Tracheal occlusion-evoked respiratory load compensation and inhibitory neurotransmitter expression in rats

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Tsai H-W, Davenport PW. Tracheal occlusion-evoked respiratory load compensation and inhibitory neurotransmitter expression in rats. J Appl Physiol 116: 1006–1016, 2014. First published February 20, 2014; doi:10.1152/japplphysiol.01256.2013.—Respiratory load compensation is a sensory-motor reflex generated in the brain stem respiratory neural network. The nucleus of the solitary tract (NTS) is thought to be the primary structure to process the respiratory load-related afferent activity and contribute to the modification of the breathing pattern by sending efferent projections to other structures in the brain stem respiratory neural network. The sensory pathway and motor responses of respiratory load compensation have been studied extensively; however, the mechanism of neurogenesis of load compensation is still unknown. A variety of studies has shown that inhibitory interconnections among the brain stem respiratory groups play critical roles for the genesis of respiratory rhythm and pattern. The purpose of this study was to examine whether inhibitory glycinergic neurons in the NTS were activated by external and transient tracheal occlusions (ETTO) in anesthetized animals. The results showed that ETTO produced load compensation responses with increased inspiratory, expiratory, and total breath time, as well as elevated activation of inhibitory glycinergic neurons in the caudal NTS (cNTS) and intermediate NTS (iNTS). Vagotomized animals receiving transient respiratory loads did not exhibit these load compensation responses. In addition, vagotomy significantly reduced the activation of inhibitory glycinergic neurons in the cNTS and iNTS. The results suggest that these activated inhibitory glycinergic neurons in the NTS might be essential for the neurogenesis of load compensation responses in anesthetized animals.

NTS; glycinergic neurons; tracheal occlusion; load compensation; vagotomy

In the face of respiratory mechanical or metabolic challenges, animals are able to compensate to the stimuli by adjusting tidal volume (VT) and/or breathing frequency to maintain appropriate minute ventilation, i.e., respiratory load compensation. Respiratory load compensation is characterized as the volumetiming (VT-T) relationship in anesthetized animals (7). The application of a respiratory-resistive load, to inspiration or expiration, increases airway resistance, which, in turn, decreases inspired or expired air volume, respectively, and results in a reflex prolongation of inspiratory time (Ti) or expiratory time (Te) of the loaded breath, respectively (27). The VT-T relationship can be abolished by vagotomy in anesthetized cats, indicating that the vagi are the principal respiratory afferents, transducing information related to the change of lung volume to the respiratory neural network to elicit the VT-T response (7, 27).

Davenport et al. (9–11) demonstrated that slowly adapting pulmonary stretch receptors (PSRs), located in the smooth muscle of the airways, innervated by fast-conducting, myelinated afferent fibers in the vagus nerves, respond to changes in lung volume and smooth muscle tone and mediate respiratory load compensation responses. PSR afferents principally terminate on the second-order interneurons, pump cells, and in the intermediate and caudal portions of the nucleus of solitary tract (NTS and cNTS, respectively) (16) and project to the lateral pons and ventral respiratory group to modify the breathing pattern (18). Therefore, the NTS is the principal structure to process peripheral respiratory afferents modulated by respiratory mechanical loads and may play a significant role in load compensation reflexes.

Afferent inputs and motor outputs of the respiratory neural network during eupneic breathing and respiratory load compensation have been studied extensively in anesthetized animals (7, 9–11, 17, 19, 26, 27). The neurotransmitters within the neurons of the respiratory neural network responsible for the neurogenesis of breathing have also been studied (1, 10, 25). However, the NTS neurons activated with respiratory load compensation stimuli and the neurotransmitters within those neurons are still unknown.

Reciprocal inhibitory connections among brain stem respiratory-related neurons have been believed to play important roles in shaping the rhythm and pattern of breathing (2, 14, 15, 22). It has been demonstrated that two-thirds of pump cells in the NTS uses GABA and glycine as neurotransmitters (13). It has been shown that trigeminal afferents modulate the respiratory neural network during eupneic breathing and respiratory load compensation (18). The neurotransmitters within the neurons of the respiratory neural network responsible for the neurogenesis of breathing have also been studied (1, 10, 25). However, the NTS neurons activated with respiratory load compensation stimuli and the neurotransmitters within those neurons are still unknown.

MATERIALS AND METHODS

Animals

Experiments were performed on 16 male Sprague-Dawley rats (320–380 g). The animals were housed in the University of Florida Animal Care Facility. They were exposed to a 12-h light/12-h dark cycle and with free access to food and water. The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Florida.

Surgical Procedure

Animals were anesthetized with urethane (1.3 g/kg ip). Adequate anesthetic depth was verified by the absence of a withdrawal reflex to a rear paw pinch. Additional urethane (20 mg/ml) was supplemented.
as necessary until the experiment was terminated. Body temperature was monitored with a rectal temperature probe (Harvard Apparatus, Holliston, MA) and maintained at 37–39°C using a heating pad. Animals were spontaneously breathing room air throughout the experiment.

Animals were placed in a supine position, and the right femoral artery was cannulated using a saline-filled catheter (polyethylene-50 tubing) connected to a pressure transducer to monitor arterial blood pressure. A saline-filled tube (polyethylene-90 tubing) was passed through the mouth into the esophagus and connected to a pressure transducer. The esophageal pressure (Pes) was monitored with a saline-filled catheter (polyethylene-50 tubing) connected to a pressure transducer. The inspiratory time (Ti) and expiratory time (Te) are shown in the trace of JEMGdia. Shadow area represents the period of ETTO.

Fig. 1. Effect of vagotomy on the respiratory load compensation responses. Breathing pattern results during control (C3–C1), occluded (O1–O3), and recovery (R1–R3) breaths. A and B: top traces are the raw diaphragm electromyography (EMGdia) signals, middle traces are the JEMGdia signals, and bottom traces are the esophageal pressure (Pes). A: breathing pattern in a group A rat with external and transient tracheal occlusions (ETTO) with intact vagi. B: breathing pattern in a group D rat with ETTO after bilateral cervical vagotomy. The inspiratory time (Ti) and expiratory time (Te) are shown in the trace of JEMGdia. Shadow area represents the period of ETTO.

Fig. 2. ETTO elicited load compensation responses in anesthetized animals with intact vagi (group B). Normalized breath-timing values during control (C3–C1), occluded (O1–O3), and recovery (R1–R3) breaths. A and B: top traces are the raw diaphragm electromyography (EMGdia) signals, middle traces are the JEMGdia signals, and bottom traces are the esophageal pressure (Pes). A: the relationship between Ti and breath number. B: the relationship between Te and breath number. C: the relationship between total breath time (Ttot) and breath number. Significant difference, *P < 0.05.
transducer to measure change in esophageal pressure (ΔPes). The analog outputs were amplified (Stoelting, Wood Dale, IL), digitized at 5 kHz (1401 computer interface; Cambridge Electronic Design, Cambridge, UK), computer processed (Spike2; Cambridge Electronic Design), and stored for analysis. Pleural pressure changes were inferred from relative ΔPes. Diaphragm electromyography (EMGdia) was recorded with bipolar Teflon-coated wire electrodes. The distal ends of the wires were bared and bent to form a hook. The bare tips of the electrodes were inserted into the costal diaphragm through a small incision in the abdominal wall. The electrode wires were connected to a high-impedance probe. The signal was fed into an amplifier (P511; Grass Instruments, Quincy, MA) and band-pass filtered (30–3,000 Hz). The analog output was digitized at 5 kHz, processed, and stored for analysis.

The trachea was exposed through a ventral midline incision and separated from surrounding soft tissue. Tracheotomy was performed in all animals. A tracheal cannula was inserted into the tracheotomy. In addition, cervical vagus nerves were separated bilaterally from surrounding tissues and carotid artery. The vagi were severed only in the vagotomy groups.

**Experimental Protocol**

The rats were divided randomly into four groups: *Group A* rats (n = 4) were surgically prepared, but breathing was not obstructed. *Group B* rats (n = 4) were surgically prepared and received 10 min of ETTO. *Group C* (n = 4) and *Group D* (n = 4) rats were surgically prepared and vagotomized, bilaterally. The *Group D* rats received 10 min of ETTO, but the tracheal occlusion was not performed in *Group C* animals. When the surgical preparation was complete, the animals were maintained anesthetized and spontaneously breathing for 90 min. Then, the ETTO or control protocols were initiated.

For the ETTO protocols (*Groups B and D*), each occlusion was applied at the end of expiration for 2–3 s by blocking the outlet of the tracheal cannula and then unblocking for at least 10 unobstructed breaths. ΔPes and EMGdia were monitored throughout the experiment to determine the onset and removal of occlusions. *Groups A and C* underwent surgical preparation and 10 min of spontaneous breathing but did not receive occlusions.

After the 10-min ETTO or control trials, the animals were maintained under anesthesia for an additional 90 min, which corresponds to the peak expression of c-Fos from the onset of stimuli (6, 12). Following the 180 min of the procedures, the animals were killed, and surrounding tissues and carotid artery. The fixed tissue was then transferred into a solution of 30% sucrose. The fixed tissue was air dried and cover slipped with anti-fading medium (DAKO).

**Immunofluorescence Double-Staining Protocol**

For each brain, every fourth section of tissue was used for immunofluorescence double-staining for c-Fos and glycine transporter 2 (GlyT2). Free-floating sections were blocked in 5% normal donkey serum in PBS (GlyT2). Free-floating sections were blocked in 5% normal donkey serum in PBS + Triton X-100 (PBS-T) for 1 h and then incubated in a mixed solution of guinea pig anti-GlyT2 (1:1,000 dilution; Millipore, Billerica, MA) and rabbit anti-c-Fos primary antibodies (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), diluted in DAKO antibody diluents (DAKO, Carpinteria, CA) for 36 h. The following day, the tissue was washed three times with PBS-T and then incubated in secondary antibodies (1:200 dilution for Alexa Fluor 488-AffiniPure donkey anti-guinea pig IgG and 1:100 dilution for Alexa Fluor 594-AffiniPure donkey anti-rabbit IgG; Jackson ImmunoResearch, West Grove, PA) for 2 h. Slices of tissue were washed with PBS-T, three times, and then mounted on glass slides, which were air dried and cover slipped with anti-fading medium (DAKO).

Negative controls were performed in the absence of the primary antibody, and the results showed no c-Fos or GlyT2-positive staining.

**Data Analysis**

**Breathing pattern.** Spike2 software (Cambridge Electronic Design) was used for the analysis of the breathing pattern. The EMGdia signals were rectified and integrated with a time constant of 50 ms (Fig. 1). The Ti, Te, total breath time (Ttot), and EMGdia amplitude for each breath were measured from the JEMGdia signals. Ti was measured from the onset to the peak of JEMGdia activity (Fig. 1). Te was measured from the peak of JEMGdia activity to the following onset of JEMGdia activity (Fig. 1). Ttot was the sum of Ti and Te. The JEMGdia amplitude was measured from baseline to peak. For the experimental group, these respiratory parameters were analyzed for the three unobstructed control breaths (C: C3, C2, C1) before occlusions, the first three occluded breaths (O: O1, O2, O3), and the three unobstructed recovery breaths (R: R1, R2, R3). For
the sham group, these respiratory parameters were measured at matched time periods. ∆Pes was measured as the difference from the baseline to the peak of ∆Pes. Ti, Te, Ttot, fEMGdia amplitude, and ∆Pes of the O and R breaths were averaged and then normalized by using mean values of the C breaths for each rat in the experimental groups.

**Immunofluorescence double-staining.** c-Fos is a transcription factor located in the nucleus of a neuron. GlyT2 is a cell membrane protein that is a specific biomarker for glycinergic neurons. A standard fluorescence microscope with appropriate filters was used to visualize and image positive fluorescence staining in these brain-tissue slides. The positive c-Fos-like immunoreactivity was defined as a red stain in the nucleus of the cell. A positive immunofluorescence double-staining of c-Fos and GlyT2 was characterized as c-Fos in the nucleus (red) and GlyT2-positive staining (green) in the cytoplasm of the same neuron. Brain regions were defined by the rat stereotaxic atlas (21).

Brain slices were collected in the iNTS, defined by the coordinates Bregma −12.5 to −13.3 mm, and cNTS, defined by the coordinates Bregma −13.3 to −14.1 mm. Three or four levels of each region (iNTS or cNTS) in each brain were used for the immunofluorescence double-staining of c-Fos and GlyT2. Immunoreactive cells at three or four levels of each region of each animal were counted manually in bilateral iNTS and cNTS by an individual blinded to the groups. There were no statistically significant differences in the numbers of a positive c-Fos neuron and colabeled c-Fos and GlyT2 neurons on either side of these regions, so the cell counts were averaged bilaterally for each level. Cell counts from the three or four slices of the same region were summed and then multiplied by 20/3 or 20/4 (depending on how many levels were used for immunofluorescence double-staining of c-Fos and GlyT2) to generate an estimate of the total cell counts in the entire iNTS or cNTS. The value of colabeled c-Fos and GlyT2, divided by the positive c-Fos cell number, represents the percentage of c-Fos-positive cells colabeled with GlyT2 in each region of each animal.

**Statistical Analysis**

All respiratory parameters are presented as mean ± SE. One-way repeated-measures ANOVA with breath as a factor (C, O, and R breaths) was used for the analysis of normalized Ti, Te, Ttot, fEMGdia amplitude, and ∆Pes, which were followed by post hoc paired t-tests. One-way ANOVA was performed to compare the immunoreactive cell numbers in each region among these four groups. A Greenhouse-Geisser correction was applied in case of violated sphericity assumptions, with reported significance levels referring to corrected degrees of freedom. The significance criterion for all analyses was set at P < 0.05.

**RESULTS**

**Breathing Pattern**

In groups A and C, there were no significant differences in Ti, Te, Ttot, fEMGdia amplitude, and ∆Pes between breaths during the experiment.

In group B, Ti, Te, and Ttot showed significant differences among O, C, and R phases (P < 0.001 for Ti; P < 0.01 for Te; P < 0.001 for Ttot). Ti was increased significantly during the O phase compared with the C and R phases (Fig. 2A). Te was also prolonged during the O phase compared with the C and R phases (Fig. 2B). The prolongation of Ti and Te contributed to a longer Ttot during the O phase compared with C and R phases (Fig. 2C). ∆Pes showed significant differences among the three phases (P < 0.001). ∆Pes was more negative during occlusions than the C and R phases. ∆Pes returned to baseline during the R phase and was not significantly different from the C phase (Fig. 3A). There was no significant difference in fEMGdia amplitude among the O, C, and R phases in group B (Fig. 3B).

In group D, there were no significant differences in Ti among the three phases (Fig. 4A). Te and Ttot showed significant differences among three phases (P < 0.001 for Te; P < 0.001 for Ttot). Tracheal occlusions resulted in a significant decrease in Te compared with the C and R phases (Fig. 4B). The shortening of Te contributed to a significant decrease in Ttot during the O phase compared with the C and R phases (Fig. 4C). ∆Pes was more negative during the O phase than the C and R phases (P < 0.001; Fig. 5A). There were no significant differences in fEMGdia amplitude among the C, O, and R phases (Fig. 5B).

**Immunofluorescence Double-Staining**

Figures 6 and 7 show the double-staining of c-Fos and GlyT2 in the cNTS in four groups. In the cNTS, no significant differences in the number of c-Fos cells were observed among groups (Fig. 8A). The number of colabeled c-Fos and GlyT2 cells showed significant differences among the four groups (P < 0.01). The number of colabeled c-Fos and GlyT2 cells in group B was significantly greater than groups A, C, and D (group A vs. group B: P < 0.01; group B vs. group C: P < 0.001; group C vs. group D: P < 0.001).
cells in group B was significantly greater than groups A and C and trended higher than group D (group A vs. group B: \( P < 0.05 \); group B vs. group C: \( P < 0.05 \); group B vs. group D: \( P = 0.111 \)). The percentage of c-Fos- and GlyT2-colabeled cells showed significant differences among the four groups (\( P < 0.001 \); Fig. 11C). The percentage of c-Fos-positive cells colabeled with GlyT2 was significantly greater in group B than groups A, C, and D (group A vs. group B: \( P < 0.001 \); group B vs. group C: \( P < 0.001 \); group B vs. group D: \( P < 0.01 \)).

**DISCUSSION**

**Breathing Pattern**

In group B, prolongation of Ti, Te, and Ttot during the O phase indicates that the respiratory load compensation responses were elicited by ETTO. In vagotomized rats, Ti was not affected by tracheal occlusions, and Te and Ttot, during the O phase, were even slightly shorter than during the C and R phases in group D, indicating that normal respiratory load compensation responses were abolished by bilateral cervical vagotomy. During the C and R phases, the breathing pattern in vagotomized animals was slower than animals with intact vags. This is a typical breathing pattern in vagotomized animals, due to the interruption of transmission ofafferent activity from PSRs during inspiration to terminate inspiratory effort (7, 27); therefore, the central brain stem is mediating the control of breathing rhythm and pattern in these rats. In addition, the shorter Te and Ttot during O1 compared with the C and R breaths suggests that in rats, other nonvagal mechanisms, such as afferents from the respiratory muscles or other nonvagal respiratory afferents, may contribute to the load-related modulation of breathing patterns in rats (5, 8, 20).

**Immunofluorescence Double-Staining**

To our knowledge, this is the first study demonstrating that inhibitory glycnergic neurons in the cNTS and iNTS were activated by respiratory load. These activated glycnergic neurons were concentrated in the medial (SoM) and ventral (SoV) solitary subnuclei of the NTS. These activated glycnergic neurons in the NTS might be considered as the second-order interneurons, possibly pump cells or pump cell-related, higher-order interneurons, based on the following reasons. First, the afferents of PSRs in the lungs activated glycinergic neurons in the NTS might be considered afferents from the respiratory muscles or other nonvagal afferents. Second, two-thirds of pump cells in the NTS uses inhibitory amino acids on the SoM and SoV and interstitial and ventrolateral subnuclei of the NTS. Second, two-thirds of pump cells in the NTS uses inhibitory GABA and glycine as neurotransmitters (13). Application of excitatory amino acids on the SoM resulted in reflex termination of inspiration and prolongation of expiration, whereas blockade of excitatory amino acids in this area reduced these changes (3, 4). Finally, these glycnergic neurons are consistent with the role of inhibitory pump cells in the brain stem respiratory network proposed by Rybak et al. (23, 24). They modeled these inhibitory pump cells to excite inspiratory-decrementing neurons in the Bötzingr complex for the termination of inspiration and prolongation of expiration and to inhibit presynaptically inspiratory neu-
Fig. 6. ETTO activated glycinergic neurons in the caudal nucleus of the solitary tract (cNTS) in anesthetized animals with intact vagi. Immunofluorescence double-staining of c-Fos (red) and glycine transporter 2 (GlyT2; green) in the cNTS (Bregma –13.8 mm) in group A animals without ETTO (B–G) and group B animals with ETTO (H–M). A: dashed box represents the area of the rat brain atlas corresponding to the regions in B–D and H–J. E–G and K–M: dashed areas in B–D and H–J, respectively. TS, solitary tract. Arrows represent immunoreactive cells. B–D and H–J: scale bars, 100 μm; E–G and K–M: scale bars, 50 μm. Abbreviations in Figs. 6 and 7: 10, dorsal motor nucleus of vagus; 12, hypoglossal nucleus; 12n, root of hypoglossal nerve; A1, A1 noradrenaline cells; A2, A2 noradrenaline cells; Amb, ambiguous nucleus; AP, area postrema; CC, central canal; Cop, copula of pyramids; Crus2, crus 2 of ansiform lobule; Cu, cuneate nucleus; cu, cuneate fasciculus; CVL, caudoventrolateral reticular nucleus; CVRG, caudoventral respiratory group; dsc, dorsal spinocerebellar tract; ECu, external cuneate nucleus; Ge5, gelatinous layer caudal spinal 5; Gr, gracile nucleus; gr, gracile fasciculus; ia, internal arcuate fibers; IOA, inferior olive, subnucleus A of medial nucleus; IOB, inferior olive, subnucleus B of medial nucleus; IOBe, inferior olive, β subnucleus; IOIC, inferior olive, subnucleus C of medial nucleus; IOD, inferior olive, dorsal nucleus; IOK, inferior olive, cap of Kooy of medial nucleus; LIRt, intermediate reticular nucleus; LIRtPC, lateral reticular nucleus, parvicell; LIRtS5, lateral reticular nucleus, sub5 part; MdD, medullary reticular nucleus, dorsal; MdV, medullary reticular nucleus, ventral; ml, medial lemniscus; mlf, medial longitudinal fasciculus; pf, pyramidal fissure; PM, paramedian lobule; PMn, paramedian reticular nucleus; Py, pyramidal tract; Ro, nucleus of Roller; ROb, raphe obscurus nucleus; RPa, raphe pallidus nucleus; rs, rubrospinal tract; RVL, rostroventrolateral reticular nucleus; RVRG, rostral ventral respiratory group; sol, solitary tract; SolC, nucleus of solitary tract, commissural; SolDM, nucleus of solitary tract, dorsomedial; SolI, nucleus of solitary tract, interstitial; SolIM, nucleus of solitary, intermediate; SolM, nucleus of solitary tract, medial; SolVL, nucleus of solitary tract, ventrolateral; sp5, spinal trigeminal tract; Sp5C, spinal 5 nucleus, caudal part; Sp5I, spinal 5 nucleus, interpolar part; ts, tectospinal tract; vsc: ventral spinocerebellar tract.
rons in the pons for the control of the breathing pattern. Therefore, the potential role of the ETTO-activated glycinergic neurons in the NTS might be related to the phase-timing changes that occur with the neurogenesis of load compensation responses.

The increased percentage of c-Fos-positive cells colabeled with GlyT2 in the cNTS and iNTS was abolished by bilateral cervical vagotomy. Kalia and Mesulam (16) have demonstrated that vagal afferents enter the lateral medulla from Bregma −12.8 to −15.3 mm and terminate in different subdivisions of the NTS from Bregma −11.8 to −18.3 mm. In the present study, the iNTS was defined as the region from Bregma −12.5 to −13.3 mm, and cNTS was from Bregma −13.3 to −14.1 mm, thus including the area of entry and termination of vagal afferents. Therefore, the present results demonstrate that bilateral cervical vagotomy could successfully block transmission of information from the PSRs in the lungs and airways to glycinergic neurons in the NTS, normally activated by ETTO. In addition, the decreased number of colabeled c-Fos and GlyT2 neurons in group D suggests that ETTO activation of glycinergic neurons in the cNTS was abolished by bilateral cervical vagotomy.
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Fig. 8. Immunofluorescence double-staining of c-Fos and GlyT2 in the cNTS in anesthetized animals with (groups A and B) or without (groups C and D) intact vagi. A: c-Fos-labeled cell number; B: colabeled c-Fos and GlyT2 cell number; C: percentage of c-Fos-positive cells colabeled with GlyT2. Significant difference, *P < 0.05; **P < 0.01; ***P < 0.001.

atory but only partially suppressed in the iNTS. The different degree of glycineric neuron suppression between cNTS and iNTS may represent different interconnections among neurons in the brain stem control network. Therefore, further research needs to be performed to differentiate the roles of the iNTS and cNTS in controlling the breathing pattern during respiratory load compensation.

In addition, ETTO were applied for three consecutive breaths, which may cause hypercapnia or hypoxia in animals. The activation of c-Fos and glycineric neurons in the NTS may be partly contributed by the changes of blood gas. However, the results showed that ETTO did not elicit activation of glycineric neurons in the NTS in vagotomized rats, demonstrating that glycineric neurons in the NTS were mainly activated by mechanical stimuli.

In conclusion, ETTO elicited load compensation responses in group B but not in group D rats. The results indicate that vagus nerves play an important role to transmit signals from PSRs to the rat brain stem for the generation for load compensation. In addition, inhibitory glycineric neurons in the cNTS and iNTS may represent different interconnections among neurons in the brain stem control network. Therefore, further research needs to be performed to differentiate the roles of the iNTS and cNTS in controlling the breathing pattern during respiratory load compensation.

DISCLOSURES
The authors have no conflicts of interest.

GRANTS
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AUTHOR CONTRIBUTIONS

REFERENCES
Fig. 9. ETTO activated glycnergic neurons in the intermediate NTS (iNTS) in anesthetized animals with intact vagi. Immunofluorescence double-staining of c-Fos (red) and GlyT2 (green) in the iNTS (Bregma −13.2 mm) in group A animals without ETTO (B–G) and group B animals with ETTO (H–M). A: dashed box represents the area of the rat brain atlas corresponding to the regions in B–D and H–J, E–G and K–M; dashed areas in B–D and H–J, respectively. Arrows represent immunoreactive cells. B–D and H–J: scale bars, 100 μm; E–G and K–M: scale bars, 50 μm. Abbreviations in Figs. 9 and 10: 4V, 4th ventricle; C1, C1 adrenaline cells; C2, C2 adrenaline cells; chp, choroid plexus; GI, gigantocellular reticular nucleus; icp, inferior cerebellar peduncle; IOPr, inferior olive, principal nucleus; IOVL, inferior olive, ventrolateral protrusion; oc, olivocerebellar tract; Pa5, paratrigeminal nucleus; PCRt, parvicellular reticular nucleus; PSol, parasolitary nucleus; R, red nucleus; SolCe, nucleus of solitary tract, central; SolG, nucleus of solitary tract, gelatinous; VL, ventrolateral thalamic nucleus.


Fig. 10. Vagotomy partially abolished the activation of inhibitory glycinergic neurons in the iNTS in anesthetized animals. Immunofluorescence staining of c-Fos (red) and GlyT2 (green) in the iNTS (Bregma –13.2 mm) in group C vagotomized animals without ETTO (B–G) and group D vagotomized animals with ETTO (H–M). A: dashed box represents the area of the rat brain atlas corresponding to the regions in B–D and H–J. E–G and K–M: dashed area in B–D and H–J, respectively. Arrows represent immunoreactive cells. B–D and H–J: scale bars, 100 μm; E–G and K–M: scale bars, 50 μm.
Fig. 11. Immunofluorescence double-staining of c-Fos and GlyT2 in the iNTS in anesthetized animals with intact vagi (groups A and B) and vagotomy (groups C and D). A: c-Fos-labeled cell number; B: colabeled c-Fos and GlyT2 cell number; C: percentage of c-Fos-positive cells colabeled with GlyT2. Significant difference, *P < 0.05; **P < 0.01; ***P < 0.001.