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Distribution of GABA, glutamate decarboxylase 67 (GAD67), Vesicular GABA transporter (VGAT) and GABA B -receptor Immunoreactivities in the Rat Esophagus

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Abstract

We investigated the distributions of gamma-aminobutyric acid (GABA), vesicular GABA transporter (VGAT), $GABA_B$-receptor ($GABA_B$-R) and glutamate decarboxylase 67 (GAD67), immunoreactivities in the rat esophagus. GABA immunoreactivity was found in the nerve fibers of the esophagus, but not in the neurons. A few GABA-immunoreactive nerve fibers ran along the muscularis mucosae and some GABA-immunoreactive nerve bundles and fibers ran along and contacted clusters of myenteric neurons. Numerous GABA-immunoreactive nerve terminals ran along the striated muscles and formed motor endplates on the muscles while GABA-immunoreactive nerve fibers contacted nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d)-positive or choline acetyltransferase (ChAT)-immunopositive neurons in the myenteric plexus. NADPH-d-positive nerve fibers often intermingled with GABA-immunoreactive motor endplates in the striated muscles. The GABA-immunoreactive nerve fibers contacting the myenteric neurons were also ChAT-immunopositive. The GABA-immunoreactive nerve fibers that formed motor endplates corresponding to α-bungarotoxin (BTX)-positive areas on the striated muscles were also ChAT-immunopositive. The average percentage of GABA-immunoreactive motor endplates to total ChAT-immunoreactive motor endplates in the upper, middle, and lower portions of the esophagus was 24.8%. VGAT immunoreactivity was seen in almost all motor endplates on the esophageal striated muscles, but not in the neurons of the myenteric plexus. Some VGAT-immunoreactive nerve terminals contacted clusters of myenteric neurons. $GABA_B$-R immunoreactivity was observed in numerous myenteric ganglia and in the proximal-to-distal part of the axons of the ganglia, but not in the striated and smooth muscles. $GABA_B$-R-immunoreactive neurons were brain nitric oxide synthase (bNOS)- or ChAT-immunopositive. The present study suggests that GABA and $GABA_B$-R may be present in the neuronal elements of the striated muscle of the rat esophagus and may play an important role in the local inhibitory system of rat esophageal motility. No GAD67 immunoreactivity was found in the nerve fibers and neurons of the esophagus. In the nucleus ambiguus and in the dorsal motor nucleus of the vagal nerves, GAD67 and VGAT immunoreactivities were shown in numerous nerve fibers on the surface of the cell bodies, but not in the cell bodies of the vesicular acetylcholine transporter-immunoreactive neurons. The present study suggests that GABA may be taken up and accumulated in the synaptic vesicles by VGAT in the nerve terminals, but not in the cell bodies of the neurons of the brainstem.

Introduction

In the rat esophagus, the muscularis externa consists almost entirely of striated muscle fibers and only partly of smooth muscle fibers at the esophagogastric junction. Esophageal motility is mainly controlled by vagal efferents from the motor neurons of the nucleus ambiguus (for review see Cunningham and Sawchenko 1990; Roman and Gonella1987). These efferents directly innervate the striated muscle via the motor endplates and neurons of the dorsal motor nucleus of the vagal nerves and indirectly regulate the smooth muscle via myenteric intrinsic neurons. On the other hand, vagal afferents are derived from sensory neurons in the nodose ganglion whose terminals perceive various stimulations from the

esophagus and relay the sensory information to the medulla oblongata (Roman 1982; for review see Cunningham and Sawchenko 1990; Conklin and Christensen 1994; Lu and Bieger 1998). In addition, the esophagus is innervated by the sensory neurons of the dorsal root ganglia and the intrinsic neurons (Conklin and Christensen 1994). The striated muscle of the rat esophagus are co-innervated via their motor endplates by both cholinergic nerve endings from the neurons of the nucleus ambiguus and nitric oxide synthase (NOS)-positive nerve terminals from intrinsic neurons containing nitric oxide (Neuhuber et al. 1994, 1998; Wörl et al. 1994; Kuramoto et al. 1999) and neuropeptides such as galanin, vasoactive intestinal polypeptide, neuropeptide Y, calcitonin gene-related peptide (CGRP), pituitary adenylate cyclase-activating polypeptide (PACAP) and enkephalin (Uddman et al. 1980; Kuramoto and Endo 1995; Kuramoto et al. 1996; Neuhuber et al. 2001; Wu et al. 2003; Kuramoto et al. 2024). Nitric oxide and these neuropeptides act on smooth muscle as inhibitory mediators in the intestine (Bult et al. 1990; Gibson et al. 1990; Tøttrup et al. 1991; for review see Furness 2000; for review see Brookes 2001). However, it is unclear whether or how these mediators are involved in the inhibition of esophageal striated muscles. Thus, the local inhibitory neuronal system involved in esophageal motility remains poorly established, although the central control of esophageal motility is remarkably well-understood (for review see Hornby and Abrahams 2000).

Gamma-aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the central and peripheral nervous systems. Previous studies found both GABA- and glutamic acid decarboxylase (GAD)-immunoreactive nerve fibers and neurons in the gastrointestinal tract (Jessen et al. 1986; Saito and Tanaka 1986; Davanger et al. 1987; Hills et al. 1987; Furness et al. 1989). GABA exerts its inhibitory actions through 3 distinct types of receptors, $GABA_A$-receptor ($GABA_A$-R), $GABA_B$-receptor ($GABA_B$-R), and $GABA_C$-receptor ($GABA_C$-R) (for review see Bowery et al. 2002; for review see Feigenspan and Bormann 1998). Previous molecular and immunohistochemical studies found $GABA_A$-R, $GABA_B$-R, and $GABA_C$-R on enteric neurons (Krantis et al. 1995; Nakajima et al. 1996; Zeiter et al. 1996; Fletcher et al. 2001). As well as in the brain, $GABA_B$-Rs are also abundantly expressed in the gastrointestinal tract (Hyland and Cryan 2010). GABA can directly and indirectly stimulate the enteric excitatory and inhibitory neurons projecting to the muscle (Krantis et al. 1980; Maggi et al. 1984). $GABA_A$-R elicits contraction through an excitatory action on cholinergic postganglionic neurons, whereas, in the small and large intestine, $GABA_B$-R induces relaxation through an inhibitory presynaptic action on cholinergic postganglionic neurons (Giotti et al. 1983; Gentilini et al. 1992; Minocha and Galligan 1993).

Nonetheless, it remains unclear whether GABA, $GABA_B$-R, VGAT exist in the inhibitory neuronal system of the rat esophagus and, if so, what functional significance they play. To clarify these issues, we immunohistochemically examined the presence and distribution of GABA, GABA-B-R, GAD67 and VGAT in the neuronal elements of the rat esophagus and discuss their possible involvement in the inhibition of esophageal motility. Furthermore, we examined the presence of VGAT, GAD67 in the cholinergic neurons of the nucleus ambiguus and in the dorsal motor nucleus of the vagal nerves.

Materials and Methods

Twenty-five male Wistar rats (Japan SLC, Shizuoka, Japan; 8 weeks old; body weight 180–200 g) were used in this study. The animals received commercial food pellets and water ad libitum. All experimental procedures were performed according to the Guidelines for Animal Care by the Japanese Red Cross Hokkaido College of Nursing (Permit Number: 33, 34, 41).

The animals were deeply anesthetized by inhalation with sevoflurane and perfused through the heart with 200 ml of physiological saline and 200 ml of 4% paraformaldehyde or 0.3% glutaraldehyde plus 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS) pH 7.4. The esophagus, brainstem and cerebellum were removed and immersed in the same fixative for 2 h at 4°C. For whole-mount preparations of esophagus, both the oral end of the esophagus and the proximal part of the stomach were ligated with a cotton thread, and the lumen was distended with a fixative solution, which was introduced with a needle. After fixation in the same fixative for 1 h, the esophagus was opened longitudinally, and the mucosal and submucosal layers were stripped away from the esophagus; the outer striated muscle layer attached to the myenteric plexus was then isolated by dissecting away the inner striated muscle layer. To prepare the cryostat sections, the esophagus was immersed overnight in PBS containing 30% sucrose at 4°C. The esophagus was divided into 3 portions (upper, middle, and lower), cut into 12 μm thick sections using a cryostat, and mounted on glass slides coated with poly-L-lysine (Sigma, St. Louis, MO, USA).

For immunohistochemistry (Table 1), the whole-mount preparations and sections of the esophagus were incubated with primary antibodies overnight at 4°C, followed by incubation for 2 h with a secondary antibody conjugated to indocarbocyanine (Cy3) or Alexa Fluor® 488. For double immunostaining, the whole-mount preparations and sections were incubated for 12 h at 4°C with a mixture of 2 primary antisera raised against different species. The immunoreacted whole-mount preparations and sections were rinsed in PBS and then incubated with a mixture of secondary antibodies conjugated to Cy3 or Alexa Fluor® 488. After PBS washes, some whole-mount preparations and sections were additionally incubated with fluorescein (FITC)-conjugated α-bungarotoxin (BTX) (1:1000; PK-CA707-00011; PromoKine, Heidelberg, Germany) as a marker of nicotinic acetylcholine receptor at the neuromuscular junctions of striated muscles and were incubated free-floating for NADPH-d as a marker of NOS (Ward et al. 1992) for 1 h at room temperature in 0.1 M PBS containing 0.2 mg/ml nitroblue tetrazolium (N-6876; Sigma), 1.0 mg/ml β-NADPH (309-50471; Oriental Yeast Co. Ltd., Tokyo, Japan), and 0.3% Triton X-100.

Table 1
List of primary antisera and secondary fluorescence conjugated antisera used for immunohistochemistry in the present study.

Primary antisera	**Host animals**	**Dilution**	**Catalogue No.**	**Source**
bNOS	Rabbit	1:4000	N31030	Transduction Laboratories, KY, USA
ChAT	Goat	1:100	AB144P	Chemicon International Inc, Temecula, CA, USA
VAChT	Goat	1:400	ABN100	Chemicon International Inc
GABA	Rabbit	1:2000	A-2052	SIGMA BIO SCIENCES, MO, USA
GAD67	Mouse	1:500	MAB5406	Millipore Corporation, Temecula, CA, USA
VGAT	Rabbit	1:600	131 002	Synaptic Systems, Goettingen, Germany
$GABA_B$-R	Guinea pig	1:4000	AB1531	Chemicon International Inc
PGP9.5	Rabbit	1:5000	RA95101	UltraClone Ltd, Isle of Wight, UK
bNOS: brain nitric oxide synthase, ChAT: choline acetyltransferase, VAChT: vesicular acetylcholine transporter, GABA: γ-aminobutyric acid, GAD: glutamate decarboxylase, $GABA_B$-R: GABA $_B$-receptor, PGP9.5: protein gene product 9.5, Cy3: indocarbocyanine				
Secondary antisera	Conjugates	Dilution	Catalogue No.	Source
Anti-goat IgG	Alexa Fluor® 488	1:100	705-545-147	Jackson ImmunoResearch, West Grove, PA, USA
Anti-guinea pig IgG	Cy3	1:250	706-165-148	Jackson ImmunoResearch
Anti-rabbit IgG	Alexa Fluor® 488	1:100	711-545-152	Jackson ImmunoResearch
Anti-rabbit IgG	Cy3	1:250	711-165-152	Jackson ImmunoResearch
Anti-goat IgG	biotin	1:500	705-065-147	Jackson ImmunoResearch
Anti-rabbit IgG	biotin	1:500	BA-1000	VECTOR LABORATORIES, Burlingame, CA, USA
Anti-mouse IgG	biotin	1:500	BA-2000	VECTOR LABORATORIES

To examine the colocalization of GABA-/ ChAT-immunoreactive nerve terminals in the neuromuscular junctions of the striated muscle, 20 sections were randomly selected from the upper, middle, and lower portions of the esophagus obtained from each of 10 animals. The number of GABA-/ ChAT-immunoreactive nerve terminals on the motor endplates of the striated muscles were counted.

Furthermore, the colocalization ratio of $GABA_B$-R/ protein gene product 9.5 (PGP9.5), brain nitric oxide synthase (bNOS)/ $GABA_B$-R- or ChAT-/ $GABA_B$-R-immunoreactive neurons in the myenteric plexus was examined.

Five areas of 2.5 mm × 2.5 mm (= 6.25 mm^2) in the whole-mount preparations were randomly selected 100 $GABA_B$-R-immunoreactive nerve cells from the 3 portions of the esophagus of 3 rats, the numbers of $GABA_B$-R-/PGP9.5-, bNOS-/ $GABA_B$-R- or ChAT-/ $GABA_B$-R-immunoreactive neurons in the myenteric plexus were counted. The data obtained from each portion of five rats were shown as mean ± S.E. and were analyzed by the non-parametric U-test.

To prepare the cryostat sections, the brainstem and cerebellum were left overnight in PBS containing 30% sucrose at 4°C. These tissues were cut about 50 μm thick or serial 20 μm thick using a cryostat and mounted on glass slides coated with poly-L-lysine (Sigma, St. Louis, Mo, USA). We examined the cryostat sections immunohistochemically by using VGAT and GAD67 antibodies whether the cholinergic neurons in the nucleus ambiguus and in the dorsal motor nucleus of the vagal nerves contained GABA or not. For immunohistochemistry, the immunofluorescent method and avidin-biotin-peroxidase complex (ABC) method (Hsu et al. 1981) was used. Some brain sections were with a staining kit purchased from Vector Laboratories (Burlingame, Calif.,USA). The tissues were incubated for 48 h at room temperature with antibodies described above, followed by incubation in biotinylated secondary antibodies and ABC for 1 h respectively at room temperature. The antigen-antibody reaction sites were visualized by incubating the section s for 15 min at room temperature with diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in Tris-HCL buffer (25mM, pH 7.6).

The specificity of the immunohistochemical staining was confirmed by replacing the primary antibody with normal serum and by using diluted antiserum pretreated with a GABA (G-012;10 μg/ml; Research Biochemicals, Natick, MA., USA) for 24 h at 4°C. No immunostaining was observed under these conditions.

Results

In the cryostat sections, a few GABA-immunoreactive nerve fibers were found near and in the muscularis mucosae (Fig. 1a) and the submucosal tissue of the rat esophagus. Numerous GABA-immunoreactive nerve fibers ran along the striated muscle fibers and terminated on them. The nerve fibers formed motor endplates on the striated muscle fibers (Fig. 1b). Thick GABA-immunoreactive nerve bundles and fibers ran among the ganglia in the myenteric plexus. Fine GABA-immunoreactive nerve fibers were seen on the neurons in the myenteric plexus and were in close contact with myenteric neurons (Fig. 1c). No GABA immunoreactivity was observed in the mucous epithelial cells, in the lamina propria, or in the neurons of the myenteric plexus of the rat esophagus.

$GABA_B$-R immunoreactivity was seen only in the neurons of the myenteric plexus in the rat esophagus (Fig. 1d). The size of the $GABA_B$-R-immunoreactive neurons ranged from small to large. The

immunoreactivity was intense at the cell surface membrane and as granular structures in the cytoplasm. No $GABA_B$-R immunoreactivity was observed in the nerve terminals, in the striated muscles or in the smooth muscle cells of the muscularis mucosae of the esophagus.

In the whole mount preparations, GABA immunoreactivity was observed in numerous nerve fibers, but not in the neurons of the rat esophagus. Thick GABA-immunoreactive nerve bundles and fibers ran along the clusters of neurons in the myenteric plexus (Fig. 2) and ramified into one or more branches that projected to a few myenteric ganglia in the myenteric plexus and to the striated muscles. Numerous GABA-immunoreactive nerve terminals formed the motor endplates on the striated muscle fibers (Fig. 3).

Double staining showed that GABA-immunoreactive nerve fibers were sometimes in close contact with NADPH-d-positive or -negative neurons in the myenteric plexus (Fig. 4a, b). NADPH-d-positive nerve fibers from myenteric neurons often intermingled with the GABA-immunoreactive motor endplates in the striated muscles. Furthermore, GABA-immunoreactive nerve fibers were sometimes in close contact with ChAT-immunoreactive or ChAT-immunonegative neurons in the myenteric plexus and were ChAT-immunopositive (Fig. 4c, d). Some GABA-immunoreactive nerve fibers eventually formed motor endplates on the striated muscles.

Double immunostaining showed that the numerous GABA-immunoreactive nerve terminals forming motor endplates on the striated muscles were both ChAT-immunoreactive (Fig. 5a, b) and BTX-positive (Fig. 5c, d). Some of the ChAT-immunoreactive or BTX-positive motor endplates were GABA-immunonegative (Fig. 5a–d). Triple staining showed that NADPH-d-positive nerve fibers intermingled with GABA-immunopositive nerve terminals and were located at BTX-positive areas (Fig. 5e–g). The percentages of ChAT-immunoreactive motor endplates with GABA immunoreactivity to total ChAT-immunopositive endplates in the upper, middle, and lower portions of the esophagus were 33.7%, 17.0%, and 23.7%, respectively (average, 24.8%) (Table 2). The differences among the number of GABA- or ChAT-positive neuromuscular junctions in the three portions of the esophagus were not significantly different. However, no GABA immunoreactivity was seen in nerve cell bodies in the myenteric plexus of the esophagus.

In the control, immunohistochemical staining was confirmed by replacing the primary antibody with absorbed with 10μg GABA. No GABA-immunostaining was observed in in the ChAT-immunoreactive motor endplates of the control sections of the rat esophagus (Fig. 5h, i).

$GABA_B$-R immunoreactivity was observed in numerous myenteric neurons of the rat esophagus. The $GABA_B$-R-immunoreactive neurons ranged in size from small to large, and the immunoreactivity was localized intensely to the cell surface membrane and to granular structures in the cytoplasm (Fig. 6). The immunoreactivity was often detectable in the proximal-to-distal part of the axons, but not in the terminals. Double immunostaining showed that the percentage of $GABA_B$-R-immunoreactive neurons to myenteric neurons with PGP9.5 immunoreactivity was 100% in the upper, middle, and lower portions of

the rat esophageal myenteric plexus. The PGP9.5 antibody was used for the neurons in the rat esophageal myenteric plexus (Kuramoto et al. 2019). Double immunostaining in the whole-mount preparations showed that numerous $GABA_B$-R-immunoreactive neurons were NOS-immunopositive (Fig. 7a, b) or ChAT-immunopositive (Fig. 7c, d). The percentages of NOS-/$GABA_B$-R-immunoreactive neurons in the upper, middle, and lower portions of the rat esophageal myenteric plexus were 64.0%, 68.6%, and 63.6% (average, 65.4%), respectively (Table 3). The percentages of ChAT-/$GABA_B$-R-immunoreactive neurons were 14.0%, 13.6%, and 9.6% (average, 12.4%) in the upper, middle, and lower portions of the rat esophageal myenteric plexus (Table 3). The differences among the number of NOS-/$GABA_B$-R- or ChAT-/$GABA_B$-R-immunopositive neuromuscular junctions in the three portions of the esophagus were not significantly different. However, no $GABA_B$-R immunoreactivity was found in the nerve terminals and neuromuscular junctions in the striated muscles, in the striated muscle cells, or in the smooth muscle cells of the muscularis mucosae.

In the whole-mount preparation of esophagus, VGAT immunoreactivity was shown mainly in the motor endplates on the striated muscles of the esophagus. The VGAT immunoreactivity was fine granular in the motor endplates (Fig. 8a, b). However, no or weak immunoreactivity was found in the nerve fibers. VGAT immunoreactivity was seen as small dots within nerve fibers and in the periphery of the nerve cells in the myenteric plexus.

In the double staining with ChAT and VGAT antibodies, almost all ChAT-immunoreactive motor endplates were VGAT-immunopositive. Some VGAT-immunoreactive nerve fibers contacted the soma of the ChAT-immunoreactive neurons in the myenteric plexus. No VGAT-immunoreactive neurons were seen in the myenteric plexus. No GAD67 immunoreactivity was observed in the neuronal elements of the esophagus.

In the double staining with vesicular acetylcholine transporter (VAChT), VGAT and GAD67 antibodies, GAD67 and VGAT immunoreactivities were not seen in the VAChT-immunoreactive neurons in the nucleus ambiguus and in the dorsal motor nucleus of vagal nerves (Fig. 9a–f). GAD67 (Fig. 9f) and VGAT (Fig. 9b, d) immunoreactivities were observed in numerous nerve fibers and in nerve terminals on the surface of the neurons, but not in the dendrites and in the cell bodies of VAChT-immunoreactive neurons (Fig. 9a, c, e) in the rat nucleus ambiguus and in the dorsal motor nucleus of vagal nerves.

In the rat cerebellum as the positive control, the GABA, GAD67 and VGAT immunoreactivities of the Purkinje cells were observed on the surface and in the periphery of the cell bodies, and in numerous axons, but not in the cell bodies and in the dendrites (Fig. 10a–d) which was in accordance with the previous reports (McLaughlin et al. 1975; Oertel et al.1981; Ottersen and Strom-Mathisen 1984; Chaundhry et al. 1998; Takayama and Inoue 2004).

Discussion

We first revealed that GABA-immunoreactive nerve fibers were ChAT-immunopositive and formed motor endplates corresponding to the BTX-positive areas of the striated muscles of the adult rat esophagus. This suggests that GABA and acetylcholine are colocalized in the motor endplates corresponding to the nicotinic acetylcholine receptor-rich portion and that GABA in the motor endplates may exert an inhibitory effect on the motility of the striated muscles of the rat esophagus. GABA in the motor endplates on the striated muscles of the vertebrates has not previously been seen. In addition, the present study showed that VGAT immunoreactivity was found in almost all motor endplates of the esophageal striated muscles. GABA is localized in the membrane of GABAergic vesicles and loads synthesized GABA into the synaptic vesicles by VGAT at the axon terminals (McIntire et al. 1997; Chaudhry et al. 1998; Fon and Edwards, 2001). The present study suggests that GABA may be present in the neuronal elements of the striated muscles of the rat esophagus, and synthesized GABA may be taken up and accumulated in the synaptic vesicles by VGAT in the nerve terminals.

Furthermore, we were unable to observe GABA-R immunoreactivity on the striated muscles of the rat esophagus. The role that GABA plays in transmission to the esophageal striated muscles is unknown. However, GABA does not directly affect the contraction of the skeletal muscle of mice (Chiou and Chang, 1994) and of the smooth muscle in several regions of the intestine of the guinea pig (Krantis et al. 1980; Krantis and Kerr 1981; Kleinrock and Kilbinger 1983). Thus, it may be unlikely that GABA directly influences the striated muscles or smooth muscles in the esophagus. Further gene and protein analyses are required to confirm the expression of other GABA-Rs in the striated muscles of esophagus.

A previous study showed that GABA had inhibitory effects on the secretion of acetylcholine at cholinergic synaptic contacts with the striated muscles by cultured retinal neurons (Agardh et al. 1985). GABA depresses excitatory cholinergic transmission in the gut by inhibiting transmitter release, an effect mediated through presynaptic $GABA_B$-Rs (Fargeas et al. 1988; Kerr et al. 1990; Gentilini et al. 1992) and modifies transmission from enteric neurons (Ong and Kerr 1983, 1984; Ohkawa 1987). However, we failed to demonstrate $GABA_B$-R immunoreactivity on pre- and postsynaptic sites in the neuromuscular junctions of the rat esophageal striated muscle because of the negligible quantity of $GABA_B$-R proteins in the nerve terminals or because of other GABA-Rs. Taken together, one possibility is that GABA from nerve terminals may have inhibitory effects on the release of GABA, acetylcholine via presynaptic $GABA_B$-R or other types of GABA-Rs.

The present study showed that NADPH-d (as a marker of NOS)-positive nerve fibers from intrinsic neurons intermingled with GABA-immunoreactive motor endplates in the esophageal striated muscles. Previous immunohistochemical studies revealed the dual innervation of motor endplates by axons that were immunoreactive for both NOS and galanin (Kuramoto et al. 1999), vasoactive intestinal polypeptide, neuropeptide tyrosine, galanin, or enkephalin (Kuramoto and Endo 1995, Kuramoto et al. 1996; Neuhuber et al. 2001; for review see Wörl and Neuhuber 2005). These neuropeptides act as inhibitory transmitters in the enteric nervous system of the intestine (for review see Furness 2000; for review see Brookes, 2001) and are found in myenteric neurons and nerve terminals on the motor endplates in the esophagus of various mammals (Wörl and Neuhuber 2005). When the endplates were orthogradely labeled by

injection of DiI into the brainstem, none had NADPH-d reactivity (Neuhuber et al. 1994) and none had galanin immunoreactivity (Wörl et al. 1998). Unilateral vagotomy did not reduce the number of NOS-immunoreactive terminals innervating the striated muscle, although more than half of the CGRP-immunoreactive motor endplates were lost (Kuramoto et al., 1999). These data suggest that the dual innervation on the motor endplates may be derived from intrinsic neurons and may inhibit the motility of esophageal striated muscles. Furthermore, we observed $GABA_B$-R immunoreactivity in the neuronal cell bodies and in the proximal-to-distal part of the axons, but not in the terminals. Our immunohistochemical method may not have been sensitive enough to detect $GABA_B$-R in the nerve terminals due to their low levels of $GABA_B$-R protein. GABA from the motor endplates may have a local inhibitory effect on the dual-innervated nerve terminals of the motor endplates on the striated muscle fibers because these esophageal myenteric neurons dually innervated the motor endplates and were positive for $GABA_B$-R.

The present study revealed that GABA-immunoreactive nerve fibers were in contact with a few NADPH-d-positive or ChAT-immunoreactive neurons in the myenteric plexus of the rat esophagus and that $GABA_B$-R-immunoreactive neurons in the myenteric plexus were NOS- or ChAT-immunopositive. This infers that GABA from the nerve fibers may have inhibitory effects on the release of neurons via postsynaptic $GABA_B$-R. Previous immunohistochemical studies demonstrated the presence of GABA or GAD (Jessen et al. 1986; Saito and Tanaka 1986; Davanger et al. 1987; Hills et al. 1987; Furness et al. 1989), $GABA_A$-R (Krantis et al. 1995), and $GABA_B$-receptor (Nakajima et al. 1996) in the neuronal elements of the intestine. GABA depresses excitatory cholinergic transmission in the gut by inhibiting transmitter release, an effect mediated through presynaptic $GABA_B$-R (Fargeas et al. 1988; Kerr et al. 1990; Gentilini et al. 1992). The inhibition of $GABA_B$-R in the neurons was mainly achieved via modulation of neurotransmitter release from presynaptic terminals and hyperpolarization of postsynaptic membranes (for review see Bowery et al. 2002). However, the present immunohistochemical method may not have been able to detect the presynaptic $GABA_B$-R in the nerve terminals due to low levels of $GABA_B$-R. Taken together, GABA from the nerve fibers contacting the esophageal myenteric neurons may have inhibitory effects on the release of GABA and acetylcholine via presynaptic $GABA_B$-R and on the release of nitric oxide or acetylcholine from intrinsic neurons via postsynaptic $GABA_B$-R.

We found no GABA-, GAD67- and VGAT-immunoreactive neurons in the rat esophageal myenteric plexus. This infers that GABA-immunoreactive nerve fibers in the rat esophagus may be extrinsic in origin. Previous studies showed innervation of dorsal motor nucleus of vagal nerves in the intrinsic neurons of the myenteric plexus and innervation of nucleus ambiguus in the striated muscles in the esophagus (Roman 1982; for review see Cunningham and Sawchenko 1990; Conklin and Christensen 1994). Because the present findings indicate that GABA-immunopositive nerve terminals are present on motor endplates that are immunopositive for ChAT, these seem to originate from the nucleus ambiguus. However, the present study showed that GAD67 and VGAT immunoreactivities were found in numerous nerve fibers on the cell bodies, but not in the dendrites and cell bodies of the VAChT-immunoreactive neurons in the rat nucleus ambiguus and in the dorsal motor nucleus of vagal nerves.

However, No GAD67-green fluorescent protein was shown in the ChAT-immunoreactive neurons of the nucleus ambiguus and the dorsal motor nucleus of vagal nerves of the transgenic mice (Gotts et al. 2015). Also, the neurons of the nucleus ambiguus and the dorsal motor nucleus of vagal nerves did not contain GAD and GABA immunoreactivity (Blessing 1990; Maqbool et al. 1991). These results suggest that GABA may be synthesized and taken up and accumulated in the synaptic vesicles by VGAT in the nerve terminals, but not in the cell bodies of the neurons.

During development, GABA disappeared from the cell bodies and dendrites of Purkinje cells and dendrites and axons of Golgi cells, became confined to the terminals as reported in previous studies (Takayama and Inoue, 2004). In the adult cerebellum, GABA is exclusively transported into the synaptic vesicles by VGAT at the axon terminals (Fon and Edwards, 2001). It is possible that the GABA expression phenomenon in Purkinje cells of the cerebellum during development also occurs in the VAChT-positive neurons in the nucleus ambiguus and dorsal nucleus of the vagal nerves.

We showed that thick GABA-immunoreactive nerve fibers divided into thinner nerve fibers and that these fine nerve fibers were in close contact with myenteric ganglia on the way to the striated muscle fibers. Tracer experiments showed that the nerve fibers derived from the nucleus ambiguus innervated the striated muscles and ramified into collateral branches that projected to one or more myenteric ganglia (Powley et al. 2013). These data suggest that the nerve fibers from the nucleus ambiguus mainly innervate striated muscles and partly innervate the myenteric ganglia.

In conclusion, we revealed the distribution of GABA, VGAT and $GABA_B$-R immunoreactivities in the rat esophagus. However, the present study showed that GAD67 and VGAT immunoreactivities were found in numerous nerve fibers on the surface of the cell bodies, but not in the dendrites and cell bodies of the VAChT-immunoreactive neurons in the rat nucleus ambiguus and in the dorsal motor nucleus of vagal nerves. These results suggest that GABA and $GABA_B$-R may be related to the local control of the inhibitory nervous system of rat esophageal motility, and that GABA may be synthesized and taken up and accumulated in the synaptic vesicles by VGAT in the nerve terminals, but not in the cell bodies of the neurons. The present study sheds new light on the local inhibitory neuronal system involved in esophageal motility.

Declarations

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Conflict of Interest: The authors declare that they have no conflict of interest.

References

1. Agardh E, Yeh HH, Herrmann R, Puro DG (1985) gamma-aminobutyric acid-mediated inhibition at cholinergic synapses formed by cultured retinal neurons. Brain Res 330:323–328
2. Blessing WW (1990) Distribution of glutamate decarboxylase-containing neurons in rabbit medulla oblongata with attention to intramedullary and spinal projections. Neuroscience 37:171–185
3. Bowery NG, Bettler B, Froestl W, Gallagher JP, Marshall F, Raiteri M, Bonner TI, Enna SJ (2002) International union of pharmacology. XXXIII. Mammalian γ-aminobutyric acid$_B$ receptors: Structure and function. Pharamcol Rev 54:247–264
4. Brookes SJ (2001) Classes of enteric nerve cells in the guinea-pig small intestine. Anat Rec 262:58–70
5. Bult H, Boeckxstaens GE, Pelckmans PA, Jordaens FH, Van Herman MYM AG (1990) Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. Nature 345:346–347
6. Chaudhry FA, Reimer RJ, Bellocchio EE, Danbolt NC, Osen KK, Edwards RH, Strom-Mathisen J (1998) The vesicular GABA transporter, VGAT, localizes to synaptic vesicles in sets of glycinergic as well as GABAergic neurons. J Neurosci 18:9733–9750
7. Chiou LC, Chang CC (1994) Pharmacological relevance of peripheral type benzodiazepine receptors on motor nerve and skeletal muscle. Br J Pharmacol 112:257–261
8. Conklin JL, Christensen J (1994) Motor functions of the pharynx and esophagus. In: Johson LR (ed) Physiology of the gastrointestinal tract. Raven, New York, pp 903–928
9. Cunningham ET, Sawchenko PE (1990) Central neural control of esophageal motility: a review. Dysphagia 5:35–51
10. Davanger S, Ottersen OP, Strom-Mathisen J (1987) Immunocytochemical localization of GABA in cat myenteric plexus. Neurosci Lett 73:27–32
11. Fargeas MJ, Fioramonti J, Bueno L (1988) Central and peripheral action of GABAA and GABAB agonists on small intestine motility in rats. Eur J Pharmacol 150:163–169
12. Feigenspan A, Bormann J (1998) GABA-gated Cl^- channels in the rat retina. Prog Retin Eye Res 17:99–126
13. Fletcher EL, Clark MJ, Senior P, Furness JB (2001) Gene expression and localization of GABA$_C$ receptors in neurons of the rat gastrointestinal tract. Neuroscience 107:181–189
14. Fon EA, Edwards RH (2001) Molecular mechanisms of neurotransmitter release. Muscle Nerve 24:581–601
15. Furness JB, Trussell DC, Pompolo S, Bornstein JC, Maley BE, Strom-Mathisen J (1989) Shape and projections of neurons with immunoreactivity for gamma-aminobutyric acid in the guinea-pig small intestine. Cell Tissue Res 256:293–301
16. Furness JB (2000) Types of neurons in the enteric nervous system. J Auton Nerv Syst 81:87–96
17. Gentilini G, Franchi-Micheli S, Pantalone D, Cortesini C, Zilletti L (1992) GABA$_B$ receptor mediated mechanisms in human intestine in vitro. Eur J Pharmacol 217:9–14

18. Gibson A, Mirzazadeh S, Hobbs AJ, Moore PK (1990) L-NG-monomethyl arginine and L-NG-nitro arginine inhibit non-adrenergic, non-cholinergic relaxation of the mouse anococcygeus muscle. Br J Pharmacol 99:602–606
19. Giotti A, Luzzi S, Spagnesi S, Zilletti L (1983) $GABA_A$ and $GABA_B$ receptor-mediated effects in guinea-pig ileum. Br J Pharamacol 78:469–478
20. Gotts J, Atkinson L, Edwards IJ, Yanagawa Y, Deuchars SA, Deuchars J (2015) Co-expression of GAD67 and choline acetyltransferase reveals a novel neuronal phenotype in the mouse medulla oblongata. Auton Neurosci 193:22–30
21. Hills JM, Jessen KR, Mirsky R (1987) An immunohistochemical study of the distribution of enteric GABA-containing neurons in the rat and guinea-pig intestine. Neuroscience 22:301–312
22. Hornby PJ, Abrahams TP (2000) Central control of lower esophagus sphincter relaxation. Am J Med 108(4A):90S–98S
23. Hsu SM, Raine L, Fanger H (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC & unlabeled antibody (PAP) procedures. J Histochem Cytochem 29:577
24. Hyland NP, Cryan JF (2010) A gut feeling about GABA: Focus on $GABA_B$ receptors. Front Pharmacol 1:124
25. Jessen KR, Hills JM, Saffrey MJ (1986) Immunohistochemical demonstration of GABAergic neurons in the enteric nervous system. J Neurosci 6:1628–1634
26. Kerr DI, Ong J, Prager RH (1990) Antagonism of $GABA_B$-receptor-mediated responses in the guinea-pig isolated ileum and vas deferens by phosphono-analogues of GABA. Br J Pharmacol 99:422–426
27. Kleinrok A, Kilbinger H (1983) γ-aminobutyric acid and cholinergic transmission in the guinea-pig ileum. Naunyn Schmiedbergs Arch pharmacol 322:216–220
28. Krantis A, Costa M, Furness J, Orbach J (1980) γ-aminobutyric acid stimulates intrinsic inhibitory and excitatory nerves in the guinea-pig intestine. Eur J Pharmacol 67:461–468
29. Krantis A, Kerr DI (1981) Autoradiographic localization of ^3H-gamma aminobutyric acid in the myenteric plexus of the guinea-pig small intestine. Neurosci Lett 23:263–268
30. Krantis A, Shabnavard L, Nichols K, de Blas AL, Staines W (1995) Localization of $GABA_A$ receptor immunoreactivity in NO synthase positive myenteric neurons. J Auton Nerv Syst 53:157–165
31. Kuramoto H, Endo Y (1995) Galanin-immunoreactive nerve terminals innervating the striated muscle fibers of the rat esophagus. Neurosci Lett 188:171–174
32. Kuramoto H, Kato Y, Sakaoto H, EndoY (1996) Galanin-containing nerve terminals that are involved in a dual innervation of the striated muscles of the rat esophagus. Brain Res 734:186–192
33. Kuramoto H, Kawano H, Sakamoto H, Furness JB (1999) Motor innervation by enteric nerve fibers containing both nitric oxide synthase and galanin immunoreactivities in the striated muscle of the rat esophagus. Cell Tissue Res 295:241–245

34. Kuramoto H, Yoshimura R, Sakamoto H, Kadowaki M (2019) Reginal variations in the number distribution of intrinsic myenteric neurons and coinnervated motor endplates on the striated muscles in the rat esophagus. Auton Neurosci 219:25–32
35. Kuramoto H, Yabe M, Morishita R, Yoshimura R, Sakamoto H (2024) Localization of sensory nerve terminals containing calcitonin gene-related peptide (CGRP) on striated muscle fibers in the rat esophagus: Evidence for triple innervation via motor endplates. Auton Neurosci 253: (in press)
36. Lu WY, Bieger D (1998) Vagovagal reflex motility patterns of the rat esophagus. Am J Physiol 274:1425–1435
37. Maqbool A, Batten TF, McWilliam PN (1991) Ultrastructural relationships between GABAergic terminals and cardiac vagal preganglionic motoneurons and vagal afferents in the cat: a combined HRP tracing and immunogold labelling study. Eur J Neurosci 3:501–513
38. Maggi CA, Manzini S, Meli A (1984) Evidence that GABAA receptors mediate relaxation of rat duodenum by activating intramural noradrenaergic-non-cholinergic neurons. J Auton Pharmacol 4:77–85
39. McIntire SL, Reimer RJ, Schuske K, Edwards RH, Jorgensen EM (1997) Identification and characterization of the vesicular GABA transporter. Nature 389:870–876
40. McLaughlin BJ, Wood JG, Saito K, Roberts E, Wu J-Y (1975) The fine structural localization of glutamate decarboxylase in developing axonal processes and presynaptic terminals of rodent cerebellum. 85:355–371
41. Minocha A, Galligan JJ (1993) Excitatory and inhibitory responses mediated by GABAA and GABAB receptors in guinea pig distal colon. Eur J Pharmacol 230:187–193
42. Nakajima K, Tooyama I, Kuriyama K, Kimura H (1996) Immunohistochemical demonstration of $GABA_B$ receptors in the rat gastrointestinal tract. Neurochem Res 21:211–215
43. Neuhuber WL, Wörl J, Berthoud H-R, Conte B (1994) NADPH-diaphorase-positive nerve fibers associated with motor endplates in the rat esophagus. new evidence for co-innervation of striated muscle by enteric neurons. Cell Tissue Res 276:23–30
44. Neuhuber WL, Kressel M, Stark A, Berthoud H-R (1998) Vagal efferent and afferent innervation of the rat esophagus as demonstrated by anterograde DiI and DiA tracing: focus on myenteric ganglia. J Auton nerv Syst 70:92–102
45. Neuhuber WL, Eichhorn U, Wörl J (2001) Enteric co-innervation of striated muscle fibers in the esophagus: Just a hangover? Anat Rec 262:42–46
46. Oertel WH, Schmechel DE, Mugnaini E, Tappaz ML, Kopin IJ (1981) Immunocytochemical localization of glutamate decarboxylase in rat cerebellum with a new antiserum. 6:2715–2735
47. Ohkawa H (1987) Effects of γ-aminobutyric acid on the non-adrenergic inhibitory potentials in circular smooth muscle cells of the guinea-pig duodenum. Biomed Res 8:185–194
48. Ong J, Kerr DIB (1983) GABAA- and GABAB-receptor-mediated modification of intestinal motility. Eur J Pharmacol 86:9–17

49. Ong J, Kerr DIB (1984) Evidence for a physiological role of GABA in the control of guinea-pig intestinal motility. Neurosci Lett 50:339–343
50. Ottersen OP, Strom-Mathisen J (1984) Glutamate- and GABA-containing neurons in the mouse and rat brain, as demonstrated with a new immunocytochemical technique. J comp Neurol 229:374–392
51. Powley TL, Mital RK, Baronowsky EA, Hudson CN, Martin FN, McAdams JL, Mason JK, Phillips RJ (2013) Architecture of vagal motor units controlling striated muscle of esophagus: peripheral elements patterning peristalsis ? Auton Neurosci 179:90–98
52. Roman C (1982) Nervous control of esophageal and gastric motility. In: Beftaccini G (ed) Handbook of experimental pharmacology, vol 59. Springer, Berlin, pp 223–278
53. Roman C, Gonella J (1987) Extrinsic control of digestive tract motility. In L.R.Johnson (Ed.) Physiology of the Gastrointestinal Tract, 2nd edn. Raven, New York, pp 507–553
54. Saito N, Tanaka C (1986) Immunohistochemical demonstration of GABA containing neurons in the guinea pig ileum using purified GABA antiserum. Brain Res 376:78–84
55. Takayama C, Inoue Y (2004) Extrasynaptic localization of GABA in the developing mouse cerebellum. Neurosci Res 50:447–458
56. Tøttrup A, Svane D, Forman A (1991) Nitric oxide mediating NANC inhibition of opossum lower esophageal sphincter. Am J Physiol 260:G385–389
57. Uddman R, Alumets J, Håkanson R, Sundler F, Walles B (1980) Peptidergic (enkephalin) innervation of the mammalian esophagus. Gastroenterology 78:732–737
58. Ward SM, Xue C, Shuttleworth CW, Bredt DS, Snyder SH, Sanders KM (1992) NADPH diaphorase and nitric oxide synthase colocalization in enteric neurons of canine proximal colon. Am J Physiol 263:G277–284
59. Wörl J, Mayer B, Neuhuber WL (1994) Nitrergic innervation of the rat esophagus: focus on motor endplates. J Auton Nerv Syst 49:227–233
60. Wörl J, Fischer J, Neuhuber WL (1998) Nonvagal origin galanin-containing nerve terminals innervating striated muscle fibers of the rat esophagus. Cell Tissue Res 292:453–461
61. Wörl J, Neuhuber WL (2005) Enteric co-innervation of motor endplates in the esophagus: state of the art ten years after. Histochem Cell Biol 123:117–130
62. Wu M, Majewski M, Wojtkiewicz J, Vanderwinden J-M, Adriaensen D, Timmermans J-P (2003) Anatomical and neurochemical features of the extrinsic and intrinsic innervation of the striated muscle in the porcine esophagus: evidence for regional and species differences. Cell Tissue Res 311:289–297
63. Zeiter DK, Li X, Broussard (1996) Identification of the $GABA_A$ receptor alpha subunit mRNA in rat intestine. Brain Res Mol Brain Res 39:241–244

Table 2 and 3

Table 2 and 3 are available in the Supplementary Files section.

Figures

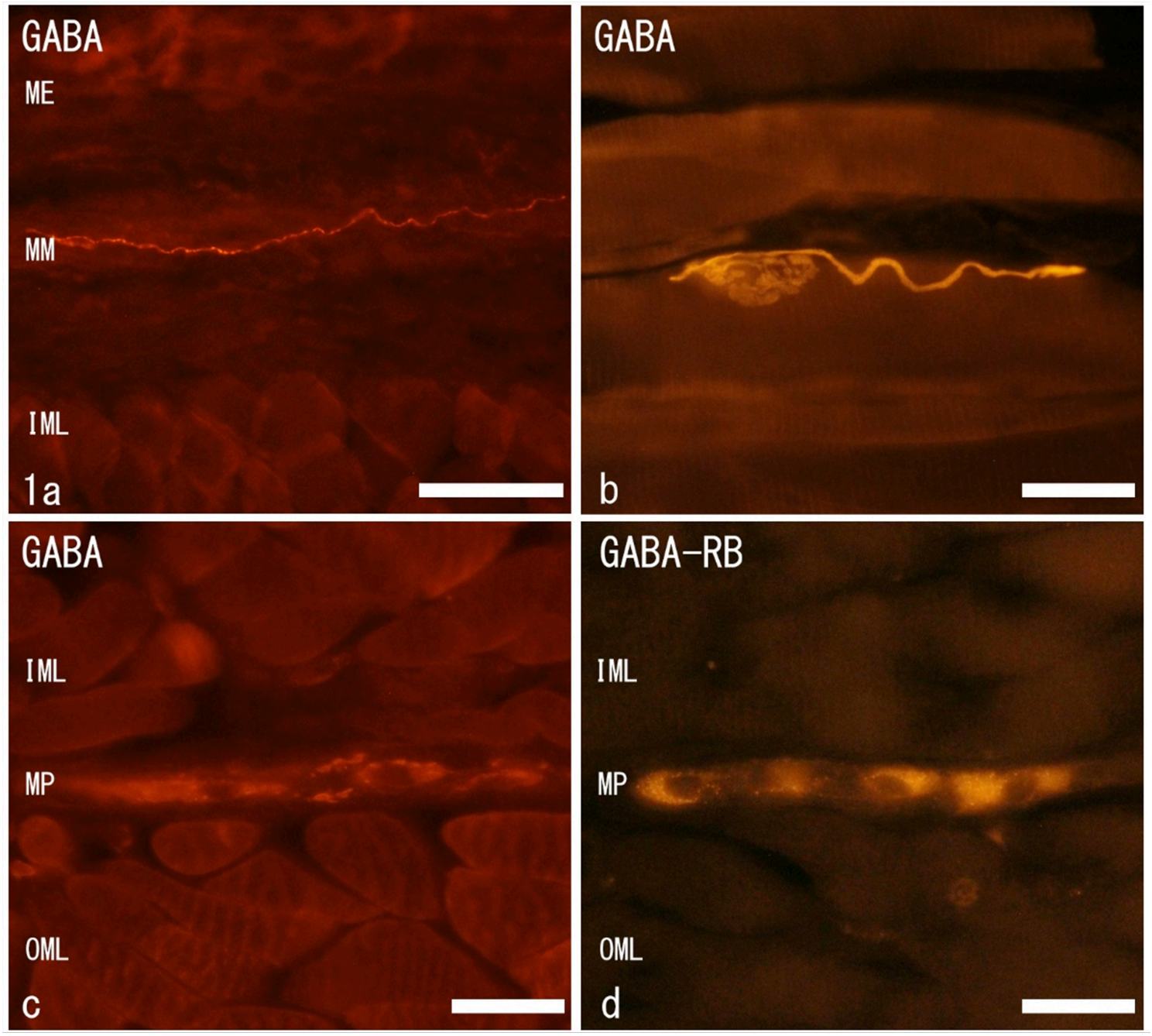


Figure 1

a–d. GABA (a-c) and GABAB-R (d) immunoreactivities in cryostat sections of rat esophagus. A GABA-immunoreactive nerve fiber runs in the muscularis mucosae (a). GABA-immunoreactive nerve fiber terminates and forms motor endplate on the striated muscles (b). GABA-immunoreactive varicose nerve fibers are seen in myenteric plexus and contact intrinsic neurons (c). GABAB-R- immunoreactive neurons are observed in the myenteric plexus (d). ME: Mucous Epithelium. MM: Muscularis Mucosae. IML: Inner Muscle Layer. OML: Outer Muscle layer. MP: Myenteric Plexus. Bar: a: 50μm, b–d: 20μm

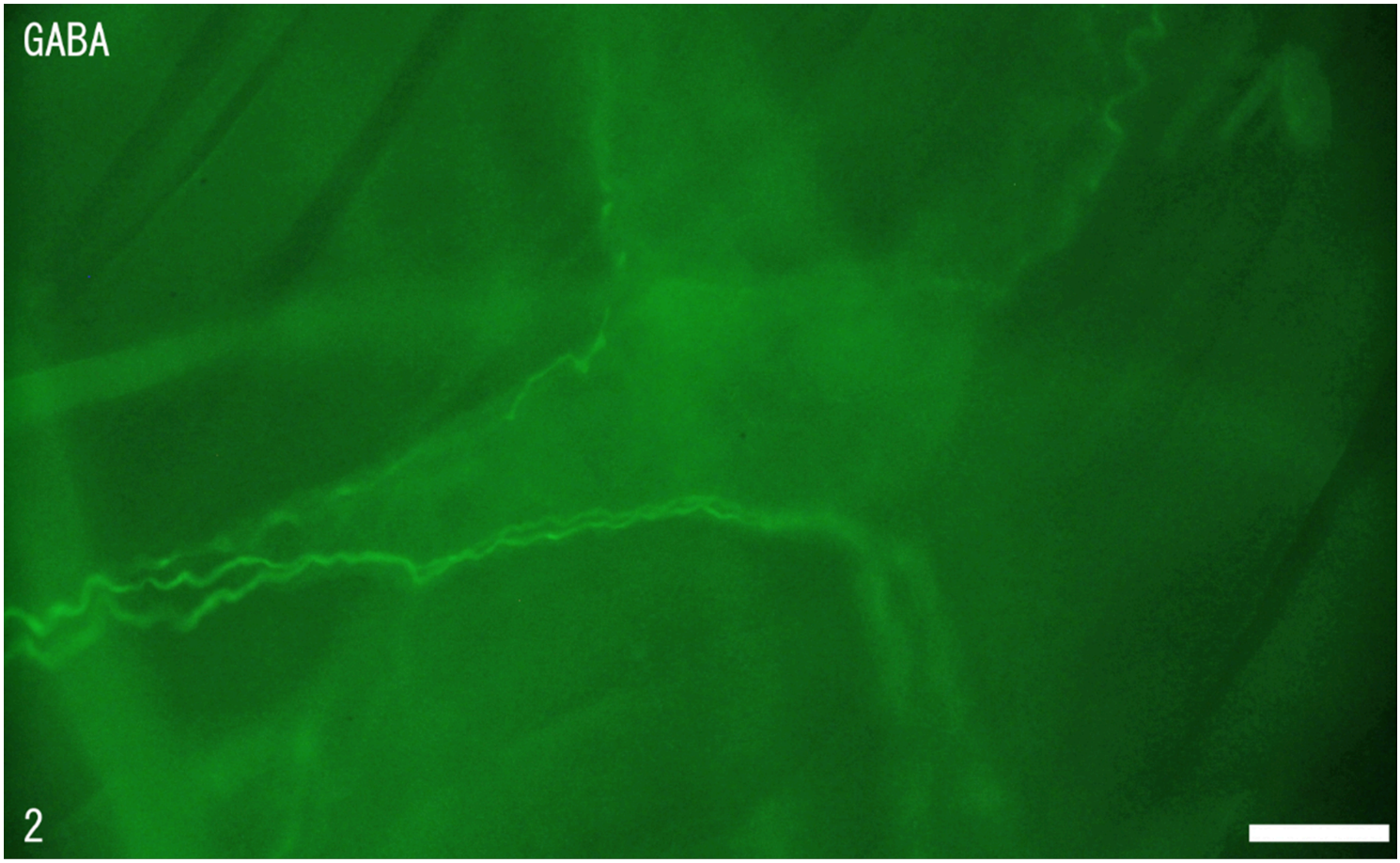


Figure 2

GABA immunoreactivity in whole mount preparations of rat esophageal myenteric plexus. GABA-immunoreactive nerve fibers closely contact and run along the intrinsic immunonegative neurons in the myenteric plexus. Bar: 20μm.

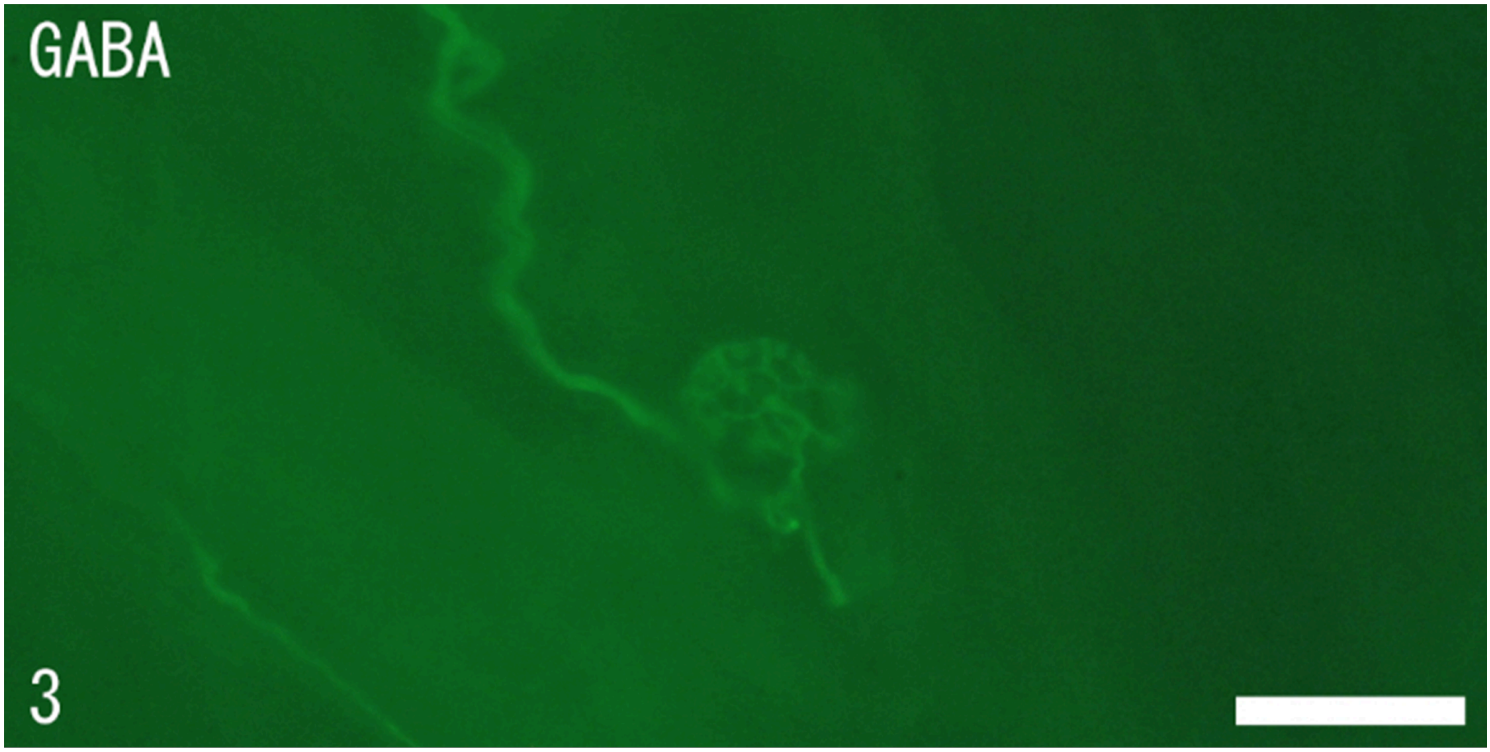


Figure 3

GABA immunoreactivity in whole mount preparations of rat esophageal striated muscles. GABA-immunoreactive nerve fiber forms motor endplate in the striated muscles. Bar: 20 μm.

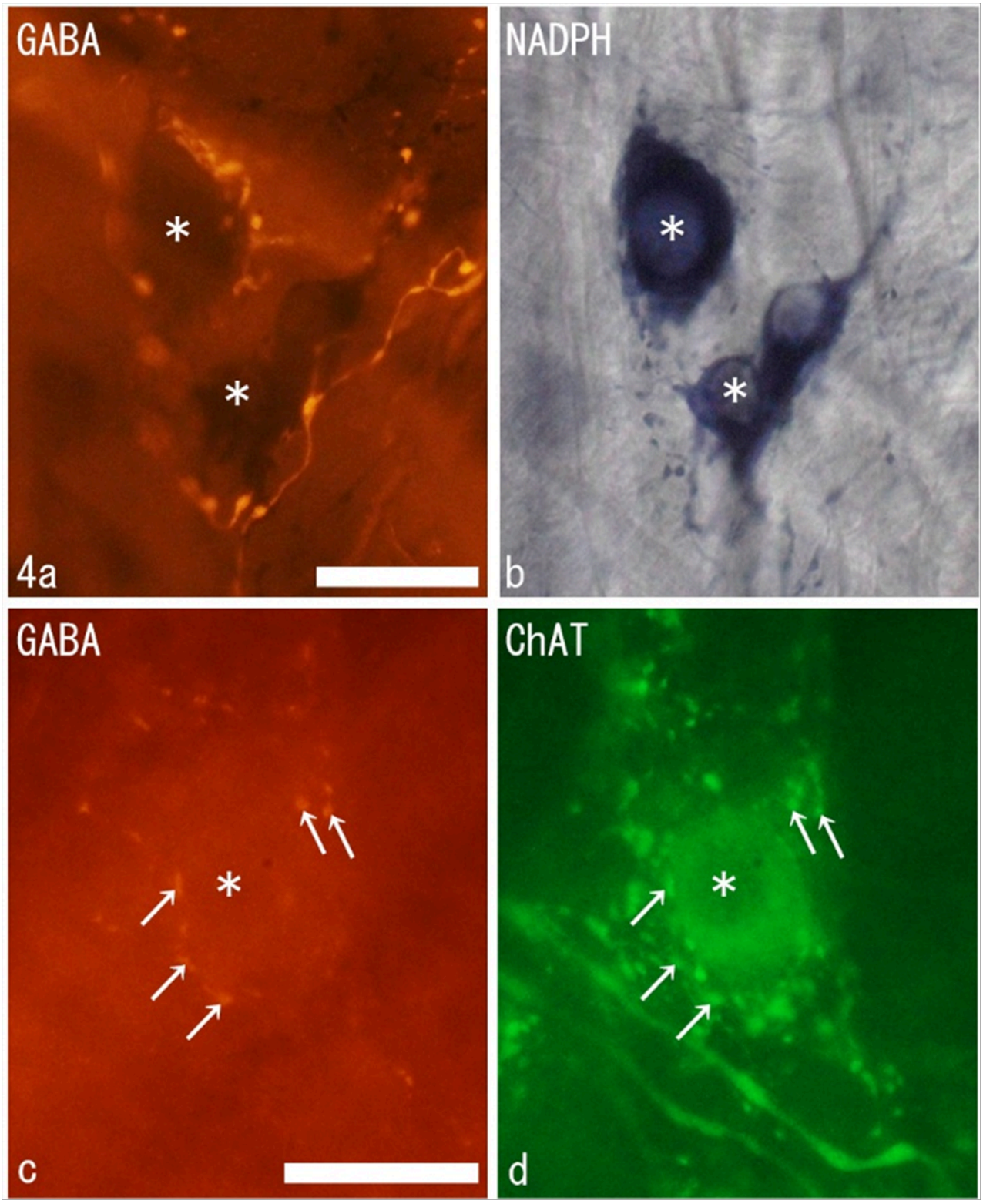


Figure 4

a–d. Double immunostaining for GABA (a, c), NADPH-d (b) and ChAT (d) in whole mount preparation of rat esophageal myenteric plexus. GABA immunoreactive nerve fibers (a) contact NADPH-d-reactive neurons (asterisks) in the myenteric plexus. Some NADPH-d-positive neurons (asterisks) were seen in the myenteric plexus (b). GABA- immunoreactive nerve fibers closely contact ChAT-immunoreactive

neurons (asterisks) in the myenteric plexus (c, d). Many of GABA-immunoreactive nerve fibers were ChAT-immunoreactive (arrows of c, d). Bar: 20 μm.

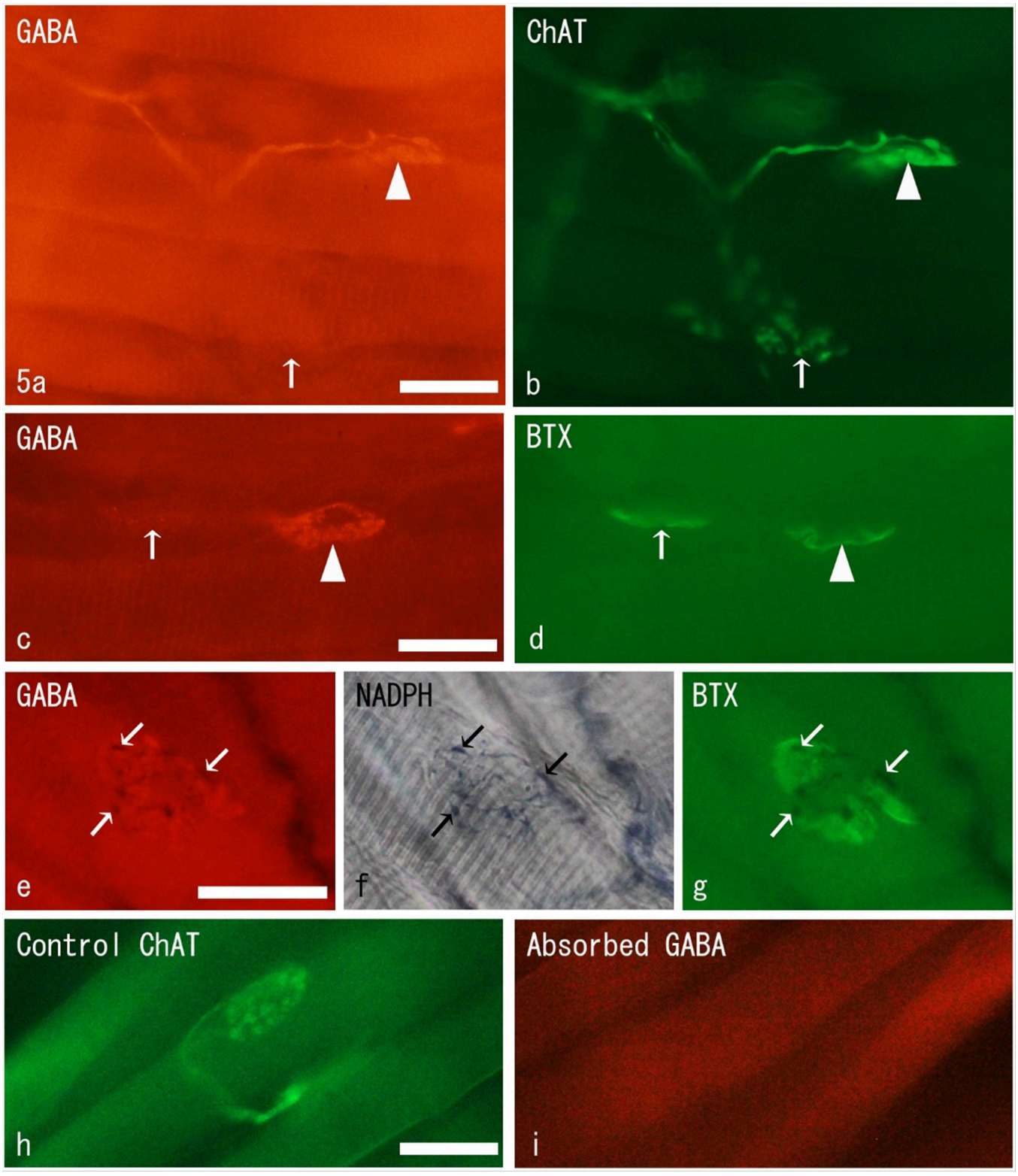


Figure 5

a–i. Double or triple staining for GABA (a, c, e), ChAT (b), α-bungarotoxin (BTX) (d, g), NADPH-d (f) in whole mount preparations and control sections of ChAT (h) and GABA absorbed with 10 μg GABA (i) of

the rat esophageal striated muscles. GABA-/ChAT- (arrowhead) or only ChAT (arrow)-immunoreactive fibers and motor endplates are seen in the striated muscles (a, b). GABA-/BTX- (arrowhead) or only BTX (arrow)-positive motor endplates are seen in the striated muscles (c, d). NADPH-d-positive nerve fibers (arrows) (f) intermingle with GABA-immunoreactive motor endplates (e) and are ramified on the BTX-positive area (g). No GABA immunoreactivity is seen in the ChAT-immunoreactive motor endplate (h, i). Bar: 20 μm.

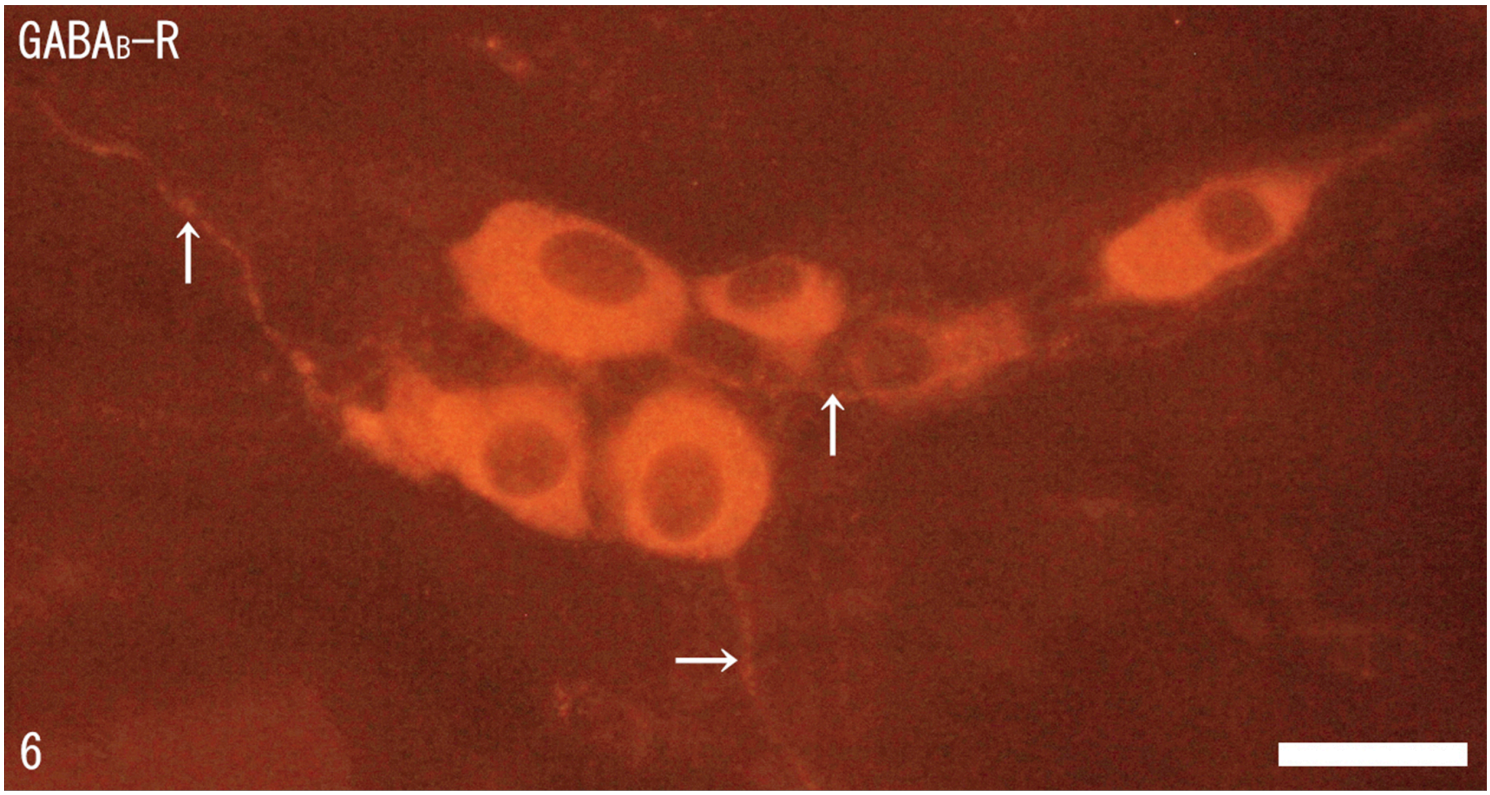


Figure 6

$GABA_B$-R immunoreactivity in whole mount preparations of rat esophageal myenteric plexus. $GABA_B$-R-immunoreactive neurons are seen in the myenteric plexus. $GABA_B$-R- immunoreactive neurons extend single long axons to the distal direction (arrows). Bar: 20 μm.

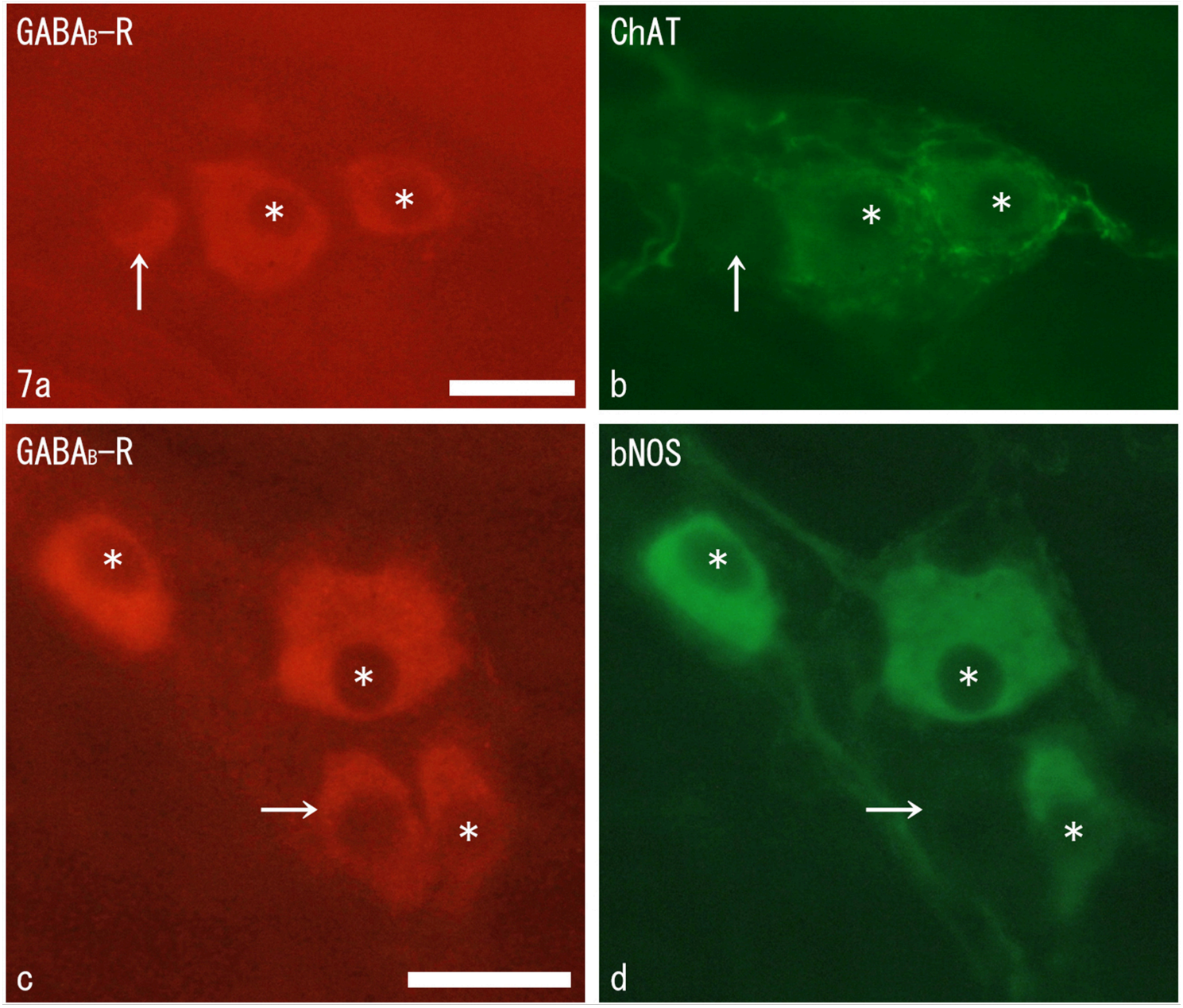


Figure 7

a–d. Double immunostaining for $GABA_B$-R (a, c), ChAT (b) and bNOS (d) in whole mount preparations of the neurons in the rat esophageal myenteric plexus. Some $GABA_B$-R-immunoreactive neurons are immunoreactive for ChAT or bNOS (asterisks of a and b, c and d) while a part of $GABA_B$-R-immunoreactive neurons is ChAT- or bNOS-immunonegative (arrow of a and b, c and d). Bar: 20 μm.

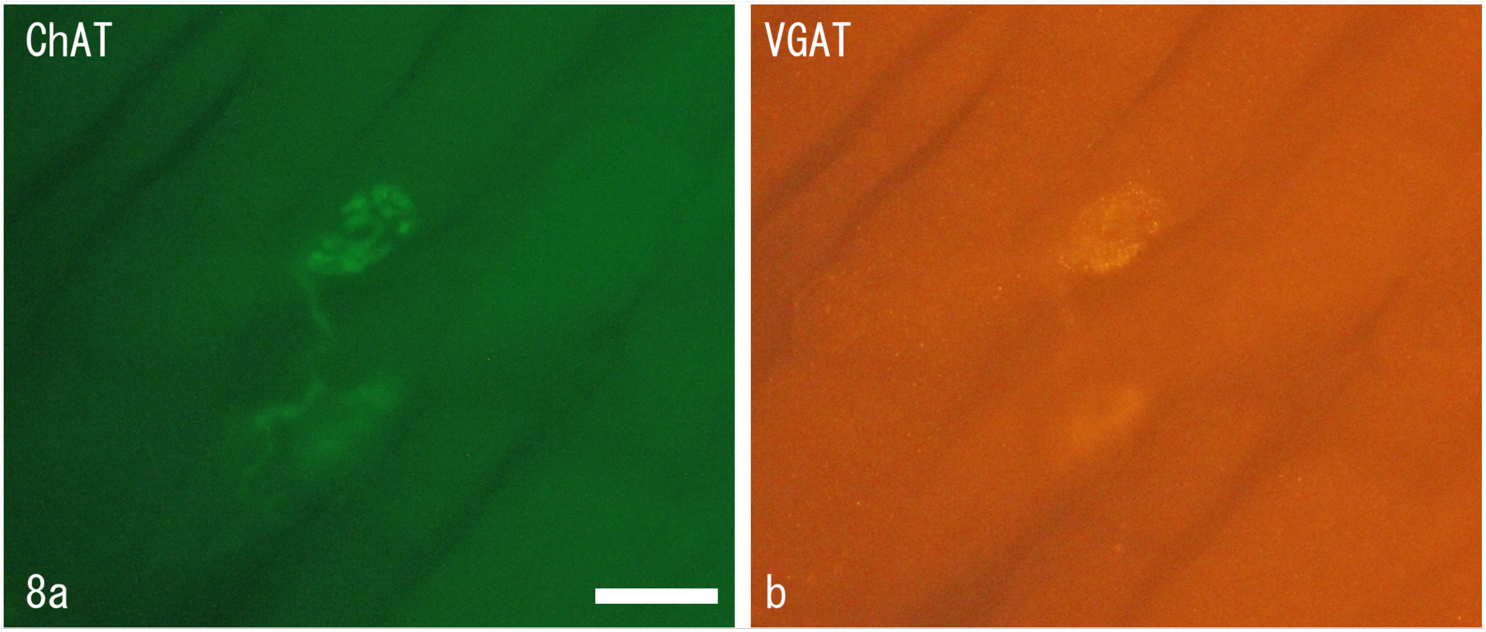


Figure 8

a–b. Double immunostaining for ChAT (a) and VGAT (b) in whole mount preparations of the nerve terminals in the rat esophageal striated muscles. VGAT immunoreactivity is seen in all ChAT-immunoreactive motor endplates. The VGAT immunoreactivity is found strongly in the nerve terminals rather than in the nerve fibers and is fine granular in the motor endplates. Bar: 20 μm.

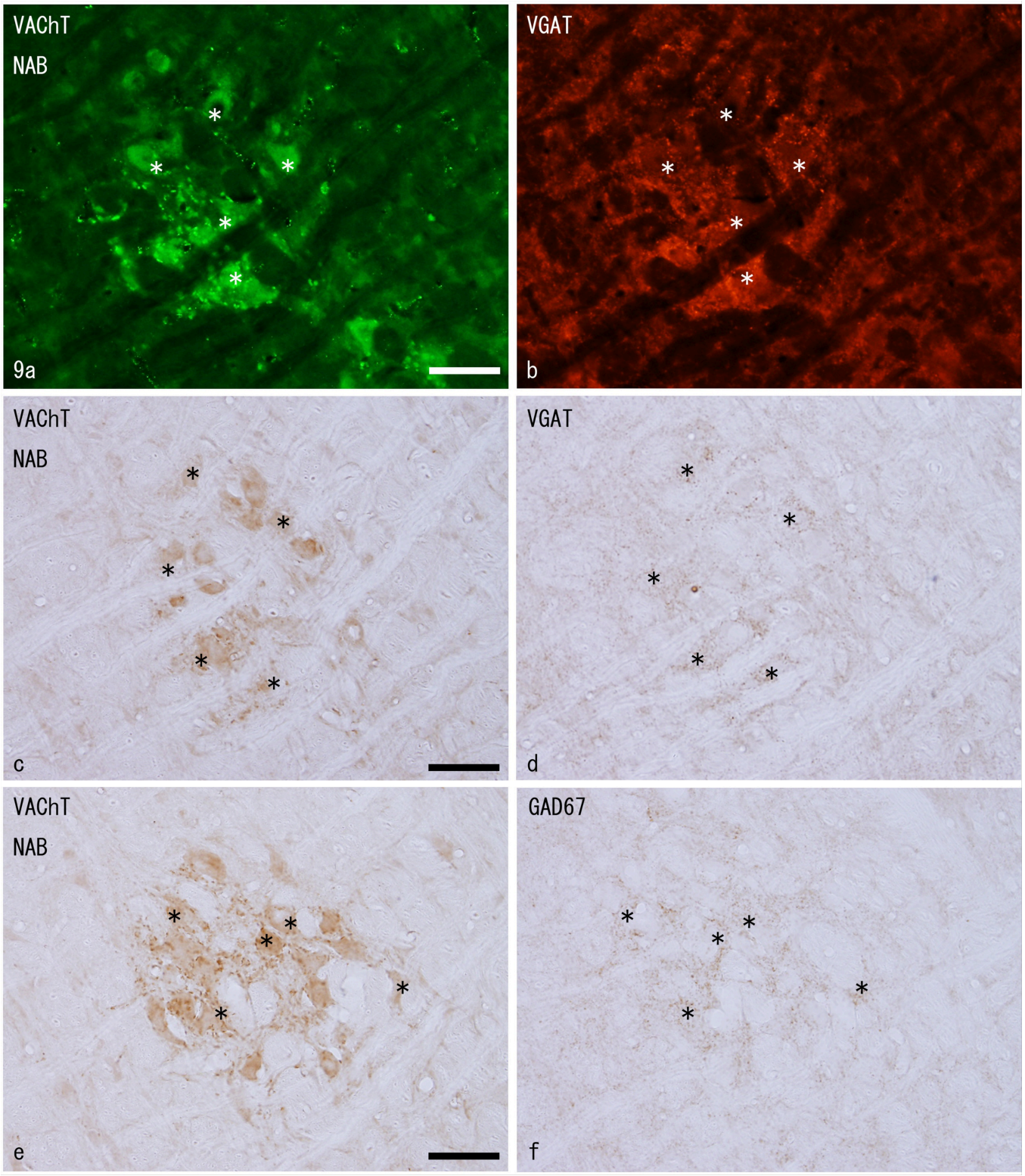


Figure 9

a–f. Double immunostaining for VAChT (a) and VGAT (b) in same section in the nucleus ambiguus of the rat medulla oblongata. Immunostaining for VAChT (c, e), VGAT (d) and GAD67 (f) using serial sections in the nucleus ambiguus of the rat medulla oblongata. VGAT (b, d) immunoreactivity is seen in the nerve fibers on the surface of and in the periphery of cell bodies (a, b asterisk), but not in the cell bodies of the VAChT-immunoreactive neurons (a, c asterisk) in the the nucleus ambiguus. GAD67 immunoreactivity (f)

is found in the nerve fibers on the surface of and in the periphery of, but not in the cell bodies of the VAChT-immunoreactive neurons (e) of the nucleus ambiguus. NAB: nucleus ambiguus of the vagal nerves. Bars: 50 μm.

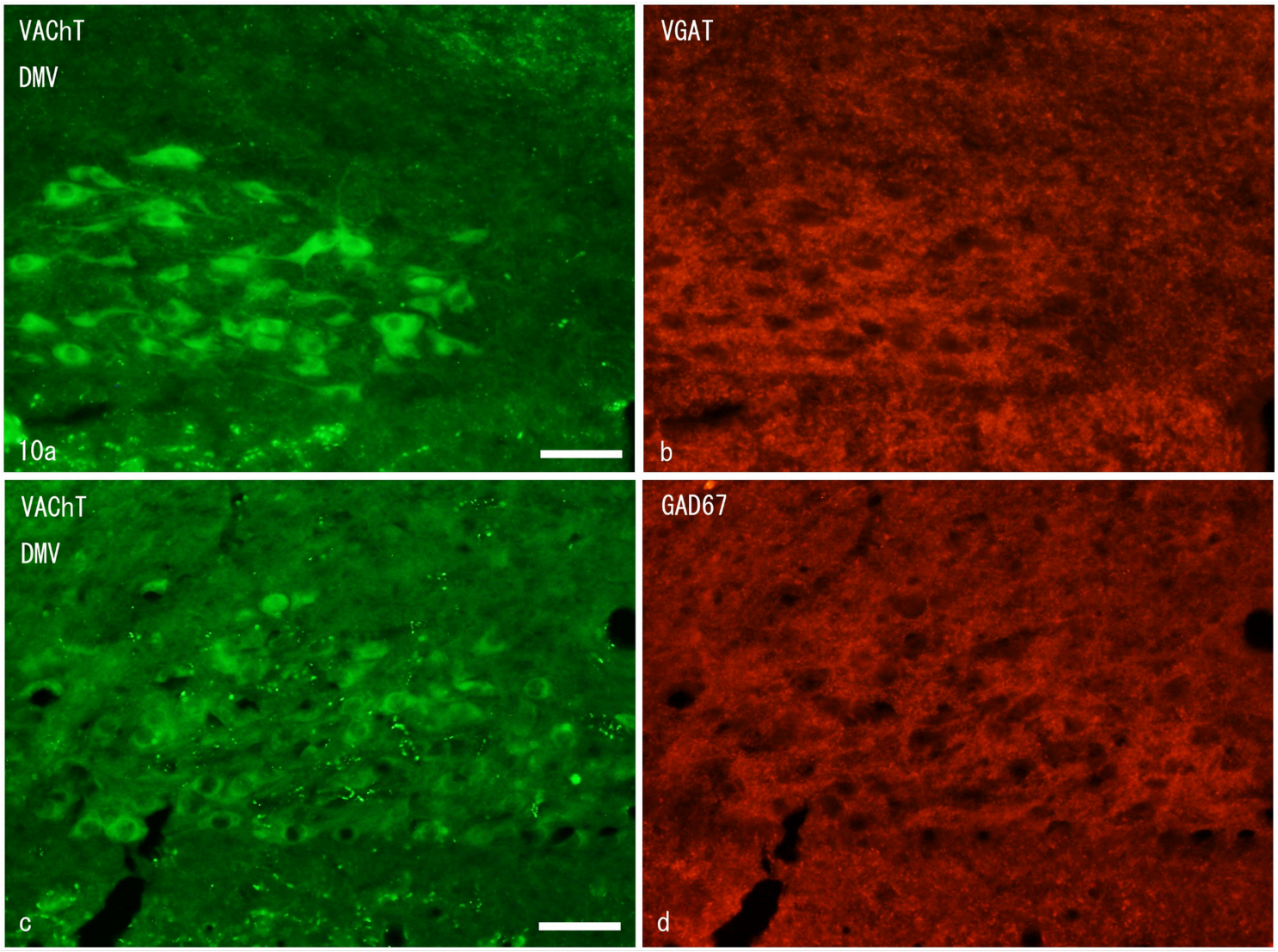


Figure 10

a–d. Double immunostaining for VAChT (a, c) and VGAT (b) or GAD67 (d) in same section in the dorsal motor nucleus of the vagal nerves of the rat medulla oblongata. VGAT- or GAD67 immunoreactivity is seen in the nerve fibers on the surface of and in the periphery nerve fibers of, but not in the cell bodies (b, d) of the VAChT-immunoreactive neurons of the dorsal motor nucleus of the vagal nerves (a, c). DMV: dorsal motor nucleus of the vagal nerves Bar: 50 μm.

Supplementary Files

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- Table23.docx