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Regional variations in the number distribution of intrinsic myenteric neurons and coinnervated motor endplates on the striated muscles in the rat esophagus

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ABSTRACT

The roles of intrinsic neurons and the significance of the coinnervated striated muscles in the esophagus are unclear. We examined the number distribution of intrinsic neurons and coinnervated motor endplates on the striated muscles in the rat esophagus using immunohistochemistry to investigate whether these neurons and coinnervated striated muscles may be relevant to the local control of esophageal motility. The number of PGP9.5-positive neurons was higher in the cervical esophagus (segment 1) and gradually decreased toward the aboral, with a moderate increase in the abdominal (segment 5). This pattern was similar to that of NOS-positive neurons, while the number of ChAT-positive neurons decreased toward the aboral, but it was not significantly different among segments 3 to 5. The number of ChAT-positive motor endplates increased toward the aboral, with the highest number in segment 5. The proportion of coinnervated motor endplates was approximately 80% in segments 1 to 4, but approximately 66% in segment 5. NPY-IR was localized in some nerve terminals among the smooth muscles of the muscularis mucosa and some NOS- or ChAT-positive esophageal intrinsic neurons. ENK-8-IR was found in some NOS- or ChAT-positive intrinsic neurons, and nerve terminals surrounding intrinsic neurons in the esophagus, but not in motor neurons at the NA or DMV. This study suggests that regional variations in the number of intrinsic neurons and coinnervated striated muscles in the rat esophagus may be involved in local regulations of esophageal motility, and that the rat esophageal intrinsic neurons may contain, at least, motor neurons and interneurons.

1. Introduction

The esophageal nervous system is primarily constructed by extrinsic nerves, including sympathetic, parasympathetic, sensory and motor nerves, and nerve fibers derived from esophageal intrinsic neurons (for review see, Roman, 1982; Christensen, 1987; Conklin and Christensen, 1994). Although the motility of the esophagus may be assumed to be coordinated by the extrinsic nerves and intrinsic neurons at the myenteric ganglia, there is little information on functional sets of the latter as excitatory and inhibitory motor neurons, interneurons, intrinsic primary afferent neurons (IPANs), intestinofugal neurons and so on, as have been identified in the myenteric plexus of the intestines (Brookes, 2001; Furness, 2006; Costa and Brookes, 2008). In the esophagus, previous chemical coding studies have revealed intrinsic neurons to be positive for proteins or peptides, such as vesicular acetylcholine transporter (VAChT) (Sang and Young, 1997), choline acetyltransferase (ChAT) (Kuramoto et al., 2004), nitric oxide synthase (NOS)/nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase (Neuhuber et al., 1994; Wörl et al., 1994), vasoactive intestinal polypeptide (VIP) (Reichel, 1998), leucine-enkephalin (L-ENK) (Wu et al., 2003), galanin (GAL) (Kuramoto and Endo, 1995), neuropeptide Y (NPY) (Reichel, 1998), substance P (SP) (Uddman et al., 1995), calretinin (Calre) (Dütch et al., 1998) and calbindin (Calb) (Kuramoto and Kuwano, 1994) and so on, and many of these neurons are suggested to

Abbreviations: ACh, acetylcholine; Calb, calbindin; Calre, calretinin; ChAT, choline acetyltransferase; DMV, dorsal motor nucleus of the vagus; ENK-8, Met-enkephalin-Alg^{6}-Gly^{7}-Leu^{8}; GAL, galanin; IGLEs, intraganglionic laminar endings; IPANs, intrinsic primary afferent neurons; IR, immunoreactivity; NA, nucleus ambiguus; NADPH, nicotinamide adenine dinucleotide phosphate; NPY, neuropeptide Y; NOS, nitric oxide synthase; PGP9.5, protein gene product 9.5; SP, substance P; VAChT, vesicular acetylcholine transporter; VIP, vasoactive intestinal polypeptide

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project their axons onto striated muscles of the esophageal external muscle layer to form coinnervation in motor endplates with nerve endings from vagal motor efferents (for review see Wörl and Neuhuber, 2005; Neuhuber and Wörl, 2016). Such an innervation pattern of the esophageal striated muscles is specific and distinct from that of ordinary skeletal muscle fibers that are innervated by only single cholinergic excitatory motor nerves, implying that esophageal striated muscle activity is locally regulated by the coinnervation of enteric nerve terminals, although the structure of the external muscle layer is different between species: it is composed of both the striated (the cranial and middle portion of the esophagus) and smooth (the middle and caudal portion) muscles in humans, cats and opossums, while it is entirely made up of the striated muscles in rats, mice, guinea-pigs and hamsters (for review see Wörl and Neuhuber, 2005; Neuhuber and Wörl, 2016). To obtain more detailed information on the morphology of the coinnervated motor endplates of the striated muscles in the esophagus is important to understand the coinnervation event likely associated with the local control system of esophageal motility. However, morphological data that underlie the possible functional roles of the esophageal intrinsic neurons remain insufficient.

In the present study, we examined in detail the distribution of intrinsic neurons throughout the rat esophagus to discuss the possible functional roles of these neurons, compared with extrinsic, especially, vagal nerves that supply predominant innervation to the esophagus (Neuhuber et al., 1998). In addition, we also investigated the distribution of the number of coinnervated motor endplates on the striated muscle fibers in the esophageal segments because a subpopulation of intrinsic neurons is probably responsible for the coinnervation of the striated muscles as described above.

2. Materials and methods

2.1. Animals

A total 38 male Wistar rats (weighing 220–250 g; SHIMIZU Laboratory Supplies, Kyoto, Japan) were used in the present study. The animals were given laboratory chow (Oriental Yeast, Tokyo, Japan) and tap water ad libitum and kept at 22 to 24 °C in plastic cages laid by clean wooden chips (2 rats per cage; 20 cm × 22 cm × 40 cm in size). All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health in the USA and Kyoto Institute of Technology. Efforts were made to minimize the number of animals used and their suffering. The Animal Experiment Committee at Kyoto Institute of Technology approved all of the animal care procedures and study protocols (authorization No. 100108).

2.2. Tissue preparation

Following an anesthesia mixture of isoflurane (inhalation) and pentobarbital sodium (60 mg/kg, i.p.), 34 animals were perfused through the heart with 150–200 mL of physiological saline, and the entire esophagus was removed. After the esophageal lumen was washed with saline, the esophagus was distended with a fixative solution containing 2% paraformaldehyde and 15% saturated picric acid in 0.1 M sodium phosphate buffer (Zamboni fixative; PB, pH 7.3) with an injection via a needle inserted into the lumen. The esophagus tissue was then immersed in the same fixative at 4 °C for 12–18 h, opened longitudinally, washed in dimethylsulfoxide (DMSO) for 10 min three times and washed in phosphate-buffered saline (PBS, pH 7.2) for 10 min three times. For wholemount preparations, the outer muscle layer attached to the myenteric plexus was isolated by separating the inner muscle and mucosal layers from the tissue. Four rats under deep anesthesia were perfused through the heart with 150–200 mL of physiological saline, followed by perfusion with 300 mL of the same fixative. Then, the esophagus and brain stem were removed and immersed for 12–18 h in the same fixative at 4 °C. After the samples were passed through DMSO and PBS, they were immersed in 0.1 M PB containing 30% sucrose and 0.1% sodium azide at 4 °C until they were transversally cut into 15-μm-thick frozen sections with a cryostat (Sakura, Tokyo, Japan).

Table 1
Characteristics of primary and secondary antibodies.

Antigen	Host	Dilution	Code and source
Primary			
PGP9.5	Rabbit (polyclonal)	1:5000	RA95101/UltraClone
NOS	Sheep (polyclonal)	1:10,000	H205/Dr.P.C. Emson
ChAT	Goat (polyclonal)	1:100	AB144P/Chemicon International
NPY	Rabbit (polyclonal)	1:10,000	22940/ImmunoStar
ENK-8	Rabbit (polyclonal)	1:8000	R0171/Prof. N. Yanaihara
Calre	Rabbit (polyclonal)	1:5000	AB5054/Millipore
Secondary			
Rabbit IgG	Donkey Cy3	1:200	67080/Jackson ImmunoResearch
Goat IgG	Donkey Alexa 488	1:100	A-11055/Molecular Probes
Sheep IgG	Donkey Alexa 488	1:100	A-11015/Molecular Probes

2.3. Immunohistochemistry

In the present study, double immunolabeling was applied in all staining procedures to estimate the number of positive intrinsic neurons and motor endplates in the esophagus. Wholemount and frozen sections 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, USA) to decrease nonspecific immunoreactions, and then washed with PBS three times for 10 min each wash. To examine the number distribution of immunoreactive intrinsic neurons and the number distribution and occurrence frequency of coinnervated motor endplates, the esophagus and brain stem were incubated for 24–48 h with a combined mixture of two primary antibodies (Table 1). The tissue was then incubated for 2 h with a combined mixture of two secondary antibodies (Table 1). All incubations were carried out at room temperature. The immunostained specimens were mounted in buffered glycerol (pH 8.2) and examined by a Zeiss fluorescence microscope equipped with appropriate filter sets (Zeiss, Germany). Digital images of the immunostained neurons were acquired as TIFF files from the fluorescence microscope connected to a digital CCD camera system (VisualixPro2, Visualix, Kobe, Japan). The images were finally processed using Microsoft PowerPoint software (Microsoft Co., Redmond, WA, USA). Details on the specificity of the NOS, ChAT and protein gene product 9.5 (PGP9.5) antisera have been described previously (Kuramoto et al., 1999; Kuramoto et al., 2004; Kuramoto and Kadowaki, 2006), and the specificity of other primary antibodies was verified by their omission.

2.4. Data analysis

After the wholemount preparation of the esophagus was immunostained, the entire length of the esophagus (5.8 ± 0.2 cm on average from 30 rats) was divided circumferentially into five portions (namely, segments 1 to 5) with equal length (Fig. 2c). The total numbers of immunoreactive neurons occurring in each segment of the esophagus were counted. The immunoreaction intensities of the antibodies used in the present study varied among intrinsic neurons of the rat esophagus. In the cell counting analysis, intrinsic neurons with very low immunoreactivity close to that seen in the background of the tissue were not included within the category of positive neurons. In addition, the total numbers of ChAT-positive motor endplates and those coinnervated by NOS-positive nerve terminals were calculated within one area of 25 mm^2 (5 mm × 5 mm) in each segment of the esophagus. Finally, the data were processed for preparing graphs using Microsoft Excel software (Microsoft Co.). The raw numbers and percentages were

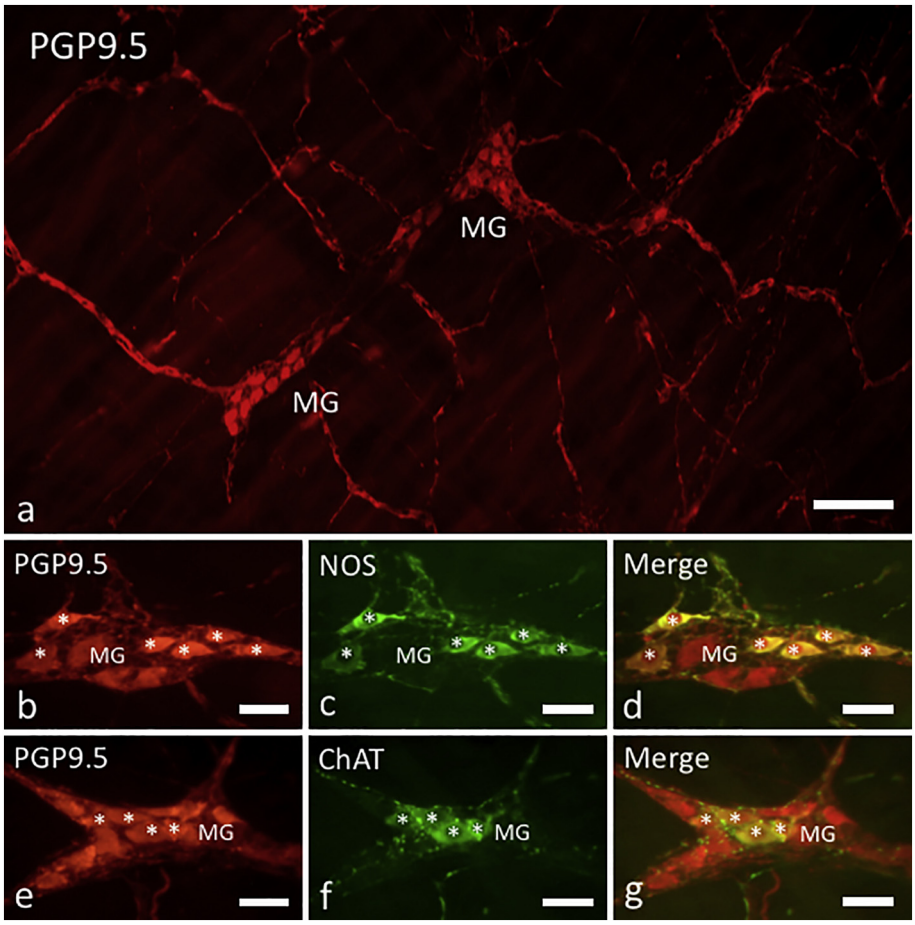

Fig. 1. Immunostained wholemount preparations of the myenteric plexus in the rat esophagus. Double immunostaining for PGP9.5 (b) and NOS (c), and PGP9.5 (e) and ChAT (f) in the myenteric ganglia. PGP9.5/NOS (d) and PGP9.5/ChAT (g) merged images. (a): A large number of cell bodies of intrinsic neurons in the myenteric ganglia (*MG*) and nerve fibers are positive for PGP9.5. *Scale* bar = 100 μm. (b–d): Six PGP9.5-positive neurons (*asterisks*) show NOS-IR in a myenteric ganglion (*MG*). *Scale* bars = 30 μm. (e–g): Four positive neurons (*asterisks*) are immunoreactive for ChAT in a myenteric ganglion (*MG*). *Scale* bars = 30 μm.

presented as the mean ± SD. Statistical analysis to evaluate the proportion of coinnervated motor endplates was performed using a one-way ANOVA followed by Tukey's multiple-comparison test. Values of $P < 0.05$ were considered to be significant.

3. Results and discussion

3.1. Motor neurons

3.1.1. Motor neurons innervating the LES

PGP9.5-immunoreactivity (IR) was found in a large number of intrinsic neurons and numerous nerve fibers in wholemount preparations of the esophagus (Fig. 1a, b, d). We estimated the total number of PGP9.5 immunoreactive neurons to be that of intrinsic neurons in the esophagus, although PGP9.5 immunostaining might not cover all esophageal intrinsic neurons. The total number of PGP9.5-positive neurons was 7207 ± 789 (n = 10 rats) in the whole esophagus. Quantification of the number of PGP9.5-positive neurons in each of five equal segments of the esophagus showed that PGP9.5-positive neurons were most frequent in segment 1 (roughly corresponding to the cervical esophagus), with a number (proportion) of 2269 ± 405 (31.5%). The number of PGP9.5-positive cells tended to decrease toward the aboral end of the esophagus, with frequencies of 1417 ± 242 (19.7%), 1141 ± 148 (15.8%) and 934 ± 132 (13.0%) in segments 2, 3 and 4, respectively, but then increased with 1445 ± 238 (20.0%) in segment 5 (the abdominal portion) (Fig. 2a). This pattern of the PGP9.5 positive neurons is similar to that observed in previous studies using the golden hamster or rat esophagus (Izumi et al., 2002; Wu et al., 2003).

The total number of NOS-positive neurons (Fig. 1c) was 4381 ± 356 (n = 5 rats) in the esophagus. The number distribution pattern of these cells was similar to that of the PGP9.5-positive neurons, and the numbers (proportions to the total number) were 1279 ± 240 (29.2%), 843 ± 86 (19.3%), 655 ± 105 (14.9%), 577 ± 73 (13.2%) and 1027 ± 42 (23.4%) in segments 1 to 5, respectively (Fig. 2b). This number distribution pattern of NOS-positive neurons is also roughly in accordance with that observed in the opossum and rat esophagus (Fang and Christensen, 1993; Wu et al., 2003). On the other hand, the total number of ChAT-positive neurons (Fig. 1f) was 2214 ± 299 (n = 5 rats), showing a decreasing tendency from the oral to aboral end of the esophagus, with 718 ± 129 (32.4%), 447 ± 94 (20.2%), 363 ± 32 (16.4%), 312 ± 46 (14.1%) and 373 ± 81 (16.9%) in segments 1 to 5, respectively, and the cell numbers between segments 3 to 5 were not so different (Fig. 2b). Thus, the increased number of PGP9.5-positive neurons in segment 5 (Fig. 2a) is considered to be due to an increase in NOS-positive neurons but not ChAT-positive neurons. This increase in NOS-positive neurons at this segment seems to depend on a close relationship with the LES because previous studies using retrograde tracing methods revealed that many NOS-positive neurons located in the myenteric plexus of the lower esophagus projected to the LES of the guinea pig and rat (Brookes et al., 1996; Kuramoto et al., 2013). Therefore, a subtype of NOS-positive intrinsic neurons in the lower esophagus, including segment 5, probably exert an inhibitory effect on LES as motor neurons because LES relaxation is caused by NO released from enteric inhibitory neurons (Yamato et al., 1992).

3.1.2. Motor neurons innervating the muscularis mucosa

To examine the distribution of NPY-IR, double immunolabeling for NPY and NOS or ChAT was performed. NPY-positive nerve fibers and terminals were located among the smooth muscles of the muscularis mucosa (Fig. 3a, d). Many of them exhibited NOS-IR (Fig. 3b), and some had ChAT-IR (Fig. 3e). Moreover, approximately 79% of NPY-positive intrinsic neurons (2954 ± 621, n = 4 rats) were NOS positive

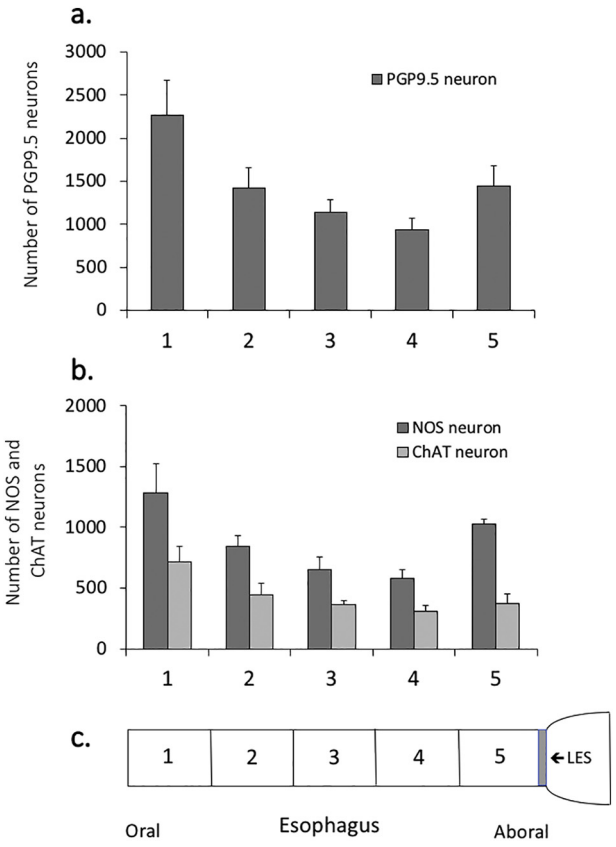

Fig. 2. Patterns of the number distribution of PGP9.5-positive neurons (a) (n = 10 rats) and NOS- and ChAT-positive neurons (b) in the whole esophagus (n = 5 rats, respectively). (a): PGP9.5-positive neurons are most frequent in segment 1 (corresponding to the cervical esophagus), decreasing in number toward the aboral end of the esophagus but increasing in number in segment 5 (the abdominal). (b): The number distribution pattern of NOS-positive neurons is similar to that of PGP9.5-positive neurons, whereas the number of ChAT-positive neurons appears to decrease toward the aboral end of the esophagus, with the cell numbers among segments 3 to 5 remaining fairly consistent. (c): The entire esophagus was circumferentially divided into five segments with equal length (1–5). Data are presented as mean ± S.D. *LES*; lower esophageal sphincter.

(Fig. 3g–i), and nearly 8% of them (2456 ± 389, n = 4) were ChAT positive (Fig. 3j–l). Additionally, NPY-positive nerve endings terminated on many motor endplates, and approximately 90% of them were NOS positive (data not shown). Most NPY-positive nerve endings likely coexist with VIP-IR and NADPH diaphorase reactivity (Reichel, 1998).

The muscularis mucosa is considered to be innervated by the submucosal ganglionic neurons in the small intestine or colon of the cat and dog (Onori et al., 1971; Furness et al., 1990). However, we found that there were very few or no nerve cell bodies in the submucosal plexus of the rat esophagus; therefore, the innervation of the muscularis mucosa is assumed to largely depend on neurons in the myenteric ganglia. The present immunohistochemical findings that both NPY/NOS- and NPY/ChAT-positive nerve fibers were present among the smooth muscles of the muscularis mucosa (Fig. 3a–f) and that both NPY/NOS- and NPY/ChAT-positive intrinsic neurons were also located in the myenteric ganglia (Fig. 3g–l) may suggest that these nerve fibers in the muscularis mucosa come from the NPY/NOS- and NPY/ChAT-positive motor neurons in the esophagus. However, we failed to verify the existence of NPY nerve fibers projecting to the esophagus from the paravertebral and prevertebral sympathetic ganglia.

3.1.3. Motor neurons innervating the striated muscle fibers

Immunohistochemistry revealed accumulations of larger bouton-like structures with ChAT-IR on a large number of striated muscles (Fig. 4a, b, c, f); thus, we considered these ChAT-positive structures to be motor endplates. Many of the motor endplates in segment 1 (Fig. 4a) appeared to be larger in size than those in segment 5 (Fig. 4b) of the esophagus. Double immunostaining for ChAT and NOS showed that the majority of the ChAT-positive motor endplates were intermingled with smaller NOS-positive nerve varicosities (Fig. 4c–e). However, some of these motor endplates had only a small number of NOS-positive varicosities among a portion of their ChAT-positive structures, and some possessed no varicosities positive for NOS (Fig. 4f–h). Motor endplates showing even a few NOS-positive nerve varicosities were categorized as coinnervated motor endplates.

Previous studies using immunohistochemistry and electron microscopy revealed that GAL- or VIP-positive nerve terminals coexpressing NOS and/or NADPH diaphorase were directly opposed to the sarcolemma of striated muscle fibers at motor endplates (Kuramoto et al., 1996; Wörl et al., 1997). Because many GAL-, VIP- and NOS-positive intrinsic neurons are present in the myenteric ganglia of the esophagus (Kuramoto et al., 1996; Wörl et al., 1997; Kuramoto et al., 1999; Wörl et al., 2002), the striated muscle fibers are deduced to receive, via motor endplates, a direct innervation of nerve terminals that could be derived from a subtype of motor neurons in the esophagus.

The total number of ChAT-immunoreactive motor endplates within an area of 25 mm^2 from five portions of the esophagus (segments 1 to 5) was 2339 ± 203 (n = 8 rats) on average. The number of motor endplates gradually increased along the aboral end of the esophagus (337 ± 28, 344 ± 30, 451 ± 51, 551 ± 71, 656 ± 78 in segments 1 to 5, respectively) (Fig. 5a). The total number of ChAT-positive motor endplates coinnervated by NOS-positive nerve terminals was 1801 ± 252 (n = 8 rats) on average in the whole esophagus, accounting for approximately 77% (1801/2339) of ChAT-positive motor endplates. On the other hand, the number of coinnervated motor endplates increased toward the aboral end of the esophagus (269 ± 27, 279 ± 41, 370 ± 61, 449 ± 82 in segments 1 to 4, respectively) but decreased somewhat in segment 5 (434 ± 82) (Fig. 5a). The proportion of coinnervated motor endplates to total motor endplates in each segment of the esophagus was estimated; the percentages in segments 1 to 4 were hardly different, with approximately 80% in each segment (80.0 ± 2.4, 80.8 ± 6.6, 81.7 ± 6.5 and 81.2 ± 8.5, respectively), but segment 5 showed a significantly lower rate of approximately 66% than any other esophagus segment ($P < 0.01$) (Fig. 5b), which may be associated with the variations in the motility of the striated muscles among the esophageal segments. Indeed, physiological data have demonstrated segmental variations in esophageal motility responses to intraluminal balloon inflation: a monophasic slow-wave response in the cervical to middle thoracic portions and a repetitive wave response in the lower thoracic to abdominal portion of the rat esophagus (Lu and Bieger, 1998). Such locally distinct responses in the upper and lower esophagus might be relevant to the differences in the numbers and coinnervation rate of the striated muscle fibers in each segment of the esophagus.

Especially, the lowest coinnervation rate of motor endplates with NOS-positive nerve terminals in segment 5, the abdominal esophagus, despite where the highest number of motor endplates were present in the esophageal segments (Fig. 5a, b) attracts our attention. Thus, among the esophagus portions, segment 5 contains the striated muscles whose motor activity could be least influenced by NOS-positive nerve terminals. A series of morphological and immunohistochemical studies by Neuhuber's research group using rats suggested a modulatory effect of enteric NOS neurons on vagal neurotransmission in esophageal striated muscles (Wörl et al., 1994; Wörl et al., 1997; Kallmunzer et al., 2008). In addition, the vagally mediated contractions of striated muscles in the hamster esophagus are inhibited by decreased acetylcholine (ACh) release from vagal motor endings via nitrergic intrinsic neurons activated by capsaicin-sensitive afferents (Izumi et al., 2003). If the striated muscle motility of the rat esophagus is inhibited by NOS nerve

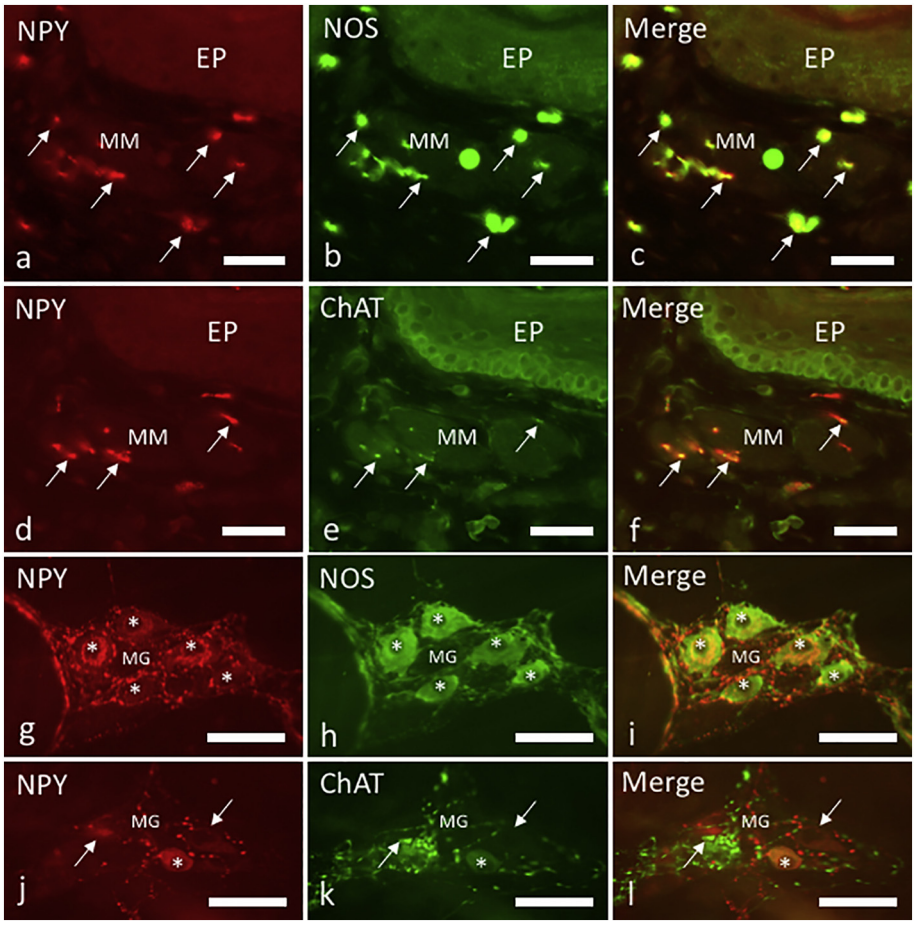

Fig. 3. Double immunolabeled frozen section preparations of the muscularis mucosa (a–f) and wholemount preparations of the myenteric ganglia (g–l). NPY/NOS (c, i) and NPY/ChAT (f, l) merged images. (a–c) and (d–f): Some nerve fibers (*arrows*) within smooth muscles of the muscularis mucosa (*MM*) show both NPY-IR and NOS-IR and both NPY-IR and ChAT-IR, respectively. *EP*: epithelium. *Scale* bars = 30 μm. (g–i): Five nerve cell bodies of intrinsic neurons are positive for both NPY and NOS (*asterisks*) in a myenteric ganglion (*MG*). *Scale* bars = 50 μm. (j–l): One NPY-positive neuron (*asterisks*) shows ChAT-IR, but two NPY-positive neurons (*arrows*) are negative for ChAT in a myenteric ganglion (*MG*). *Scale* bars = 50 μm.

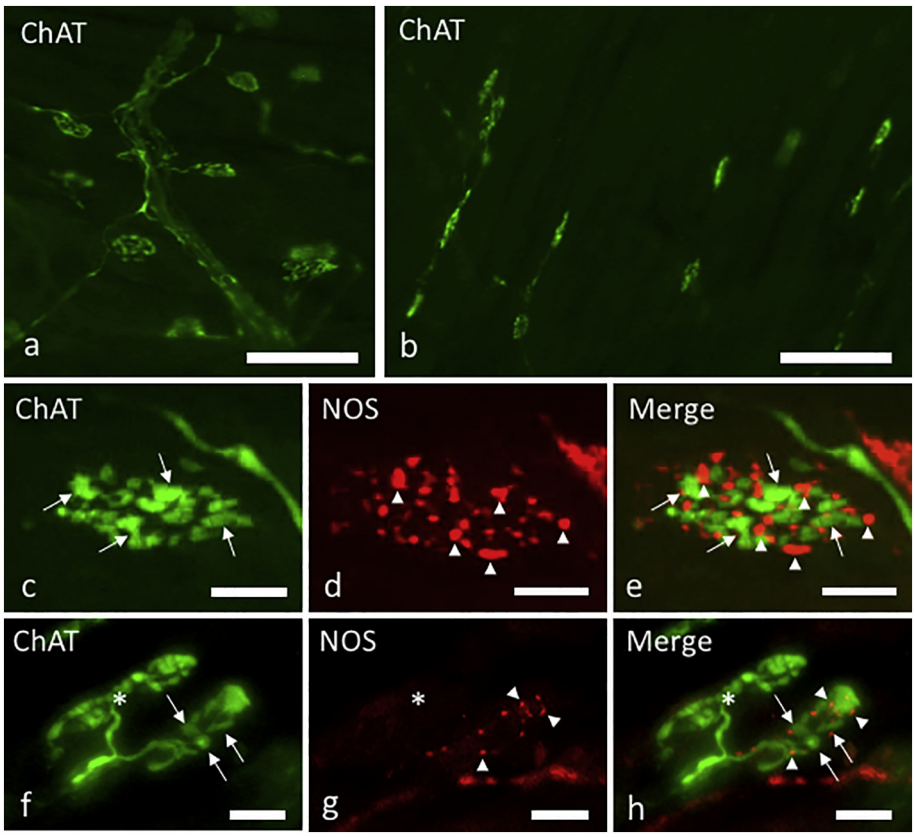

Fig. 4. Motor endplates on the striated muscle fibers in immunostained wholemount preparations of the rat esophagus. Double immunostaining for ChAT (c, f) and NOS (d, g) in the outer striated muscle layer. ChAT/NOS merged images (e, h). (a, b): Many motor endplates are seen in segment 1 (a) and segment 5 (b). A majority of motor endplates in segment 1 (a) appear to be larger in size than those in segment 5 (b). *Scale* bars = 100 μm. (c–e): A motor endplate with larger ChAT-positive bouton-like structures (*arrows*) is intermingled with numerous smaller NOS-positive nerve varicosities (*arrowheads*). Both structures never overlap with each other. *Scale* bars = 20 μm. (f–h): A few NOS-positive small varicosities (*arrowheads*) are present among the ChAT-positive endings (*arrows*) in a motor endplate at the right side, but no NOS-positive varicosities are seen in a motor endplate at the left side (*asterisks*). *Scale* bars = 20 μm.

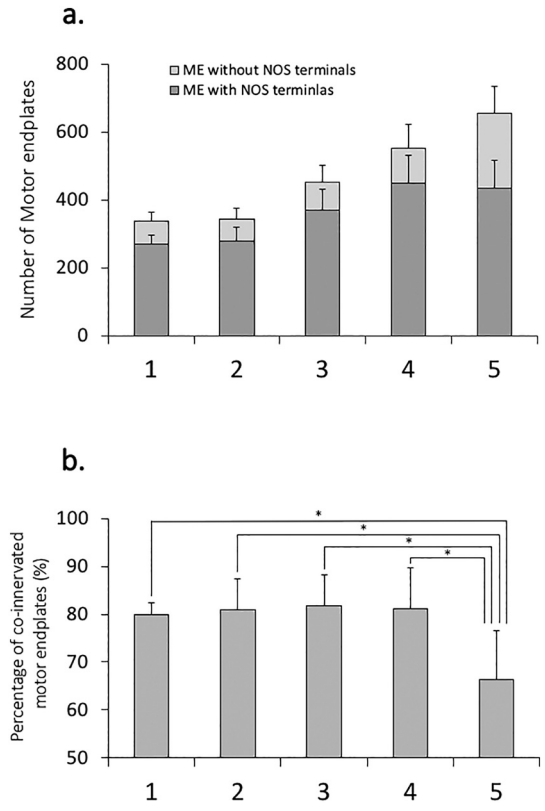

Fig. 5. Number distributions of ChAT-positive motor endplates and those innervated by NOS-positive terminals (a) and proportions of the innervated motor endplates (b) in the whole esophagus (n = 6 rats). (a): The number of motor endplates gradually increases along the aboral end of the esophagus with the highest number in segment 5, while the number of coinnervated motor endplates increases up to segment 4 of the esophagus but decreases somewhat in segment 5. (b): The proportions of coinnervated motor endplates to total motor endplates are not significantly different among segments 1 to 4, with approximately 80% in each of these segments, but segment 5 shows a significantly lower proportion than any other segment of the esophagus (*$P < 0.01$), with approximately 66% using a one-way ANOVA followed by Tukey's multiple-comparison test. Data are presented as mean ± S.D. *ME*; motor endplate.

terminals, as in the hamster esophagus, the striated muscles of segment 5 may be speculated to have the most powerful contraction activity among those of the other segments. If this hypothesis is true, the lower esophageal portion with this possible potent contraction might be the pivotal site responsible for a clearance system to egest chemical components such as acid and pepsin refluxed from the stomach. However, since the tunica muscularis in the lower esophageal part of humans is almost entirely composed of smooth muscles unlike that of rats, the mechanism of neural transmission involving muscle motility in the lower esophageal portion is considered to be different between these two species; therefore, the assumed relationship between the contraction intensity of coinnervated striated muscles and clearance system in the rat lower esophagus would not apply to that in the human esophagus. Interestingly, clinical data have revealed that the amplitude of esophageal contraction in the lower esophagus in patients with reflux esophagitis is significantly lower than that in healthy subjects (Sugiura et al., 2001), implying that the failure of the muscle contraction in the lower esophagus may lead to a decline in the clearance ability, resulting in the development of esophageal mucosal damage. This observation suggests that the ability to contract the muscles at the lower esophageal portion is closely related to the clearance event. If the rat lower esophagus has a similar motility behavior that causes reflux at the human lower esophagus despite the differences in the musculature component in the lower esophageal portion between species, investigating motility and responsiveness to noxious stimuli such as acid and capsaicin using the rat esophagus may be worthwhile to understand the mechanistic development of reflux-induced diseases including gastroesophageal reflux disease (GERD).

3.2. Interneurons

Immunoreactivity of Met-enkephalin-Arg^6-Gly^7-Leu^8 (ENK-8), which is derived from preproENK A containing also Met-ENK and Leu-ENK (Noda et al., 1982), was found in some of the intrinsic neurons and nerve fibers in the myenteric plexus of the esophagus (Fig. 6a). Approximately 68% of ENK-8-positive intrinsic neurons (453 ± 22, n = 3 rats) were immunoreactive for ChAT (Fig. 6b), and approximately 24% of them (536 ± 69, on average, n = 3 rats) were positive for NOS (data not shown). Some ENK-8 nerve terminals with ChAT-IR surrounded ENK-8-positive or negative intrinsic neuronal cell bodies (Fig. 6a–c). ENK-8-positive varicose nerve endings showing NOS-IR were also localized on some ChAT-positive motor endplates (data not shown).

To determine the origin of these ChAT/ENK-8-positive nerve terminals surrounding intrinsic neurons, the compact portion of the nucleus ambiguus (cNA) and dorsal motor nucleus of the vagus (DMV) in the medulla oblongata, which provide vagal cholinergic motor efferents for the esophagus, were examined using double immunolabeling for ENK-8 and ChAT. Almost all motor neurons in both the cNA (Fig. 6d–f) and DMV nuclei (Fig. 6g–i) were positive for ChAT, but negative for ENK-8, although numerous ENK-8-positive nerve terminals were observed around the cell bodies of their motor neurons (Fig. 6d, g). Therefore, a part of the ChAT/ENK-8 positive nerve terminals innervating intrinsic neurons in the esophageal ganglia (Fig. 6a–c) are suggested to originate from a subclass of ACh/ENK-8 containing intrinsic neurons as interneurons in the esophagus, but neither the cNA nor DMV, where the absence of ENK-8 containing neurons is consistent with the finding in a previous report (Murakami et al., 1987). In addition, as our previous study showed that many NOS-positive nerve terminals surrounded intrinsic NOS-negative and -positive neurons (Kuramoto and Kadowaki, 2006), a subtype of intrinsic NOS-positive neurons may be also interneurons. However, the questions of whether the presumed types of interneurons that are responsible for the excitatory or inhibitory reflex pathway and involved in esophageal motility are of ascending or descending polarity, how these neurons communicate with other kinds of intrinsic neurons or each other and what other chemical codings they express remain to be settled.

3.3. Sensory neurons

The sensory system in the esophagus is generally accepted to be primarily composed of both vagal and spinal primary afferents, which are mechanosensitive and/or nociceptive (Wörl and Neuhuber, 2005; Kollarik and Brozmanova, 2009). Especially, the close relationship is present between the vagal sensory afferents and esophagus: a large number of sensory neurons with calbindin (Calb)-IR (Kuramoto and Kuwano, 1994) or calretinin (Calre)-IR (Dütch et al., 1998) in the nodose ganglion have been demonstrated to project to the esophagus to form intraganglionic laminar endings (IGLEs) in the myenteric ganglia, which are deduced to be vagal mechanoreceptive structures (Zagorodnyuk and Brookes, 2000; Zagorodnyuk et al., 2003). Thus, we investigated whether the IGLEs are related with numerous cholinergic nerves distributed in the esophagus using double immunolabeling of ChAT and Calre as a marker of the IGLEs. As a result, no Calre-positive IGLEs in the ganglia exhibited ChAT-IR (Fig. 6j–l), indicating that the Calre-positive IGLEs (Fig. 6j) are quite different from ChAT-positive nerve fibers (Fig. 6k), although very few varicose nerve endings were positive for both IRs. This suggests that almost all of cholinergic nerves in the esophagus are neither sensory nor derived from, at least, the vagal afferents.

On the other hand, the intestines have been shown to contain

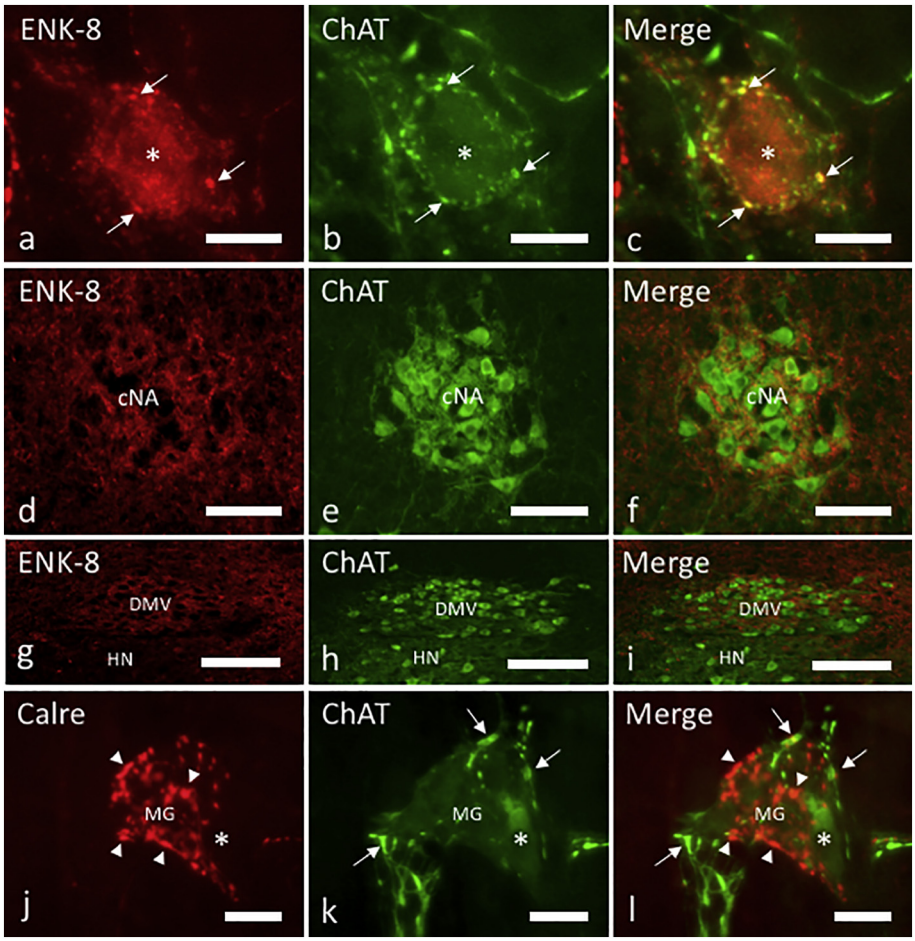

Fig. 6. Double immunolabeled wholemount preparations of the myenteric ganglia (a–c and j–l) and frozen section preparations of the cNA (d–f) and DMV (g–i) in the medulla oblongata. ENK-8/ChAT (c, f, i) and Calre/ChAT (l) merged images. (a–c): Several nerve endings (*arrows*) showing both ChAT-IR and ENK-8-IR surround one ChAT/ENK-8-positive nerve cell body (*asterisks*). *Scale* bars = 20 μm. (d–f): Almost all of the cell bodies of the motor neurons in the cNA are ChAT positive, but there are no neurons showing ENK-8-IR in the cNA. *cNA*: compact portion of nucleus ambiguus. *Scale* bars = 100 μm. (g–i): Almost all motor neurons in the DMV are positive for ChAT, but no ENK-8 neurons are seen in the DMV. *DMV*: dorsal motor nucleus of the vagus, *HN*: hypoglossal nucleus. *Scale* bars = 200 μm. (j–l): In a myenteric ganglion (MG), Calre-positive IGLEs (*arrowheads*) are entirely different from ChAT-positive nerve fibers (*arrows*), and one ChAT-positive neuron (*asterisks*) is negative for Calre. *Scale* bars = 30 μm.

intrinsic primary afferent neurons (IPANs) as the first neurons of the intrinsic reflex pathways, which can be activated by adequate mechanical and chemical stimuli (Kirchgessner et al., 1992; Bertrand et al., 1997; Kunze et al., 1998). However, whether distinctive sensory neurons such as IPANs exist within the esophageal myenteric plexus has remained unknown because the esophagus receives predominant innervation from vagal afferents (Neuhuber et al., 1998; Chang et al., 2003) as well as spinal afferents. Remarkably, stretching of the subdiaphragmatic esophageal wall of mice in longitudinal or transverse directions has been shown to cause stretch-activated LES relaxation or contraction, respectively, suggesting that these effects are mediated via mechanosensitive neurons in the myenteric plexus (Jiang et al., 2009). Moreover, an in vitro experiment demonstrated that a subtype of esophageal inhibitory NO-containing motor neurons is mechanosensitive (Dong et al., 2015). Therefore, intrinsic NO-containing mechanosensitive neurons seem to be present in the subdiaphragmatic esophagus, suggesting that a subclass of NO-containing neurons located in the abdominal esophagus may play both motor and mechanosensory roles. Mazzuoli and Schemann (2009) have physiologically and pharmacologically identified mechanosensitive neurons in the myenteric plexus of the guinea-pig ileum, a new population of which have rapidly adapting discharge to deformation, contain interneurons and motor neurons, and differ from IPANs in chemical coding and responses to stimuli.

3.4. Other neurons

Our finding that PGP9.5-positive intrinsic neurons were most frequent in segment 1, the cervical esophagus (Fig. 2a), was unexpected and surprising. Intriguingly, previous studies using retrograde tracing and immunohistochemistry demonstrated the presence of esophageal neurons that project to the trachea in the guinea pig and mouse, most of which were NOS/VIP immunopositive (Moffatt et al., 1998; Balentova et al., 2013). Furthermore, Mazzone and McGovern (2010) showed by electrophysiological and immunohistochemical methods that a subset of esophageal cholinergic neurons with calretinin-IR innervated cholinergic neurons within the ganglia of the guinea-pig trachea. This evidence suggests that viscerofugal neurons, which project to the trachea or bronchus, are present within the myenteric ganglia of the esophagus, similar to the intestinofugal neurons in the guinea-pig small intestine (Kuramoto and Furness, 1989) and the rat large intestine (Luckensmeyer and Keast, 1995), and that a subpopulation of them may act on an efferent limb in a reflex pathway between the trachea or bronchus and esophagus. Clinically, the involvement of cough in GERD has been considered to be markedly critical: a stimulus such as acid refluxing to the esophagus in patients with chronic cough and GERD likely sensitizes the cough reflex that is mediated by the vagal afferents innervating the esophagus (Kollarik and Brozmanova, 2009). Direct activation from the esophageal viscerofugal neurons may be one of the neural pathways that cause the cough reflex because a population of cholinergic esophageal neurons is suggested to innervate intrinsic tracheal cholinergic ganglia to cause contraction of the smooth muscles in the guinea-pig trachea (Mazzone and McGovern, 2010). Although why segment 1, the cervical esophagus, showed the highest number of intrinsic neurons in the esophagus and whether a large number of them may supply their nerve fibers to the trachea or bronchus are unclear, we predict that a number of intrinsic cholinergic and/or nitrergic neurons located especially at segments 1 and 2 of the esophagus where the trachea is attached, may project to the trachea to participate in the cough reflex system. To clarify this hypothesis, further studies with

ingenious methods using neuronal anterograde or retrograde tracers are needed.

In conclusion, the present results showing regional variations in the number of intrinsic myenteric neurons and coinnervated motor endplates in the rat esophagus suggest functional roles of esophageal intrinsic neurons as, at least, motor neurons (innervating the striated and smooth muscles in the esophagus) and interneurons and local regulation of striated muscle motility. The variations could be involved in the formation of esophageal segmental organizations with vagal motor efferents, contributing to differences in local movement among the esophageal segments. Although we can consider the intrinsic neurons present in the esophagus, including motor and sensory neurons, interneurons, and viscerofugal neurons as described above, as being in the intestines, the number of intrinsic neurons that play these roles in each segment of the esophagus is unknown.

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