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Expression of Sex Steroid Hormone Receptors in Vagal Motor Neurons Innervating the Trachea and Esophagus in Mouse

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The medullary vagal motor nuclei, the nucleus ambiguus (NA) and dorsal motor nucleus of the vagus (DMV), innervate the respiratory and gastrointestinal tracts. We conducted immunohistochemical analysis of expression of the androgen receptor (AR) and estrogen receptor α (ERα), in relation to innervation of the trachea and esophagus via vagal motor nuclei in mice. AR and ERα were expressed in the rostral NA and in part of the DMV. Tracing experiments using cholera toxin B subunit demonstrated that neurons of vagal motor nuclei that innervate the trachea and esophagus express AR and ERα. There was no difference in expression of sex steroid hormone receptors between trachea- and esophagus-innervating neurons. These results suggest that sex steroid hormones may act on vagal motor nuclei via their receptors, thereby regulating functions of the trachea and esophagus.

Key words: sex hormone receptors, nucleus ambiguus, dorsal motor nucleus of the vagus, trachea, esophagus

I. Introduction

Respiratory and gastrointestinal tract functions are affected by sex steroid hormones. For example, women experience various gastrointestinal symptoms, including nausea, heartburn, and constipation, during pregnancy [46], and some women experience similar symptoms prior to menstruation [21, 42, 48]. There are also sex-based differences in the incidences of respiratory and gastrointestinal diseases such as bronchial asthma [1, 11], gastroesophageal reflux, and functional dyspepsia [28, 40]. Furthermore, changes in the hormonal state of women during the menstrual cycle can affect the intensity of gastrointestinal symptoms [41, 49]. However, the mechanisms underlying the influence of sex steroid hormones on the respiratory and gastrointestinal tracts have not been elucidated.

Medullary vagal motor nuclei, consisting of the nucleus ambiguus (NA) and dorsal motor nucleus of the vagus (DMV), are the major motor nuclei of the vagus nerve. The NA is the origin for special visceral efferent fibers, while the DMV is the origin of general visceral efferent fibers. Vagal motor nuclei control the motion of the respiratory and gastrointestinal tracts, glandular secretion, and blood flow. Sex steroid hormone receptor expression has been shown in vagal motor nuclei [6, 14, 37, 45, 47, 50, 52], which suggests that sex steroid hormones may regulate functions of the respiratory and gastrointestinal tracts via these nuclei. However, the relationship of expression of sex steroid hormone receptors in vagal motor nuclei and their

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projection targets has not been examined. Therefore, we analyzed expression and sex differences of the androgen receptor (AR) and estrogen receptor α (ERα) in vagal motor nuclei, and investigated the immunohistochemical neuronal characteristics and associated innervation of the trachea and esophagus in 9-week-old mice.

II. Materials and Methods

Animals

Male and female C57BL6/J mice aged 10 weeks old were purchased from Shimizu Laboratory Supplies Co. Ltd. (Kyoto, Japan) and housed in plastic cages with standard bedding and continuous access to food and water. Five mice were used in each experiment. The temperature was maintained at 22°C with a 12-hr light:dark cycle. All experimental procedures were authorized by the Committee for Animal Research, Kyoto Prefectural University of Medicine.

Tissue preparations for immunohistochemistry

Animals were anesthetized with sodium pentobarbital and perfused via the left ventricle with 20–30 ml physiological saline followed by 100 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB: pH 7.4). Brains were immediately removed and immersed in the same fixative for 8 hr. After immersion in 25% sucrose in 0.1 M PB for 3 days at 4°C for cryoprotection, brains were quickly frozen and cut into 20-μm coronal sections using a cryostat (CM3050 S; Leica, Nussloch, Germany). These sections were washed several times with PBS for 5 min each.

AR or ERα immunohistochemistry: immunoperoxidase histochemistry

Brain sections from male and female mice were used in this experiment. Endogenous peroxidase activity was eliminated from the sections by incubation with 2% H_2O_2 in PBS with 0.3% Triton X-100 for 30 min, and the sections were then rinsed with PBS three times for 5 min each. After blocking nonspecific binding components with 1% normal goat serum and 1% BSA in PBS containing 0.1% Triton X-100 for 1 hr at room temperature, the sections were incubated with rabbit anti-AR (1:200; Epitomics, Burlingame, CA, USA) or rabbit anti-ERα (1:5000; Millipore Corp, Billerica, MA, USA) antibodies for 48 hr at 4°C. Immunopositive products were detected with a streptavidin-biotin (SAB) kit (Nichirei, Tokyo, Japan), followed by diaminobenzidine (DAB) development. The preparations were analyzed using an Olympus Optical microscope (Tokyo, Japan).

Double immunofluorescence for AR or ERα and ChAT or CGRP

Brain sections from male mice were washed with PBS and blocked for 1 hr at room temperature with 2% bovine serum albumin (BSA) containing 0.3% Triton X-100 in PBS. They were then incubated for 48 hr at 4°C with the following primary antibodies: rabbit anti-AR (1:200; Epitomics) or rabbit anti-ERα (1:5000; Millipore Corp) and goat anti-choline acetyltransferase (ChAT) (1:1000; Millipore Corp) or mouse anti-calcitonin gene-related peptide (CGRP) (1:8000; Abcam, Cambridge, UK). After the primary antibody was removed by rinsing, sections were incubated with secondary antibodies for 1 hr at room temperature. The following secondary antibodies were used for double-staining: anti-rabbit Alexa Fluor (AF) 488 (1:1000; Invitrogen, Grand Island, NY, USA) and anti-goat AF 546 (1:1000; Invitrogen) or anti-mouse AF 546 (1:1000; Invitrogen).

Cholera toxin B subunit (CTb) tracing and double immunofluorescence for CTb and ChAT, AR or ERα

Male mice were anesthetized with isoflurane. The extrathoracic trachea and esophagus were exposed and a single injection (200 nl) of a 2% solution of cholera toxin B subunit (CTb) conjugated to AF 488 (CTb 488, Molecular Probes, Eugene, OR, USA) was then injected into the tracheal adventitia or/and (in the same animal) a single injection (200 nl) of a 2% solution of CTb conjugated to AF 555 (CTb 555, Molecular Probes) was injected into the cervical esophagus wall between the muscularis and mucosal layers through a glass micropipette [29, 35]. Great care was taken to prevent unwanted spread of dye to neighboring tissues. After 5 days, animals were perfusion fixed and tissue was processed as described below. Coronal sections of 20 μm were washed with PBS and blocked for 1 hr at room temperature with 2% BSA containing 0.3% Triton X-100 in PBS. They were then incubated with goat anti-ChAT (1:1000; Millipore Corp.), rabbit anti-AR (1:200; Epitomics) or rabbit anti-ERα (1:5000; Millipore Corp) primary antibodies for 48 hr at 4°C. After the primary antibody was removed by rinsing, sections were incubated with anti-goat or rabbit AF 633 (1:1000; Invitrogen) secondary antibody for 1 hr at room temperature.

Image analyses and statistics

After staining, sections were mounted on aminosilane-coated glass slides and covered with a glass microcover slip. Immunohistochemical staining was observed under a light microscope (BX 50; Olympus) and photographs of the NA and DMV were captured using a CCD camera (DP 21; Olympus). Immunofluorescent staining was viewed and captured using a LSM-510 META confocal laser-scanning microscope (Carl Zeiss, Jena, Germany). Digital photomicrographs were processed with Zeiss LSM Image Browser software (Carl Zeiss). For the NA, 3 sections from each mouse were selected at levels −6.80 mm, −6.90 mm, and −7.00 mm from the Bregma. For the DMV, 4 sections from each mouse were selected at levels −7.00 mm, −7.20 mm, −7.40 mm, and −7.60 mm from the Bregma, as described elsewhere [39]. The number of immunopositive cells with clearly visible transected round nuclei was

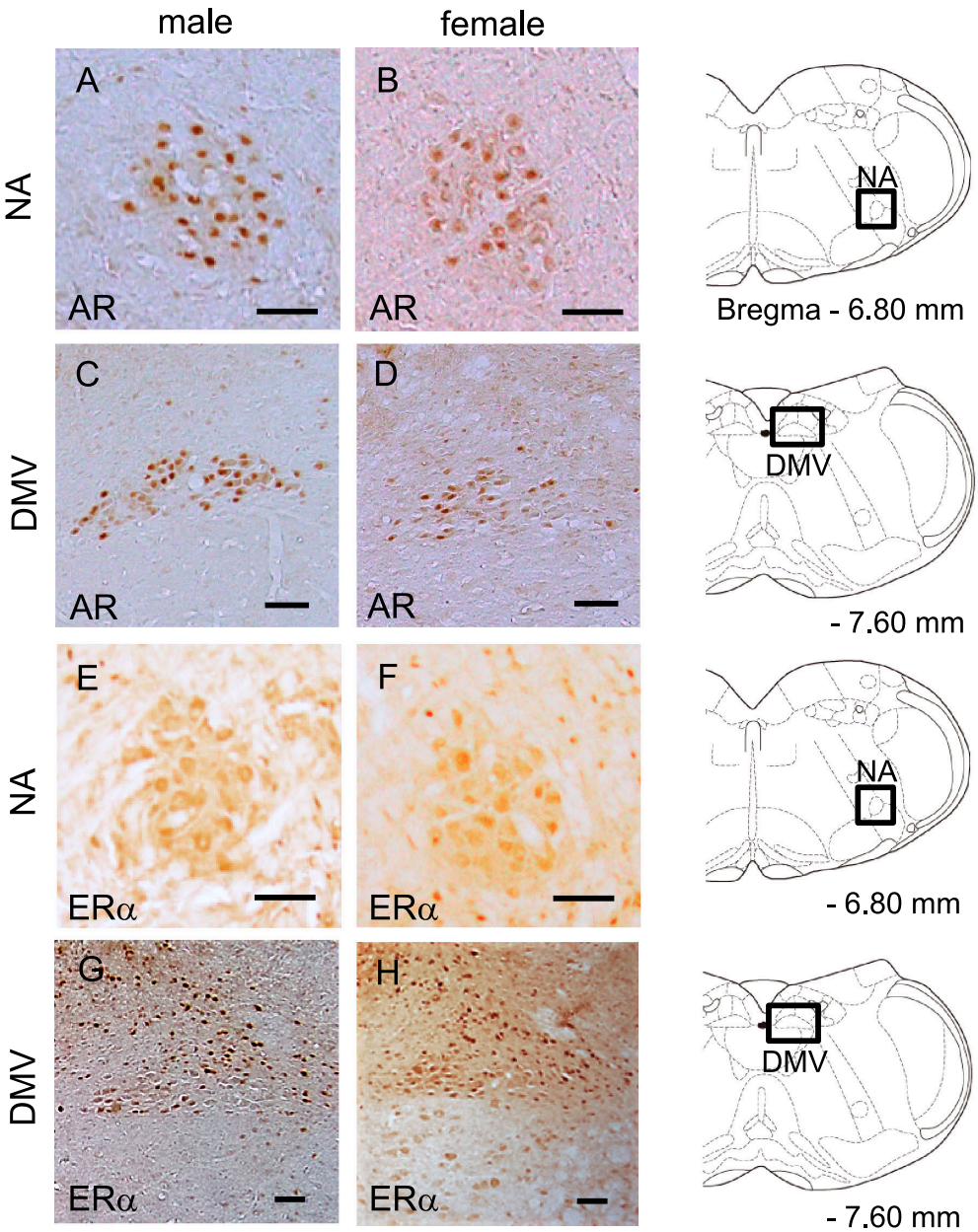

Fig. 1. AR immunoreactivity in the NA at −6.80 mm (**A, B**) and DMV at −7.60 mm (**C, D**) in male (**A, C**) and female (**B, D**) mice. ERα immunoreactivity in the NA at −6.80 mm (**E, F**) and DMV at −7.60 mm (**G, H**) in male (**E, G**) and female (**F, H**) mice. Bar=50 μm.

Table 1. *Numbers of AR-immunoreactive neurons in vagal motor nuclei in male and female mice*

	Number of neurons	
	Male (n=5)	Female (n=5)
NA	163.4±3.2	145.0±5.3*
DMV	294.8±5.7	234.0±7.2*

* $P<0.05$ vs. male mice.

Table 2. *Numbers of ERα-immunoreactive neurons in vagal motor nuclei in male and female mice*

	Number of neurons	
	Male (n=5)	Female (n=5)
NA	162.0±4.4	158.0±5.2
DMV	522.2±6.8	555.4±5.2*

* $P<0.05$ vs. male mice.

counted. All values are expressed as means±SEM. The significance of a sex difference or a difference between the group injected with CTb 488 into the trachea and CTb 555 into the esophagus was evaluated by Student t-test.

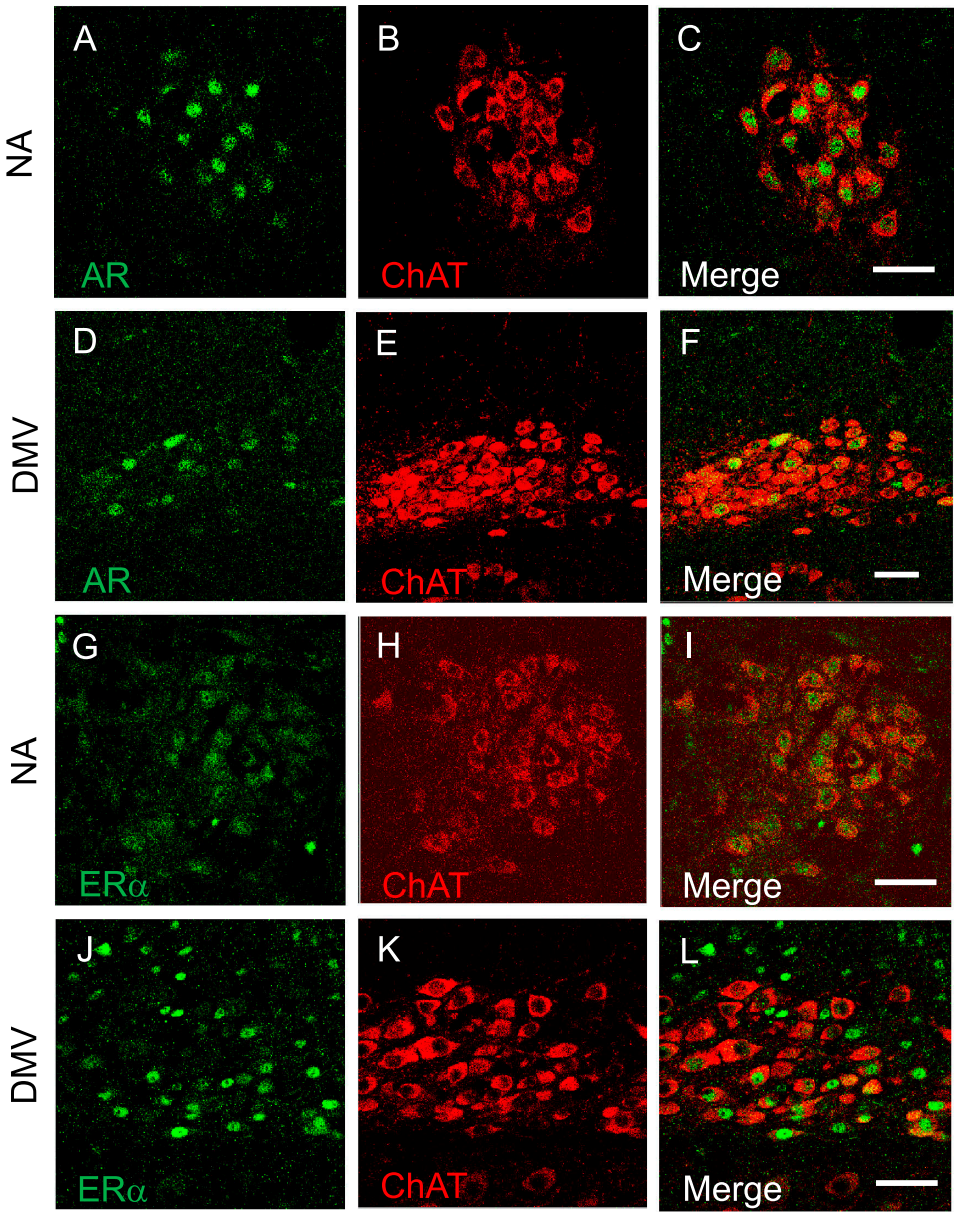

Fig. 2. Double immunostaining for sex hormone receptors (green) and ChAT (red) in the NA (**A–C, G–I**) and DMV (**D–F, J–L**). (**A, D**) AR-immunoreactive neurons. (**G, J**) ERα-immunoreactive neurons. (**B, E, H, K**) ChAT-immunoreactive neurons. (**C**) Merged image of **A, B**. (**F**) Merged image of **D, E**. (**I**) Merged image of **G, H**. (**L**) Merged image of **J, K**. Bar=50 μm.

III. Results

Localization and sex differences of AR or ERα expression in the NA and DMV

AR and ERα were expressed in the rostral NA and part of the DMV in males and females (Fig. 1). AR was localized predominantly in the nucleus and the intensity tended to be stronger in males than in females (Fig. 1A–D). ERα was localized both in the nucleus and cytoplasm in the NA (Fig. 1E, F), whereas ERα was localized predominantly in the nucleus in the DMV (Fig. 1G, H). A sex difference was not apparent in the intracellular distribution of ERα (Fig. 1E–H). Males had higher counts of AR-immunoreactive cells than females in the NA ($P<0.05$, Table 1) and DMV ($P<0.05$, Table 1). In contrast, females had higher counts of ERα-immunoreactive cells than males in the DMV ($P<0.05$, Table 2). No sex difference was noted in counts of ERα-immunoreactive cells in the NA (Table 2).

Colocalization of AR and ChAT in the NA and DMV

AR and ChAT immunoreactivities were strongly prevalent in the NA (Fig. 2A–C, Table 3). In the DMV, 38.8±0.9% of ChAT-immunoreactive neurons were positive for AR, and almost all AR-immunoreactive neurons were positive for ChAT (Fig. 2D–F, Table 3).

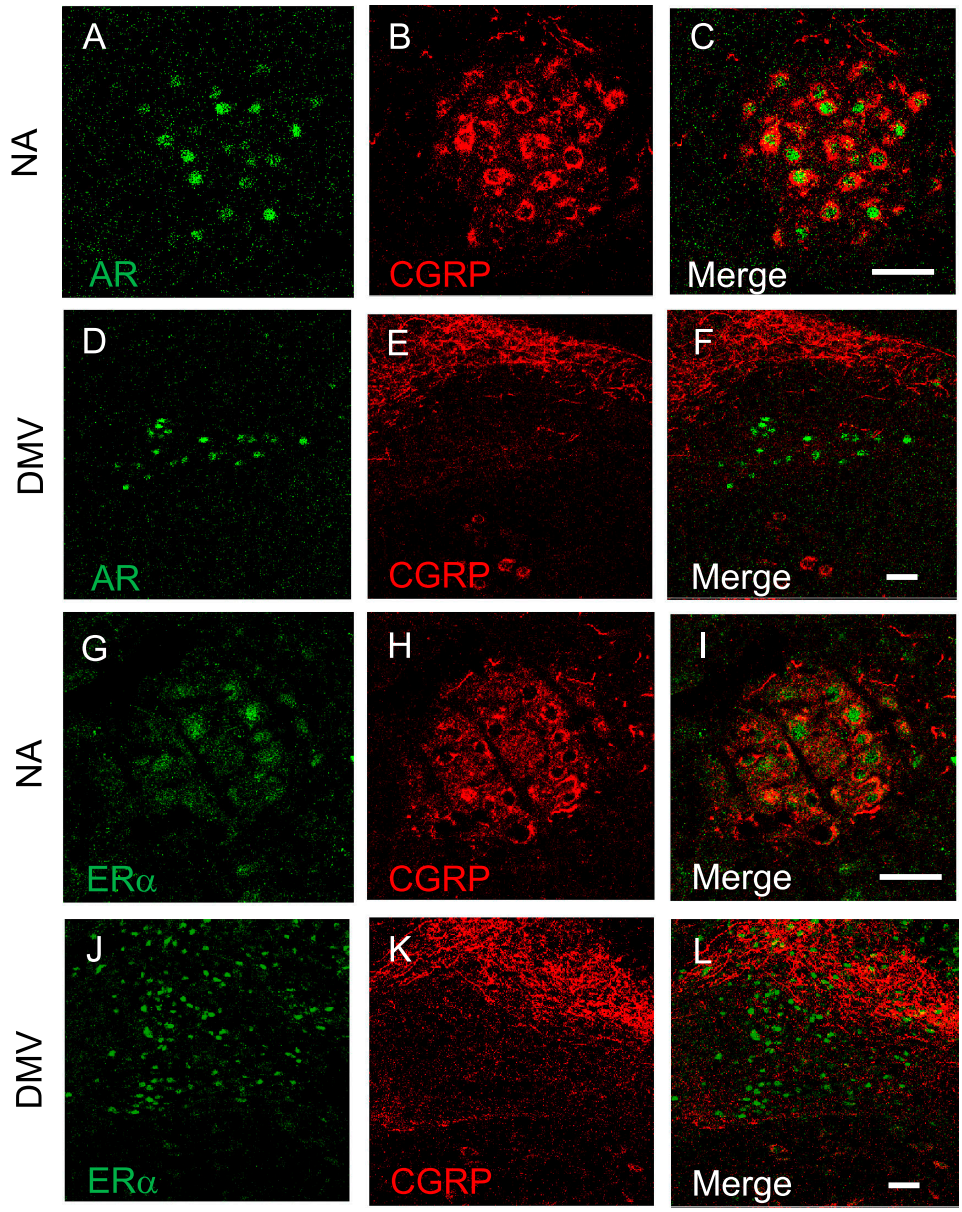

Fig. 3. Double immunostaining for sex hormone receptors (green) and CGRP (red) in the NA (**A–C, G–I**) and DMV (**D–F, J–L**). (**A, D**) AR-immunoreactive neurons. (**G, J**) ERα-immunoreactive neurons. (**B, E, H, K**) ChAT-immunoreactive neurons. (**C**) Merged image of **A, B**. (**F**) Merged image of **D, E**. (**I**) Merged image of **G, H**. (**L**) Merged image of **J, K**. Bar=50 μm.

Table 3. *Percentages of sex hormone receptors and ChAT-immunoreactive neurons in vagal motor nuclei*

	Percentages (%)			
	AR/ChAT	ChAT/AR	ERα/ChAT	ChAT/ERα
NA	94.5±0.6	96.8±0.8	96.5±0.7	95.5±2.1
DMV	38.8±0.9	95.5±0.6	84.3±1.6	78.2±1.0

Table 4. *Percentages of sex hormone receptors and CGRP-immunoreactive neurons in the NA*

	Percentages (%)			
	AR/CGRP	CGRP/AR	ERα/CGRP	CGRP/ERα
NA	95.0±1.6	97.8±0.8	93.9±1.0	94.9±1.6

Colocalization of ERα and ChAT in the NA and DMV

ERα and ChAT immunoreactivities were strongly prevalent in the NA (Fig. 2G–I, Table 3). In the DMV, 84.3±1.6% of ChAT-immunoreactive neurons were positive for ERα, and 78.2±1.0% of ERα-immunoreactive neurons were positive for ChAT (Fig. 2J–L, Table 3).

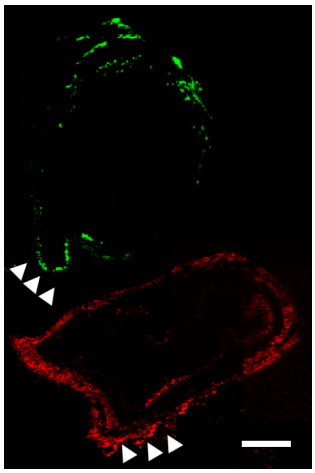

Fig. 4. Diffusion of CTb 488 (green) to the trachea and CTb 555 (red) to the esophagus in 24 hr after injections. Arrowheads indicate CTb injection sites in the trachea and esophagus. Bar=250 μm.

Colocalization of AR or ERα and CGRP in the NA and DMV

AR/ERα and CGRP immunoreactivities were strongly prevalent in the NA (Fig. 3A–C, 3G–I, Table 4). No CGRP expression was detected in the DMV (Fig. 3D–F, 3J–L).

Spread of tracers in the trachea and esophagus

CTb 488 in the extrathoracic trachea and CTb 555 in the cervical esophagus were not found 5 days after injections. Therefore, diffusion of CTb was examined in 24 hr after injections in the trachea and esophagus. At 24 hr after injection, CTb 488 was seen in an almost total ring of the tracheal wall, and CTb 555 was seen in the circumferential esophageal wall (Fig. 4). There was no spread of dye to neighboring tissues.

Colocalization of ChAT and CTb in the NA and DMV

In the NA and DMV of mice with injection into the trachea or esophagus, all CTb-labeled neurons had positive ChAT immunoreactivity (data not shown). CTb-labeled neurons were localized among the ChAT-immunoreactive neurons in the rostral NA, whereas no CTb-labeled neurons were detected among the ChAT-immunoreactive neurons in the caudal NA (Fig. 5).

Colocalization of AR and CTb in the NA and DMV

In the NA of mice with injection into the trachea or esophagus, all CTb-labeled neurons had positive AR immunoreactivity (Fig. 6A, E, Table 5). In the DMV of both groups, a small population of CTb-labeled neurons was detected. The rates of AR expression in CTb-positive neurons in the trachea and esophagus injection groups were 53.3±13.3% and 50.3±16.8%, respectively, with no significant difference between the groups (Fig. 6B, F, Table 5).

Colocalization of ERα and CTb in the NA and DMV

In the NA, CTb-labeled neurons were all positive for ERα in mice with injections into the trachea and esophagus (Fig. 6C, G, Table 6). In the DMV, a small population of CTb-labeled neurons was detected in both groups. The rates of ERα expression in CTb-positive neurons of the trachea and esophagus injection groups were 47.7±14.6% and 39.3±11.0%, respectively, with no significant difference between the groups (Fig. 6D, H, Table 6).

Collateral innervation of the trachea and esophagus from neurons in the NA and DMV

In the NA, we observed neurons that were labeled with two CTb tracers (CTb 488 in the trachea, CTb 555 in the esophagus) (Fig. 7). Of CTb 555-labeled neurons, 24.4±1.6% were labeled with CTb 488, while 33.4±5.1% of CTb 488-labeled neurons were labeled with CTb 555 (Table 7). In contrast, no CTb co-labeled neurons were detected in the DMV.

IV. Discussion

In this study, we investigated sex steroid hormone receptor expression in mouse vagal motor nuclei and examined the immunohistochemical characteristics of sex steroid hormone receptor-positive neurons and their innervation into the trachea and esophagus. Several studies have shown expression of sex steroid hormone receptors in vagal motor nuclei, with AR found to be expressed in the rat NA [14, 47, 52] and ERs expressed in rodent vagal motor nuclei [6, 37, 45, 50]. Our results are generally consistent with these findings. However, investigations of the detailed distribu-

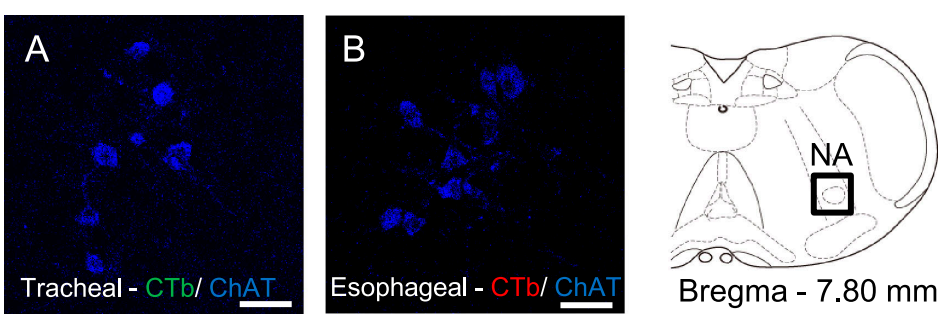

Fig. 5. Double immunostaining for ChAT (blue) and CTb 488 (green) (**A**) or CTb 555 (red) (**B**) in the NA at −7.80 mm. CTb 488- or CTb 555-labeled neurons were not observed in the caudal NA. Bar=50 μm.

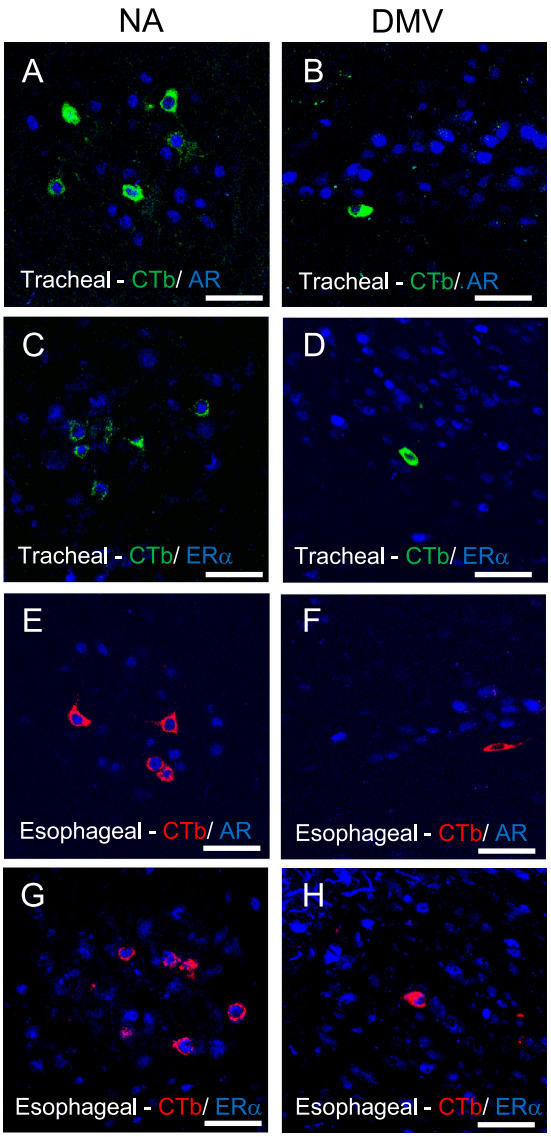

Fig. 6. Double immunostaining for sex hormone receptors (blue) and CTb (green or red) in the NA (**A, C, E, G**) and DMV (**B, D, F, H**). (**A–D**) Representative photomicrographs of the distribution of AR or ERα in tracheal neurons retrogradely labeled with CTb 488 in the NA and DMV. (**E–H**) Representative photomicrographs of the distribution of AR or ERα in esophageal neurons retrogradely labeled with CTb 555 in the NA and DMV. Bar=50 μm.

Table 5. *Numbers and percentages of AR- and CTb-positive neurons in vagal motor nuclei*

	Number of neurons		Percentages (%)
	CTb and AR	CTb	AR/CTb
NA			
Tracheal	27.0±2.7	27.0±2.7	100
Esophageal	32.4±4.0	32.4±4.0	100
DMV			
Tracheal	2.2±0.7	4.2±0.7	53.3±13.3
Esophageal	2.2±0.8	4.2±1.0	50.3±16.8

No significant differences between the trachea and esophagus injection groups.

Table 6. *Numbers and percentages of ERα- and CTb-positive neurons in vagal motor nuclei*

	Number of neurons		Percentages (%)
	CTb and ERα	CTb	ERα/CTb
NA			
Tracheal	28.0±4.0	28.0±4.0	100
Esophageal	35.8±1.5	35.8±1.5	100
DMV			
Tracheal	2.2±0.7	4.4±0.5	47.7±14.6
Esophageal	1.6±0.4	3.8±0.6	39.3±11.0

No significant differences between the trachea and esophagus injection groups.

Table 7. *Numbers and percentages of CTb 488- and CTb 555-positive neurons in the NA*

	Number of neurons			Percentages (%)	
	Double positive	CTb 488	CTb 555	CTb 488/555	CTb 555/488
NA	9.0±1.0	27.8±2.6	36.6±2.7	24.4±1.6	33.4±5.1

tion of sex hormone receptors in vagal motor nuclei are limited. In this study, we showed that sex steroid hormone receptors are expressed in the mouse rostral NA, and that ARs are expressed in the mouse DMV. In addition, we examined sex differences of AR and ERα expression in mouse vagal motor nuclei. AR intensity tended to be stronger in males than in females. In contrast, a sex difference

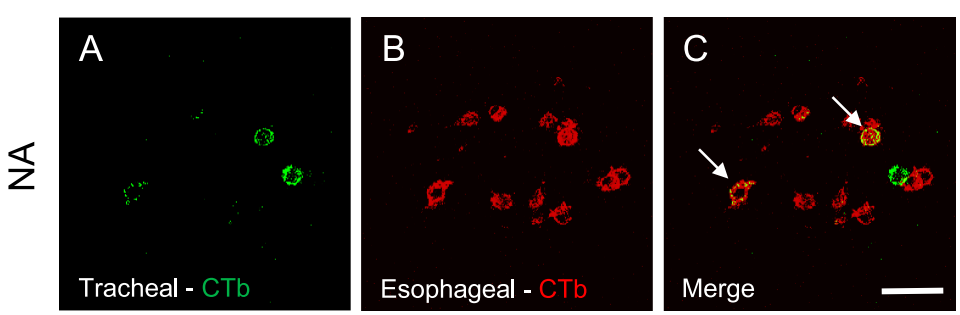

Fig. 7. Representative photomicrographs showing dual retrograde labeling of neurons in the NA following administration of CTb 488 (green) to the trachea and CTb 555 (red) to the esophagus. (**A**) CTb 488-immunoreactive neurons and (**B**) CTb 555-immunoreactive neurons in the NA. (**C**) Merged image of **A, B**. Double-labeled neurons appear yellow (arrow). Bar=50 μm.

was not apparent in the intracellular distribution of ERα. Males had higher counts of AR-immunoreactive cells in the NA and DMV, whereas females had higher counts of ERα-immunoreactive cells in the DMV. Previous studies have compared AR expression in the NA [52] and ER expression in the DMV [45] among male and female rats. Our results are generally consistent with these findings. To the best of our knowledge, this is the first report on the sex difference of AR-immunoreactive cell counts in vagal motor nuclei. Our results may provide a new avenue to explain the mechanism of sex differences in respiratory and gastrointestinal functions.

We determined the immunohistochemical characteristics of sex hormone receptor-positive neurons. Previous studies have suggested that the NA is immunoreactive for ChAT [44] and CGRP [26, 25, 34, 43], while the DMV is immunoreactive for ChAT alone [31]. Our findings are consistent with these reports. In the NA, ChAT- and CGRP-immunoreactive neurons were co-expressed in sex hormone receptor-positive neurons, whereas no CGRP expression was detected in the DMV and most sex hormone receptor-positive neurons were positive for ChAT. This difference in the immunohistochemical characteristics between neurons in the NA and DMV may reflect their roles as origins for special and general visceral efferent fibers, respectively.

Retrograde tracing showed the topographical organization of the mouse vagal motor nuclei, including projections to the extrathoracic trachea and cervical esophagus. In the mouse NA, rostral neurons projected to the extrathoracic trachea and cervical esophagus, whereas no clear topographical organization was observed in the DMV. Subnuclei of the NA have been studied in rats and dogs [2, 5, 22–24], and neurons innervating the respiratory tract and esophagus are predominant in the rostral NA, which is termed the compact formation [5, 12, 13, 15–17, 19, 18, 27, 30, 32, 33, 51]. Although subnuclei of the mouse NA have not been studied in detail, our results are similar to these findings. Some studies of DMV subnuclei have shown that rostral neurons in the DMV innervate the respiratory tract and cervical esophagus [13, 15–17, 20, 30], whereas no topographical organization has been observed in other studies [3, 36].

Dual retrograde tracing experiments showed that NA neurons innervate the trachea and the esophagus. We performed experiments with care to avoid diffusion to surrounding tissues, based on the methods of Mazzone *et al.* [35], and our results were reproducible. McGovern *et al.* [36] did not observe double innervation of the trachea and esophagus by NA neurons in guinea pigs; however, approximately 5% of neurons were double-labeled using two retrograde tracers in the current study. Cheng *et al.* [9] reported that NA neurons innervate the heart and esophagus in rats, and Hisa *et al.* [23] showed that NA neurons innervate the thyroarytenoid and lateral cricoarytenoid muscles in dogs. McGovern *et al.* [36] found that approximately 40% of ChAT-immunoreactive rostral NA neurons in guinea pigs were positive for CGRP, and there was a difference in the expression rate for CGRP between trachea- and esophagus-innervating neurons. In contrast, we found that ChAT- and CGRP-immunoreactive rostral NA neurons were highly colocalized (data not shown), which indicates a possible species difference. Furthermore, this finding shows that the immunohistochemical characteristics of mouse NA neurons are homogeneous, which is not in conflict with our results of double innervation of the trachea and esophagus by NA neurons. In addition, no significant difference in sex hormone receptor expression was observed between trachea- and esophagus-innervating neurons in the NA, which is also not in conflict with double innervation of the trachea and esophagus by NA neurons. The function of this innervation may be associated with coughing or asthmatic response in patients with gastroesophageal reflux [10], which is thought to be caused by simultaneous transmission of efferent signals to the trachea and esophagus, following transmission of afferent signals caused by gastric acid-induced chemical stimulation of the NA via the nucleus tractus solitarius.

Sex steroid hormones such as androgen and estrogen have been shown to affect mucous secretion and smooth muscle contraction in the respiratory or gastrointestinal tracts [4, 7, 8, 38], but the mechanisms are unknown. The current study shows that some NA and DMV neurons innervating the trachea and esophagus express AR and/or ERα, which suggests that androgens and estrogens may activate receptors in vagal motor nuclei, in order to regulate functions of the trachea and esophagus. This mechanism may explain gastrointestinal symptoms such as heartburn during pregnancy and before menstruation. It is possible that fluctuating blood estrogen activates ERα in vagal motor nuclei, thereby affecting the motion of the esophagus, as well as blood flow and glandular secretion. Similarly, this may be related to findings that sex hormones affect the occurrence and progression of bronchial asthma and reflux esophagitis.

The results of this study suggest that sex steroid hormones may regulate functions of the trachea and esophagus similarly by directly acting on vagal motor nuclei, via activation of sex hormone receptors. These results improve understanding of the sex steroid hormone-mediated mechanism for regulating functions of the respiratory and gastrointestinal tracts. The next step is to evaluate changes in the results and functions of the trachea and esophagus after castration or ovariectomy.

V. Disclosure Statement

The authors have nothing to disclose.

VI. Acknowledgments

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