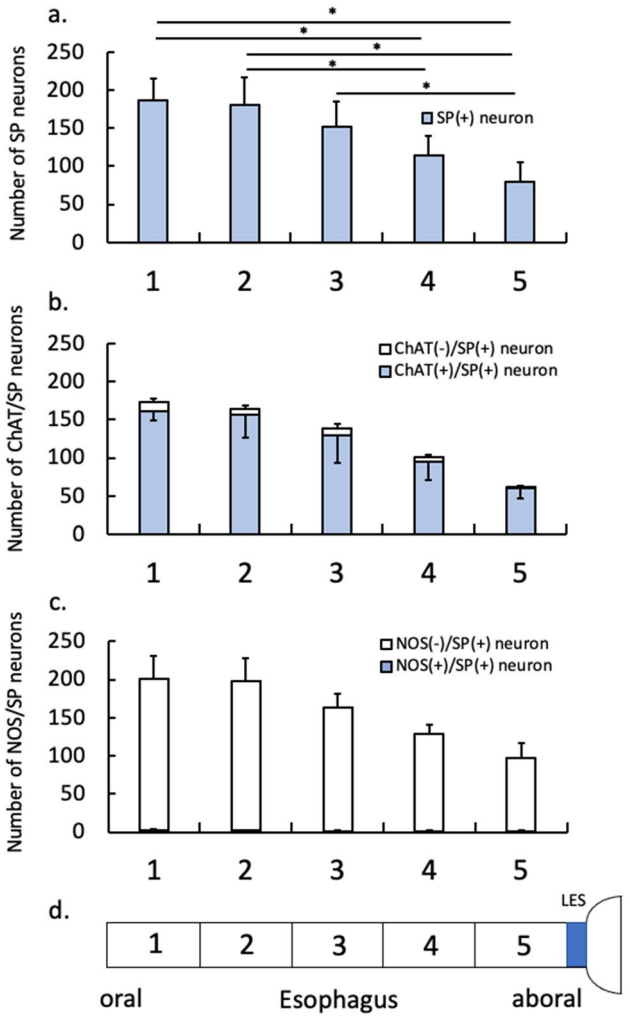

Fig. 2 Distribution pattern of SP neurons and those of ChAT/SP and NOS/SP neurons per 1 cm^2. **a** The number of SP neurons ($n = 8$ rats) is highest in segment 1 (cervical esophagus) and decreases gradually from the oral to the aboral end. Significant differences among the esophageal segments were determined using one-way ANOVA followed by Tukey's multiple-comparison test. *$P < 0.05$. **b** Most (95%) of SP neurons are ChAT-positive, and the distribution pattern of ChAT/SP neurons ($n = 4$ rats) is like that of SP neurons. **c** There are very few (1%) NOS-positive SP neurons ($n = 4$ rats) in any segment. **d** The whole esophagus was divided into five segments of equal length (named segments 1–5, from the cervical to abdominal end). Data are shown as mean ± SD. LES, lower esophageal sphincter

other hand, the number of SP neurons with NOS-IR was only 9.0 ± 3.9 in the entire esophagus (Fig. 1c–c″), and the number 1 cm^2 in segments 1–5 was 2.1 ± 1.5, 1.6 ± 0.4, 1.3 ± 1.0, 1.3 ± 0.5, and 1.5 ± 1.3, respectively ($n = 4$ rats; Fig. 2c). The percentage of NOS/SP neurons of the total SP neurons was 1.1 ± 0.8%, 0.8 ± 0.2%, 0.8 ± 0.2%, 1.1 ± 0.4%, and 1.4 ± 1.0% in segments 1–5, respectively (average for the entire esophagus: 1.0 ± 0.4%). There were less than 0.3% intrinsic neurons with CGRP-IR (approximately 20 on average, $n = 6$ rats) out of the total intrinsic neurons (7207 ± 789, described above), and almost all of them were ChAT-positive (data not shown).

Distribution of SP nerve fibers or endings in the esophagus

In wholemount preparations, varicose SP nerve fibers or terminals passed through the ganglia or between nerve cells in many myenteric ganglia (Fig. 1a), as shown by Kressel and Radespiel-Troger (1999). In frozen section preparations, they were found in the mucosal and muscularis external layers, with many in the muscularis mucosa and myenteric plexus, some in the external muscle layers, and a few in the lamina propria and submucosal tissue (Fig. 3a). Among the smooth muscles of the muscularis mucosa, SP nerve endings were most frequent in segment 1 (10.0 ± 2.0), followed closely by segment 5 (9.5 ± 3.0) and then by segments 2 (7.1 ± 0.6), 3 (7.1 ± 1.2), and 4 (7.1 ± 1.7) (Fig. 4a). The number of ChAT/SP nerve endings per 10^4 μm^2 in the muscularis mucosa was 9.0 ± 2.4, 6.0 ± 0.6, 5.9 ± 0.8, 5.8 ± 1.9, and 9.1 ± 3.0 in segments 1–5, respectively (Fig. 4b; average 7.1 ± 1.2; $n = 3$ rats). The percentage of ChAT/SP nerve endings of the population of SP nerve endings was 88.4 ± 3.1%, 87.4 ± 3.1%, 87.5 ± 1.6%, 87.9 ± 7.2%, and 86.8 ± 1.2% in segments 1–5, respectively (average 87.4 ± 1.4%). On the other hand, the number of NOS/SP nerve endings per 10^4 μm^2 in each segment was 2.2 ± 0.3, 1.6 ± 0.1, 1.4 ± 0.1, 1.7 ± 0.6, and 1.8 ± 0.2 (average 7.1 ± 1.2; Fig. 4c), and the percentage of NOS/SP nerve endings of the population of SP nerve endings in each segment was 22.1 ± 2.2%, 21.6 ± 0.4%, 18.7 ± 1.7%, 22.5 ± 3.7%, and 20.7 ± 3.7% (average 21.2 ± 1.6%).

Many SP-positive nerve endings were distributed in the LES smooth muscle, with 7.1 ± 1.0 per 10^4 μm^2 (Fig. 6), which was comparable to densities in the muscularis mucosa of segments 2, 3, and 4 (Fig. 4). Double-immunolabeling showed that the percentage of SP nerve endings with ChAT-IR (Fig. 5a–a″) or NOS-IR (Fig. 5b–b″) out of the total population of SP nerve endings in the LES was 67.9 ± 2.9% ($n = 3$ rats) or 30.3 ± 5.5% ($n = 3$ rats), respectively (Fig. 6), and the density of ChAT or NOS nerve endings was higher than that of SP nerve endings.

In the wholemount preparations, many SP nerve endings were seen around the cell bodies of intrinsic neurons in the myenteric ganglia, but only small numbers showed ChAT-IR (Fig. 1d–d″). In many cases, tracing or detecting projections and terminals that emerged from intrinsic SP or ChAT neurons was extremely difficult because of very weak immunoreactions. In addition, NOS/SP-positive nerve endings around cell bodies of intrinsic neurons were scarce (data not shown).

When the esophageal wholemounts were double-immunostained for SP and ChAT or NOS, the number of SP nerve

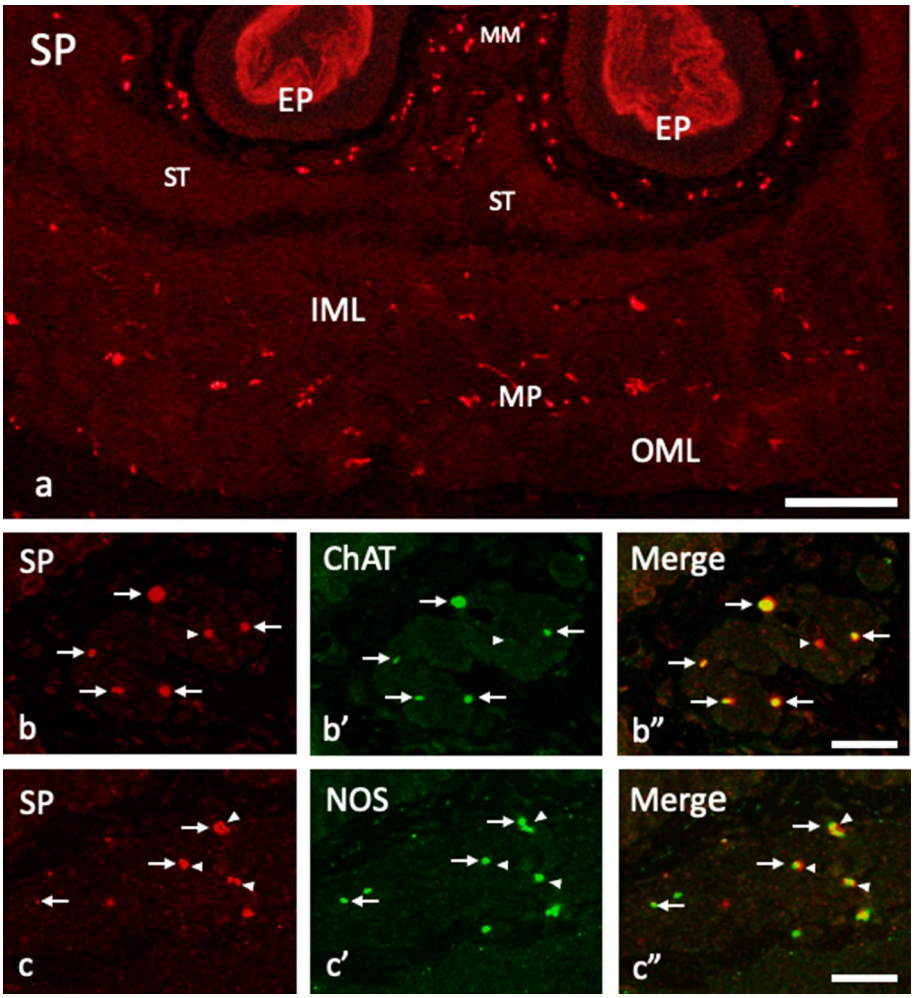

Fig. 3 Frozen transverse sections in segment 5 of the esophagus double-immunolabeled with SP antibodies (AB1566 and Y150). **a** Many SP nerve terminals ($n = 6$ rats) are present in the muscularis mucosa (MM) and myenteric plexus (MP), but there are few SP nerve terminals in the inner (IML) and outer muscle layers (OML). The submucosal tissue (ST) has very few SP terminals. Scale bar, 100 µm. EP, epithelium. **b**–**b″** Many SP nerve endings ($n = 3$ rats) are ChAT-positive (arrows), and one SP nerve ending is ChAT-negative (arrowheads). Scale bar, 20 µm. **c**–**c″** Some SP nerve endings ($n = 3$ rats) are positive for NOS (arrows), but several nerve endings show no NOS-IR (arrowheads). Scale bar, 20 µm

fibers surrounding single blood vessels was from 2 to 8, and in selected 50 blood vessels it was approximately 180 and 240 (average approximately 150, $n = 4$ rats). The majority (average approximately 75%, $n = 2$ rats) of them showed ChAT-IR (Fig. 5c–c″), and some (average approximately 13%) were NOS-positive (Fig. 5d–d″). However, some perivascular SP nerve fibers were ChAT- or NOS-negative.

Substance P (SP)-immunoreactivity (IR) in motor endplates of the esophageal striated muscles

Fine varicose SP nerve fibers or endings were localized on approximately 4.7% (on average, $n = 3$ rats) of the total number of motor endplates that occurred in a 1 cm^2 area in five segments. They were intermingled among vagal motor coarse ChAT nerve terminals and were NOS- or ChAT-negative (Fig. 7a–a″, b–b″), indicating that the varicose SP nerve terminals were separate from the vagal cholinergic motor terminals or enteric nitrergic nerve endings on the motor endplates. Further, most of the SP nerve fibers were CGRP-positive (Fig. 7c, c′). In addition, vagal motor ChAT-positive coarse nerve terminals on motor endplates themselves often exhibited weak-to-intense SP-IR in response to any of the three SP antibodies (Fig. 7b, b′); the SP-positive vagal motor ChAT nerve terminals was between approximately 10% and 30% (26% on average) of the total vagal motor nerve terminals in individual wholemount from three rats immunolabeled by each of three SP antibodies.

Fast Blue (FB)-labeled neurons in the DRG, NG, and medulla oblongata by retrograde tracing

Masses of FB crystals and many striated muscles intensely labeled with FB were seen at the FB-injection sites in the abdominal esophageal wall. After FB was injected, labeled neurons showing white–bluish fluorescence were found in the DRG (T8) and NG, and in the compact nucleus ambiguous (NA) and dorsal motor nucleus of the vagus (DMV) in the medulla oblongata. The FB-labeled neurons were more numerous in the NG (134 ± 51 on average, $n = 3$ rats) than in the DRG (34 ± 12 on average, $n = 3$ rats). In addition, a number of FB-labeled neurons were present

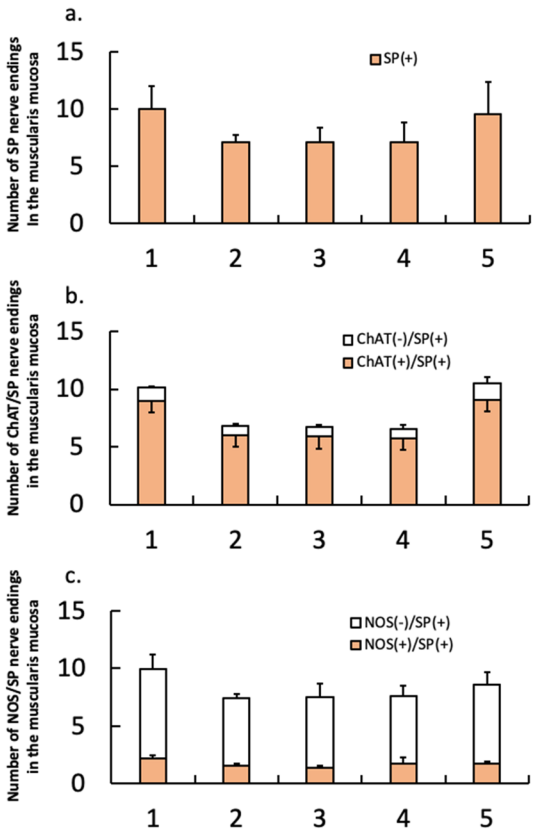

Fig. 4 Densities of SP nerve endings and ChAT/SP and NOS/SP nerve endings per 10^4 μm^2 in the muscularis mucosa. **a** There is a higher density of number of SP nerve endings in segment 1 (cervical esophagus) and 5 (abdominal) than in other segments. **b** The density pattern of ChAT/SP nerve endings is similar that of SP nerve endings, with the higher densities in segments 1 and 5. The percentage of ChAT/SP nerve endings of the total population of SP nerve endings is higher than 85% in all segments. **c** The number of NOS/SP nerve endings is lower than that of ChAT/SP nerve endings, and the percentage of NOS/SP nerve endings of all SP nerve endings is approximately 20%. Data are shown as mean ± SD

in the NA and DMV. Double-immunostaining showed that approximately 55% and 73% of the FB-labeled neurons in the DRG were SP- and CGRP-positive (Fig. 8a–a″), respectively, and almost all the SP/FB neurons exhibited CGRP-IR (Fig. 8a–a″). While, in the NG, approximately 7% and 58% of the FB-labeled neurons were positive for SP and CGRP, respectively (Fig. 8b–b″). In both the NA and DMV, almost all FB-labeled neurons were ChAT-positive, but SP-negative (Fig. 8c–c″, d–d″).

Staining patterns and specificity of three SP antibodies used

The staining patterns of intrinsic SP neurons, and SP nerve fibers or endings throughout the rat esophagus were basically similar between the three SP antibodies used, as shown in Table 1. Thus, the rabbit SP antibodies (AB1566 and Y150) were applied to double-immunolabel neuronal cell bodies, nerve fibers or endings (Fig. 1), and blood vessels (Fig. 5c–c″, d–d″) in the myenteric plexus of wholemounts, and muscularis mucosa (Fig. 3b–b″, c–c″) and LES (Fig. 5a–a″, b–b″) of cryostat sections. On the other hand, the rabbit SP antibodies (Y150) and guinea-pig SP antibody (GHC7451) were used for triple-immunostaining in the motor endplates (Fig. 7a–a″, b–b″, c–c″), and double-immunostaining combined with FB in the DRGs (Fig. 8a–a″) and NG (Fig. 8b–b″), and in the NA (Fig. 8c–c″) and DMV (Fig. 8d–d″) of the medulla oblongata. As a result of SP antibodies preabsorbed with a full-length of SP, neither of the rabbit SP antibodies (Y150 and AB1566) (Fig. 7d, f) or the guinea-pig SP antibody (GHC7451) (Fig. 7e) showed SP-IR in neuronal cell bodies and nerve fibers in the myenteric plexus of wholemount preparations.

Discussion

Characteristics of intrinsic SP neurons in the rat esophagus

Notably, approximately 95% of intrinsic SP neurons in the rat esophagus were ChAT-positive but NOS-negative, indicating that they are almost cholinergic. By contrast, almost all esophageal intrinsic GAL- or NPY-positive neurons (99% or 98%, respectively) exhibited NOS-IR, with only a few that were ChAT-positive (unpublished data). In addition, GAL or NPY nerve endings were localized on the majority of motor endplates (70% or 81%, respectively), and almost all or most (98% or 88%, respectively) of them showed NOS-IR (unpublished data), suggesting that the NOS/GAL and NOS/NPY nerve terminals on motor endplates are derived from esophageal intrinsic NOS/GAL and NOS/NPY neurons (Wörl and Neuhuber 2005). On the other hand, the presence of only a few SP nerve endings on motor endplates (4.7%) may indicate that intrinsic SP neurons participate very little in direct action through motor endplates of the esophageal striated muscles. We therefore assume that intrinsic SP neurons in the rat esophagus are little involved in direct transmissional effects to the vagal ChAT motor nerve endings or the striated muscles via the motor endplates, and instead serve other roles in the esophagus.

Intrinsic SP neurons as motor neurons

The smooth muscles of the muscularis mucosa in the small and large intestines of cats and dogs are innervated by submucosal neurons (Onori et al. 1971; Furness et al. 1990). However, the smooth muscles of the muscularis mucosa in the rat esophagus are probably innervated by myenteric

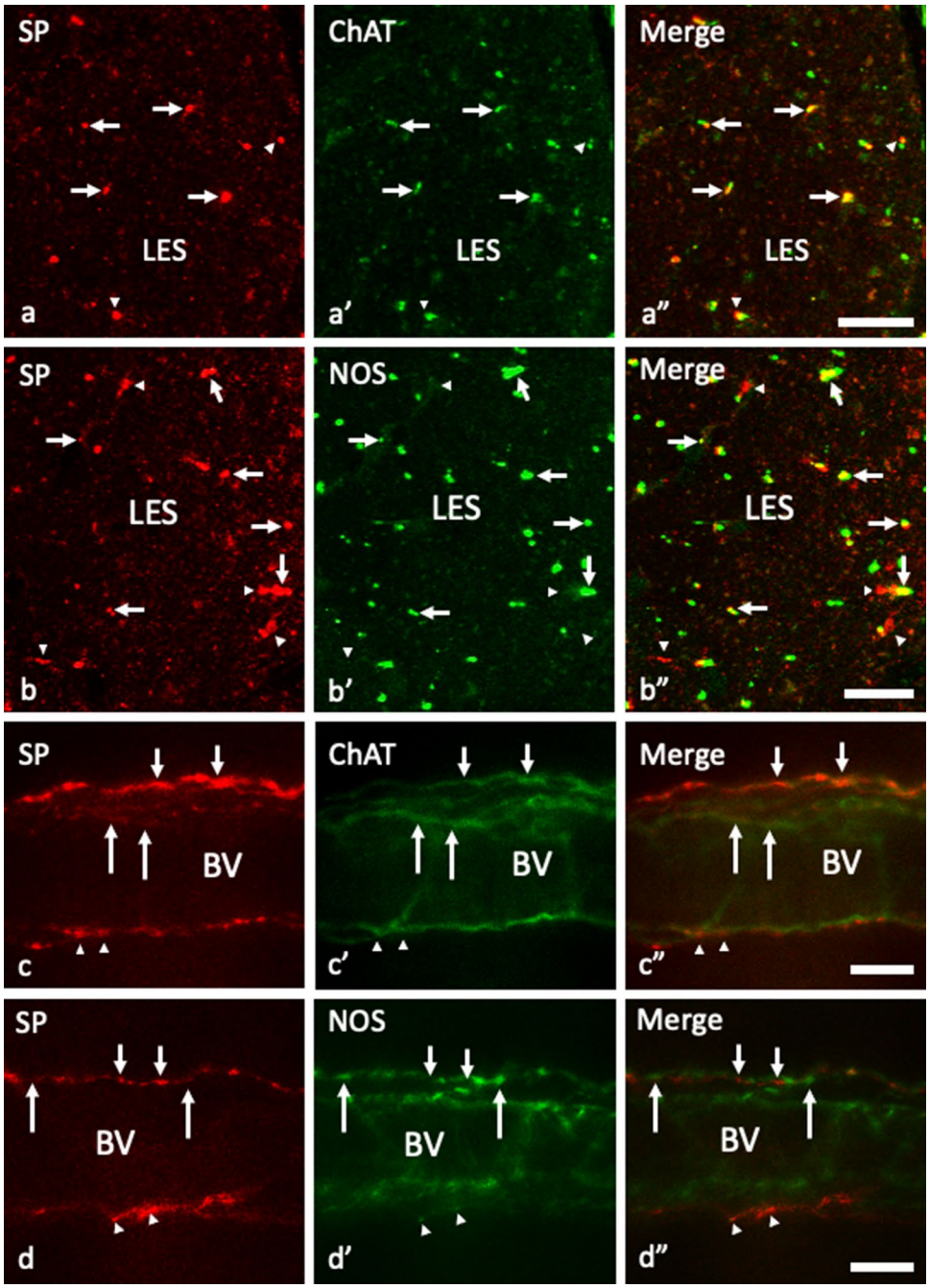

Fig. 5 Frozen transverse sections of the LES (**a**–**a″**, **b**–**b″**) and wholemounts including blood vessels (BV) (**c**–**c″**, **d**–**d″**), both of which were double-immunolabeled using SP antibodies (AB1566 and Y150). **a**–**a″** In the muscularis mucosa (MM), some SP nerve endings are ChAT-positive (arrows) and a few are ChAT-negative (arrowheads). **b**–**b″** In the lower esophageal sphincter (LES), some SP nerve endings are NOS-positive (arrows) and some are NOS-negative (arrowheads). **c**–**c″** Some nerve fibers, indicated by short arrows, long arrows, and arrowheads around a blood vessel (BV), are positive for both SP and ChAT. **d**–**d″** One SP nerve fiber (arrowheads) is NOS-positive, but another SP nerve fiber (short arrows) is NOS-negative. Some ChAT nerve fibers, including one indicated by long arrows, are SP-negative. Scale bars, 20 µm

neurons, because there are very few or no nerve cells in the esophageal submucosa (cf. Furness 2006; Neuhuber and Wörl 2016). SP nerve terminals in the muscularis mucosa are common to rats and other species, including cats, humans, pigs, opossums, and dogs (Leander et al. 1982; Domoto et al. 1983; Wattchow et al. 1987; Christensen et al. 1989; Sandler et al. 1993). Double-immunolabeling revealed that over 80% of the SP nerve fibers in the muscularis mucosa were positive for ChAT, and in all esophageal segments this percentage was > 90%. Retrograde tracing revealed that SP neurons in the DRGs or NG that projected to the esophagus were ChAT-negative (data not shown), implying that a large subclass of the ChAT/SP myenteric neurons throughout the esophagus innervate the muscularis mucosa.

Pharmacological investigations have demonstrated that, like ACh in guinea pigs and rats (Kamikawa et al. 1982; Bieger and Triggle 1985), SP causes contraction to the esophageal muscularis mucosa of opossums (Christensen and Percy 1984; Daniel 1989). Thus, a subset of intrinsic ChAT/SP neurons in the esophagus is assumed to project to the muscularis mucosa as excitatory motor neurons to regulate local esophageal movement.

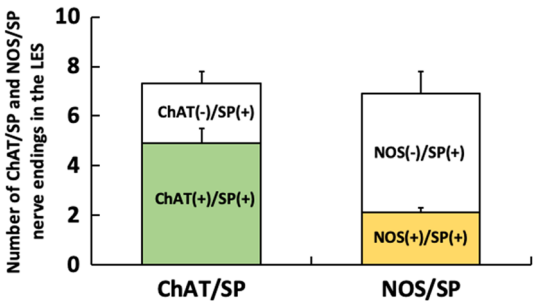

Fig. 6 Densities of SP nerve endings and ChAT/SP ($n = 3$ rats) and NOS/SP nerve endings ($n = 3$ rats) per 10^4 μm^2 in the LES. The number of ChAT/SP nerve endings is more than double that of NOS/SP nerve endings

Intriguingly, we observed a higher density of ChAT/SP nerve fibers in the muscularis mucosa in segments 1 and 5 (the cervical and abdominal esophagus), both of which may be important portions that are related to gastroesophageal reflux. The condition in segment 1 may be due to the necessity for smoother deglutition at the cervical esophagus, in cooperation with the external striated muscles, thus making breathing easier and/or preventing reflux or microaspiration from stomach contents into the pharynx or trachea. Esophageal segment 5 is thought to possess potent contractility among the five esophageal segments, as it has most striated muscle cells in the external muscle layer (Kuramoto et al. 2019). The striated muscles may therefore allow muscularis mucosa-induced local motility in the abdominal esophagus,

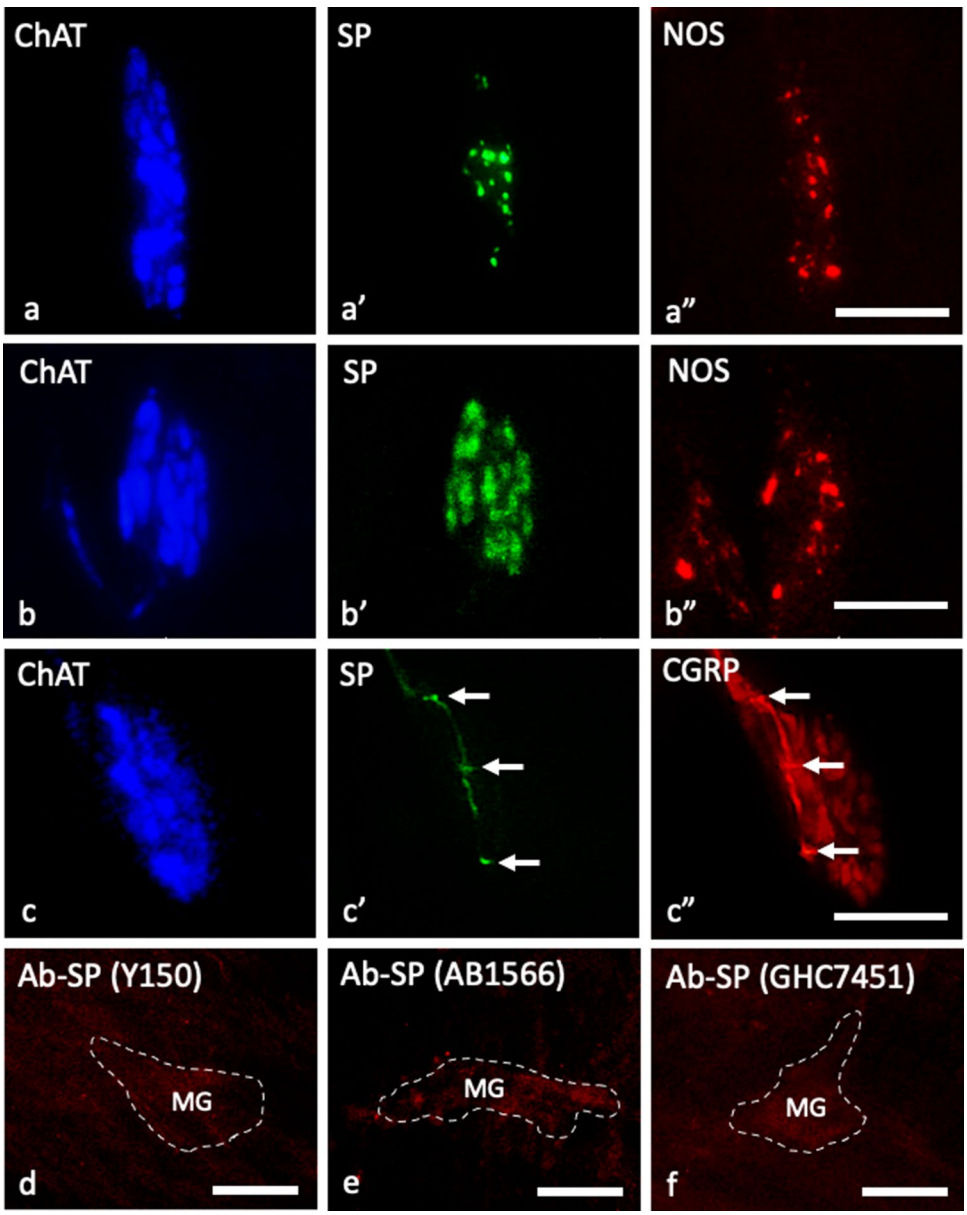

Fig. 7 Motor endplates of striated muscles in esophageal wholemount preparations that were triple-immunolabeled (**a–a″**, **b–b″**) using rabbit SP antibodies (Y150 and GCH7451) and a guinea-pig SP antibody (**c** and **c″**). **a–a″** Fine varicose SP (**a′**) and NOS (**a″**) nerve terminals are intermingled among vagal ChAT coarse motor nerve endings (**a**) on a motor endplate, but their nerve endings are completely separated. Scale bar, 20 μm. **b–b″** Vagal ChAT motor endings (**b**) show SP-IR (**b′**), where only NOS nerve endings (**b″**) are present, but fine SP nerve endings are not localized (**b′**). Scale bar, 20 μm. **c**, **c′** A motor endplate with vagal coarse motor nerve terminals showing CGRP-IR (**c**, **c″**) includes a fine CGRP varicose nerve fiber that is also SP-positive (arrows) (**c′**). Scale bar, 20 μm. **d–f** Wholemount preparations immunolabeled with the SP antibodies of Y150 (**d**), AB1566 (**e**), and GHC7451 (**f**) preabsorbed with SP antigen. In any of the pretreated SP antibody, reactions in nerve cells and nerve fibers in the myenteric plexus are not different from those in background, and SP-positive neuronal cell bodies or nerve fibers as seen in Fig. 1 are not found. Areas surrounded by dashed lines indicate the myenteric ganglia (MG). Scale bars, 100 μm. Ab-SP, SP antibody preabsorbed with SP antigen

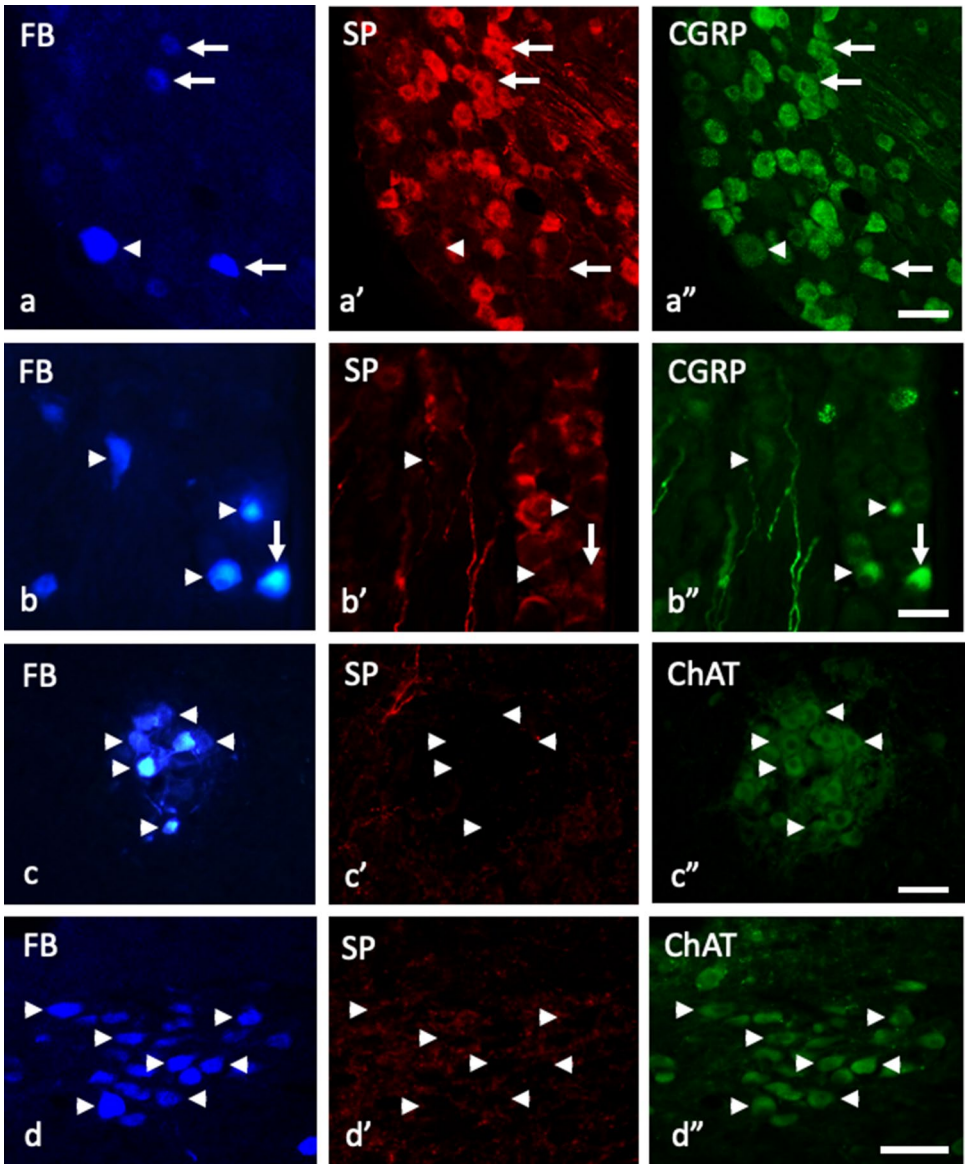

Fig. 8 Frozen sections of the DRG (T8) (**a**–**a″**), NG (**b**–**b″**), and medulla oblongata (**c**–**c″**, **d**–**d″**) of rats injected with FB into the abdominal esophagus, which were immunostained with SP antibodies (Y150 and GCH7451). **a**–**a″** In the DRG (T8), FB-labeled neurons (arrows) (**a**) are both SP (**a′**) and CGRP-positive (**a″**), but a FB-labeled cell (arrowheads) (**a**) is SP-negative (**a′**) and CGRP-positive (**a″**). Scale bar, 30 µm. **b**–**b″** In the NG, one FB-labeled neuron (arrows) (**b**) is positive for both SP (**b′**) and CGRP (**b″**), but other FB-labeled cells (**b**) indicating by arrowheads are SP-negative (**b′**), but CGRP-positive (**b″**). Scale bar, 30 µm. **c**–**c″** All FB-labeled neurons (arrowheads) (**c**) in the NA are SP-negative (**c′**), but ChAT-positive (**c″**). Scale bar, 50 µm. **d**–**d″** All FB-labeled neurons (arrowheads) (**d**) in the NA are negative for SP (**d′**), but positive for ChAT (**d″**). Scale bar, 50 µm

which may subserve to flush out refluxed material into the stomach.

Immunohistochemical studies have revealed SP nerve fibers in the LES of some species, including humans, pigs, cats, and dogs (Aggestrup 1985; Aggestrup et al. 1985; Parkman et al. 1989; Sandler et al. 1991), which is consistent with our finding in the rat LES. Furthermore, SP-induced LES contraction has been pharmacologically demonstrated in many species, including opossums, pigs, dogs, cats, and rabbits (Mukhopadhyay 1978; Aggestrup 1985; Aggestrup et al. 1985; Dobreva et al. 1994; Kohjitani et al. 1996), although administration of SP and NK1R agonists to the ferret LES induced a biphasic response (initial brief contraction followed by prolonged relaxation; Smid et al. 1998a, b). Likewise, ACh and its agonists cause LES contraction (Coruzzi et al. 1985; Farre and Sifrim 2008; Liu et al. 2011). These findings suggest that both SP and ACh play a key role in the LES in many animals. The present immunohistochemical study demonstrated that both ChAT/SP and NOS/SP nerve terminals were found in the LES, with the former considerably more frequent than the latter. However, why segment 5 has a higher density of ChAT/SP nerve terminals in the muscularis mucosa despite having the least ChAT/SP neurons of all the esophageal segments is unclear. This may be explained by the possibility that not only ChAT/SP neurons in the lower esophagus (segments 4 and 5) but also those in the local part of the LES and/or those in a proximal stomach are involved in the LES innervation. This interpretation is based on a retrograde tracing experiment that demonstrated that a small number of ChAT neurons in the abdominal esophagus, the local part of the LES, and the proximal stomach project to the LES of guinea pigs (Brookes et al.

1996), although whether they contain SP remains unclear. Thus, it is presumed that a subgroup of esophageal ChAT/SP neurons activate, as motor neurons, the smooth muscles of the LES via SP and ACh released from their nerve endings.

Intrinsic SP neurons as interneurons

Considering that NOS neurons are surrounded by many NOS nerve terminals, we previously proposed that a subpopulation of intrinsic NOS neurons in the esophagus may be interneurons (Kuramoto and Kadowaki 2006). In contrast, the present study suggest that a subgroup of ChAT/SP neurons are also interneurons, since a few ChAT/SP nerve terminals surrounded the cell bodies of myenteric neurons in the esophagus. Since most intrinsic SP neurons exhibited ChAT-IR, we expected to find ChAT/SP nerve endings surrounding more myenteric neurons. However, we did not observe clear profiles of nerve endings showing both SP-IR and ChAT-IR around intrinsic neurons. This may have been because of the very weak SP-IR and ChAT-IR in nerve endings surrounding neuronal cell bodies in the myenteric ganglia, even if those endings are derived from ChAT/SP intrinsic neurons.

Because we could not find ChAT-positive SP neurons in the DRGs and NG via the retrograde tracing experiment (unpublished data), we assume that the ChAT/SP nerve terminals surrounding myenteric neurons are derived from a subclass of intrinsic ChAT/SP neurons that act as interneurons, which may contribute to local excitatory reflexes for esophageal motility. However, whether these neurons are ascending or descending interneurons is unclear. In the myenteric plexus of the small intestine in guinea pigs, most ascending and descending interneurons exhibit ChAT-IR, with the former also containing SP/TK-IR (Brookes 2001; Furness 2006).

Intrinsic SP neurons as vasomotor neurons

Our finding that SP nerve fibers were present in the perivascular area of the rat esophagus is consistent with previous research on the esophagi of guinea pigs, cats, and pigs (Leander et al. 1982). Predominant ChAT/SP perivascular nerves running throughout the esophagus suggest that they are derived from a subset of intrinsic ChAT/SP neurons that function as vasomotor neurons, because intrinsic cholinergic secretomotor/vasodilator neurons have been identified in the small intestine of guinea-pigs (Furness et al. 2003). However, some SP perivascular nerve fibers were ChAT- or NOS-negative, indicating that they are derived from sensory neurons in DRGs or NG, where there are numerous SP-containing sensory neuronal cell bodies (Green and Dockray 1987; Helke and Niederer 1990). Neurotransmitters such as ACh, SP, ATP, and 5-HT act on vascular endothelial cells to produce endothelium-dependent relaxing factor, which leads to blood vessel relaxation; in addition, this factor itself is nitric oxide (cf. Burnstock 1990; Toda and Okamura 1990). Thus, esophageal perivascular ChAT/SP nerve fibers may regulate local blood flow within the esophagus via their relaxation action, as has been speculated for the gut (Vanner and Surprenant 1996).

Substance P (SP)-varicose nerve endings on motor endplates

The appearance of SP-IR in the vagal motor nerve endings on some motor endplates was unexpected, because no SP-containing neurons have yet been confirmed in the NA, which projects to the esophageal striated muscles (Ljungdahl et al. 1978; Warden and Young 1988), being consistent with the results of our FB-retrograde tracing experiment that no FB neurons labeled within the NA were positive for SP. The SP-IR may be present because the vagal nerve endings on motor endplates take up SP that is released from SP nerve terminals that run close to or inside the motor endplates. However, this is unlikely, because it is generally thought that there is no uptake mechanism for neuropeptides, since they diffuse and are proteolyzed by extracellular peptidases after being released from the nerve terminals (Schwartz and Javitch 2013). Moreover, the released SP disappears rapidly, since it is cleaved by several peptidases (Michael-Titus et al. 2002). Another possibility is that substances reacting to polyclonal SP antibodies may be localized in a part of the vagal motor ChAT-positive coarse nerve terminals on a subclass of motor endplates. However, even though the source of the SP is unclear, we should focus on the fact that all three SP antibodies used in this study exhibited SP-like immunoreaction within the vagal motor endings.

In the present study, we detected fine varicose SP nerve fibers intermingling within only a few motor endplates on the esophageal striated muscles, which were ChAT-negative. The finding that neuronal cell bodies in the DMV were ChAT-positive, but SP-negative suggests that the SP nerve endings on the motor endplates are not derived from the DMV. Thus, we propose two possibilities regarding their origin: one is esophageal intrinsic neurons, and the other is sensory neurons in the DRGs or NG. The first possibility may seem more feasible, but the SP nerve fibers were neither ChAT- nor NOS-positive, indicating that they are not derived from esophageal SP intrinsic neurons with ChAT-IR or NOS-IR. However, the possibility that they are intrinsic cannot be excluded, because SP nerve fibers may originate from intrinsic neurons that contain only SP without either ChAT- or NOS-IR. The second possibility is that the SP nerve fibers may be extrinsic, derived from sensory neurons in the DRGs or NG, where most SP-containing sensory neurons co-express CGRP and/or TRPV1 (Franco-Cereceda

et al. 1987; Banerjee et al. 2007; Tan et al. 2008). In fact, the double-immunolabeling we performed here revealed that most varicose SP nerve terminals on the motor endplate were positive for CGRP. On the other hand, we found very few CGRP neurons (< 0.3% of the intrinsic neurons) in the myenteric plexus, almost all of which were ChAT-positive. Considering that the varicose CGRP/SP nerve endings on motor endplates were negative for ChAT, it does not seem likely that the intrinsic CGRP/ChAT neurons supply their nerve endings on motor endplates. Therefore, most SP nerve terminals on the motor endplates probably originate from sensory neurons in the DRGs or NG. Taken together, these results suggest that the fine varicose SP nerve endings on the motor endplates of the rat esophageal striated muscles have both intrinsic and extrinsic origins. Although the roles of these SP nerve terminals remain unclear, we postulate that they modulate the motility of the striated muscles via either presynaptic excitation or postsynaptic inhibition by SP, as has been suggested for skeletal muscles (Wali 1985; Ganguly et al. 1987). In addition, CGRP colocalizing with SP may inhibit esophageal motility, because CGRP is suggested to negatively modulate nerve-evoked ACh release in neuromuscular preparations of rats (Kimura et al. 1997). In addition, CGRP is considered as a motoneuron-derived trophic factor that increases ACh receptor synthesis at vertebrate neuromuscular junctions (New and Mudge 1986). The varicose CGRP/SP nerve endings on motor endplates, which are assumed to be derived from sensory ganglia, may contribute to modulation of ACh transmission or detect chemical circumstances within motor endplates to regulate esophageal motility.

The concept that motor endplates on a small subpopulation of esophageal striated muscles receive triple innervation by vagal motor efferents, intrinsic neuronal endings, and sensory afferents is particularly interesting, because although it would represent only a minor innervation to the striated muscle, it suggests that the esophageal motor endplates may be integrating sites at which motor and sensory signals traveling via neuronal transmission accumulate to contribute to regulation of local reflexes to ensure esophageal striated muscle movement. Further investigation is necessary to clarify this question.

Conclusion

This immunohistochemical study suggests that intrinsic SP neurons in the rat esophagus act as motor neurons, interneurons, and vasomotor neurons. Our investigation of the colocalization of SP-IR and ChAT-IR or NOS-IR revealed that most SP neurons exhibited ChAT-IR. This is in marked contrast to the situation in which the majority of intrinsic GAL or NPY neurons are NOS-positive (unpublished data), subsets of which probably innervate esophageal striated muscles. A subgroup of myenteric SP neurons that act as motor neurons is assumed to mainly innervate smooth muscles in the muscularis mucosa and LES. The higher density of SP or ChAT/SP nerve endings in the muscularis mucosa in segments 1 and 5 (the cervical and abdominal esophagus, respectively) may be related to the local regulation of reflux of the stomach contents via subserving the motility of the external striated muscles. On the other hand, the localization of fine varicose SP nerve endings, unlike NOS-, VIP-, GAL-, M-ENK-, or NPY-containing nerve terminals (cf. Neuhuber and Wörl 2016), on only a few motor endplates of the striated muscles suggests that intrinsic SP neurons are rarely associated with direct effect of SP to striated muscles or vagal motor nerve endings via the motor endplates. Intrinsic nerve cell bodies were surrounded by numerous SP nerve terminals, but a small number of these nerve terminals were ChAT-positive, implying that they are derived from a subset of ChAT/SP intrinsic neurons that function as interneurons to contribute to local excitatory reflexes for esophageal motility. Many SP nerve fibers were present along the esophageal blood vessels, and many of these showed ChAT-IR and may be from intrinsic ChAT/SP neurons, which may be involved, as vasomotor neurons, in regulating local blood flow via their relaxation action.

The localization of only a few (approximately 4.7%) of fine varicose SP nerve endings, most of which are positive for CGRP and presumed to be derived from the DRGs or NG, on the motor endplates of the esophageal striated muscles appears to provide a new physiological phase to the motor endplates. The esophageal striated muscles are triply innervated by vagal motor efferents, esophageal intrinsic neuronal endings, and sensory afferents via the motor endplates. These may then be integrating sites at which motor and sensory signals traveling via neuronal transmission accumulate to regulate local reflexes and ensure esophageal striated muscle movement.

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Data availability The data that support the findings of this study are available from the corresponding author, HK, upon reasonable request.

Declarations

Conflict of interest The authors have no conflict of interest to declare.

Ethical approval All experiment procedures were carried out according to the Guide for the Care and Use of Laboratory Animals of the National Institute of Health in the USA and the guidelines of Kyoto Institute of Technology. The Animal Experiment Committee at Kyoto Institute of Technology has approved the animal care procedures and protocols on the experiment (authorization no. 1001176 in 2021).

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