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THE EFFECT OF TRACHEAL OCCLUSION ON RESPIRATORY LOAD COMPENSATION: CHANGES IN NEURONS CONTAINING INHIBITORY NEUROTRANSMITTER IN THE NUCLEUS OF THE SOLITARY TRACT IN CONSCIOUS RATS

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Abstract

Respiratory load compensation Volume-Timing (Vt-T) relationships have been extensively studied in anesthetized animals. There are only a few studies in conscious animals although consciousness and behavior play a critical role in modulation of breathing. The aims of the study were to determine the effect of intermittent and transient tracheal occlusions (ITTO) elicited load compensation responses and the changes in activation of inhibitory glycinergic neurons in the nucleus of solitary tract (NTS) in conscious rats. The results showed that ITTO elicited an increase in expiratory time (Te) but did not affect inspiratory time (Ti) and diaphragm activity (EMGdia). An increase in total breathing time (Ttot) was due exclusively to the increase in Te. In addition, glycinergic neurons were activated in the intermediate NTS (iNTS) but not in the caudal NTS (cNTS). These results suggest that the activated glycinergic neurons in the iNTS may be important for the neurogenesis of load compensation responses in conscious animals.

Introduction

Respiratory load compensation has been extensively studied in anesthetized animals. There are relatively few studies of load compensation in the conscious state although consciousness and behavior play a critical role in the modulation of breathing pattern when animals encounter breathing challenges.

Respiratory load compensation responses are characterized as Volume-Timing (Vt-T) relationships in anesthetized animals. A respiratory load is a mechanical opposition to ventilation such as airway resistance, lung compliance or thoracic mechanical impedance. Applying a single inspiratory or expiratory load decreased volume of inspired (Vi) and expired air (Ve) and resulted in a prolongation of inspiratory time (Ti) and expiratory time (Te), respectively; the timing parameters were not affected during unloaded breaths (Clark

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and von Euler, 1972; Zechman et al., 1976). This is a vagal-dependent reflex and mediated primarily by slowly adapting pulmonary stretch receptors (PSRs) in lungs and airways. PSRs respond to changes in lung volume or transmural pressure across the airways and synapse with second order interneurons in the nucleus of solitary tract (NTS) that project to the pontine respiratory group (PRG) and ventral respiratory group (VRG) to modify breathing pattern (Davenport et al., 1981a; Davenport et al., 1981b; Davenport and Wozniak, 1986). In addition to the Vt-T relationships, respiratory load compensation is also characterized by an increase of diaphragm activity and an recruitment of abdominal muscle motor output (Koehler and Bishop, 1979; Lopata et al., 1983). In anesthetized animals, the load compensation responses are principally mediated by the neurons in the brainstem respiratory neural network (Pate and Davenport, 2012; Tsai and Davenport, 2014).

In conscious animals, respiratory load compensation involves cortical (primary sensorimotor cortices, supplementary motor and premotor cortex) and subcortical neural structures (thalamus, globus pallidus, caudate, cerebellum and limbic system) to voluntarily modify breathing pattern (Davenport and Vovk, 2009). It has been demonstrated that there is a gating system in the thalamus for the perception of respiratory load sensation in humans (Chan and Davenport, 2008). In addition, neurons in the thalamus were activated by tracheal occlusions in rats which may be related to the gating process of respiratory load sensation (Pate and Davenport, 2013). A gated signal will be processed and integrated by the limbic system (affective dimension) and somatosensory cortex (discriminative dimension) (von Leupoldt and Dahme, 2005, 2007). The affective dimension is responsible for the processing of the emotional component of the respiratory inputs (Davenport and Vovk, 2009). The discriminative dimension is related to the determination of the spatial, temporal and intensity perception of the respiratory inputs (von Leupoldt and Dahme, 2005; von Leupoldt et al., 2008). The motor cortex receives the integrated sensory information and sends projections to the brainstem or directly to the motor neurons in the spinal cord to allow voluntarily modification of the reflexive pattern of load compensation (Davenport and Vovk, 2009).

Conscious animals respond to respiratory load differently from anesthetized animals. In conscious dogs, single tracheal occlusion at the end of expiration prolonged T_i and decreased breathing frequency (Phillipson, 1974). Conscious newborn lambs responded to a single expiratory load by dilating the larynx and prolonging T_e , followed by a return to baseline during the post-load breath (Watts et al., 1997). Application of two consecutive external inspiratory loads to conscious goats caused prolonged T_i and augmented respiratory output during the two loaded breaths (Hutt et al., 1991). In awake ponies, sustained external inspiratory loads for 4 minutes resulted in an increase in T_i , decrease in T_e and augmentation of diaphragm activity during the first loaded breath and only had minimal changes after first loaded breath (Forster et al., 1994). In conscious humans, application of resistive loads for an entire breath resulted in a prolongation of T_i , T_e and total breathing time (T_{tot}) (Calabrese et al., 1998). The conflicting results between these studies may be due to different experimental designs, including differences in load strengths, load durations, load applications, species and age. Previous studies used external respiratory loads to elicit load compensation responses and applied the load to a single phase of respiration (inspiration or expiration) or a single entire breath. To our knowledge, respiratory load compensation

elicited by transient and intrinsic tracheal occlusions in conscious animals has not been studied. In our laboratory, we developed a surgical strategy to produce intrinsic, transient tracheal occlusions (ITTO) without changing lung compliance. The first purpose of the present study was to determine the ITTO-elicited load compensation responses in conscious animals.

Additionally, respiratory drive in anesthetized animal is dominated by the reciprocal interconnections within the brainstem neural network. In the model proposed by Rybak (Rybak et al., 2007; Rybak et al., 2008), the brainstem respiratory neural network consists of synaptic connections between the pons, ventral medulla and nucleus of solitary tract. Inhibitory reciprocal interconnections between the post-I, aug-E in the BöTC and pre-I, early-I in the pre-BöTC are proposed to generate the respiratory rhythm. The pons has excitatory projections to the respiratory neurons in the dorsal and ventral medulla to modulate the rhythm and pattern of breathing. The NTS is a site of integrating and processing respiratory peripheral afferents from lungs and airways for further modification of breathing pattern to generate appropriate ventilation for the body's demand. PSRs in the lungs and airways are the primary receptors sensing the changes in lung volume or transmural pressure and synapse on the pump cells in the NTS and project to other respiratory neurons in the medulla and pons for the reflex control of breathing pattern (Davenport et al., 1981a). In addition, It has been reported that application of excitatory amino acids on the medial subnucleus of solitary tract (SolM) containing pump cells caused reflex termination of inspiration and prolongation of expiration, while blockade of excitatory amino acid in this area reduced these changes (Bonham et al., 1993; Bonham and McCrimmon, 1990).

It has been demonstrated that glycinergic neurons in the NTS were activated by tracheal occlusions in anesthetized animals (Tsai and Davenport, 2014). Therefore, the inhibitory glycinergic neurons in the NTS are critically important for the neurogenesis of load compensation responses. In conscious animals, the breathing pattern is principally modulated by cortical and subcortical structures implying that consciousness and behavior are important for the neurogenesis of conscious load compensation responses. Therefore, the second purpose of this study was to determine the distribution and activation of inhibitory glycinergic neurons in the NTS responding to ITTO in conscious animals. We hypothesized that glycinergic neurons in the NTS would be activated by ITTO in conscious animals. We used immunofluorescence double staining of c-Fos and glycine transporter 2 (GlyT2) to test our hypothesis.

Materials and Methods

Animals

Experiments were performed on 11 male Sprague-Dawley rats (320–380g). The animals were housed in the University of Florida animal care facility. They were exposed to a 12 hr light/12 hr 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

The Sprague-Dawley rats were randomly divided into experimental (n=5) and sham (n=6) groups. All animals were anesthetized using inhaled isoflurane gas (2–5% in O₂) and received a tracheal cuff and bipolar electrodes in diaphragm. Adequate anesthetic depth was verified by the absence of a withdrawal reflex to a rear paw pinch. Buprenorphine (0.04–0.05 mg/kg body weight) and carprofen (5mg/kg body weight) were administered preoperatively via subcutaneous injection. The eyes were coated with petroleum ointment to prevent drying. Incision sites were shaved and sterilized with povidine-iodine topical antiseptic solution. Body temperature was monitored with a rectal probe and maintained at 37–39°C using a heating pad.

The trachea was exposed through a ventral incision. The trachea was separated from surrounding tissue. An inflatable cuff (Fine Science Tools; Foster City, CA, USA) was sutured around the trachea, two cartilage rings caudal to the larynx. The cuff was connected to a saline-filled syringe via a rubber tube. An exploratory laparotomy was performed and bipolar electrodes were implanted into the diaphragm. The rubber tube from the cuff and electrodes were routed subcutaneously to the dorsal neck surface and externalized through an incision between the scapulae, and were fixed in the skin using closing sutures. The ventral neck incision and abdominal incision were closed using an interrupter suture pattern.

Following surgery, rats were administered normal saline (0.01–0.02 ml/g body weight). Postoperative analgesia was provided with buprenorphine (0.01–0.05 mg/kg body weight) and carprofen (5 mg/kg body weight) every 24 h for at least three days. Animals were allowed a full week recovery before training protocol began.

Experimental Protocol

One week after the surgery, the rats were placed in a restrainer for 3 hours per day for two days for habituation. On post-operative day 10, experimental group rats were placed in a restrainer for 90 min, followed by 10 minutes of ITTO then 90 min post-occlusion eupneic breathing in the restrainer; sham group rats were placed in a restrainer for 3 hours without receiving ITTO. ITTO was initiated in the beginning of inspiration and prolonged for 2–3 seconds. During the experiment, diaphragm EMG (EMG_{dia}) were recorded by bipolar electrodes implanted in diaphragm and the signal was fed into an amplifier (P511, Grass Instruments; Quincy, MA) and band-pass filtered (30–3000 Hz). The analog output was digitized at 5 kHz, processed and stored for analysis. The trachea cuff was connected to a pressure transducer to measure tracheal occluder pressure. A pressure of about 800 mmHg could completely close the lumen of trachea without damaging it. The analog outputs were amplified (Stoelting; Wood Dale, IL), digitized at 5 kHz (Cambridge Electronics Designs 1401 computer interface; Cambridge, UK), computer processed (Spike2, Cambridge Electronics Design; Cambridge, UK) and stored for analysis.

Following the time in the restrainer, the animals were euthanized and perfused. Brains were harvested and fixed in 4% paraformaldehyde for 24 h and then transferred into a solution of 30% sucrose in PBS. The fixed tissue was coronally sectioned into 40 µm thick with a cryostat (HM101, Carl Zeiss) for immunofluorescence analysis.

Immunofluorescence Double-Staining Protocol

Every fourth section of tissue was used for immunofluorescence double-staining for c-Fos and GlyT2 in each brain. Brain sections were blocked in 5% normal donkey serum in PBS-Triton X-100 (PBS-T) for 1h, and then incubated in a mixed solution of guinea pig anti-GlyT2 (1:1000 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 hrs. After incubation with primary antibodies, PBS-T was used to wash brain sections. After three times of wash, brain tissue was 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 hrs. Slices of tissue were washed with PBS-T three times and then mounted on glass slides. Sides were air-dried and cover-slipped with anti-fading medium (DAKO; Carpinteria, CA). 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—Breathing pattern was analyzed by Spike 2 software (Cambridge Electronics Design; Cambridge, UK). Raw diaphragm EMG signals were rectified and integrated using a time constant of 50 ms (Figure 1). Ti, Te, Ttot and integrated EMGdia ($\int$ EMGdia) amplitude for each breath were measured from the $\int$ EMGdia traces. The onset to the peak of $\int$ EMGdia activity was defined as Ti (Figure 1). The peak of $\int$ EMGdia activity to the following onset of $\int$ EMGdia activity was defined as Te (Figure 1). Ttot was the sum of Ti and Te. The $\int$ EMGdia amplitude was measured from baseline to peak. In experimental group animals, these respiratory parameters were analyzed for the control breaths (C: C3, C2, C1) prior to occlusions, the first three occluded breaths (O: O1, O2, O3) and the following three recovery breaths (R: R1, R2, R3). These respiratory parameters were measured at matched time periods in sham group animals. Ti, Te, Ttot and $\int$ EMGdia amplitude of occluded and recovery breath were averaged and then normalized by using mean values of the control breaths for each rat in experimental group.

Immunofluorescence double-staining—A standard fluorescence microscope with appropriate filters was used to visualize and image positive fluorescence staining in brain tissue slides. c-Fos is a sensitive biomarker for neuronal activation (Dragunow and Faull, 1989). GlyT2 is a specific marker of glycinergic neurons (Poyatos et al., 1997). c-Fos in the nucleus (red) and GlyT2 (green) in the cytoplasm of the same neuron was determined as a positive immunofluorescence double-staining of c-Fos and GlyT2. Brain regions were defined by the Rat Stereotaxic Atlas (Paxinos and Watson, 1998).

Forty brain slices were collected from intermediate NTS (iNTS: bregma, -12.5 to -13.3 mm) and caudal NTS (cNTS: bregma, -13.3 to -14.1 mm), twenty slices for each region. In each brain, three or four levels of each region (iNTS or cNTS) were used for the immunofluorescence double-staining of c-Fos and GlyT2. An individual blinded to the groups manually counted immunoreactive cells in each region bilaterally. There were no

significant differences in the numbers of positive c-Fos neurons and co-labeled c-Fos and GlyT2 neurons on either side of these regions so the cell counts were averaged at each level.

An estimate of the total cell counts in iNTS or cNTS was generated by multiplying the sum of cell counts from the three or four slices of each region to 20/3 or 20/4 (depending on the number of levels used for double-staining). The percentage of c-Fos positive cells co-labeled with GlyT2 was generated from dividing the cell counts of co-labeled c-Fos and GlyT2 by the positive c-Fos cell count in each region of each animal.

Statistical analysis

All respiratory parameters are represented as mean $\pm$ SE. One-way repeated measures analysis of variance (RMAVOVA) with breath as a factor (control, occluded and recovery breaths) was used for the analysis of normalized Ti, Te, Ttot and $\int$ EMGdia amplitude. Post-hoc paired t-tests were used for further analysis. The differences of immunoreactive cell numbers in each region between experimental and sham groups were analyzed by one-way ANOVA. 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

Figure 1 illustrates the breathing pattern in conscious animals. There were no significant differences in Ti (Figure 2A) and $\int$ EMGdia amplitude (Figure 2D) between O, C and R phases. There were significant differences in Te (Figure 2B; $p < 0.001$) and Ttot (Figure 2C; $p < 0.001$) between three phases. Te was significantly prolonged during O3 but not O1 and O2 breaths when compared to C and R phases ($p < 0.05$) (Figure 2B). Prolongation of Te contributed to the significant increase of Ttot during O2 and O3 when compared to C and R phases ($p < 0.05$) (Figure 2C). There were no significant differences in Ti, Te, Ttot and $\int$ EMGdia at matched time points in the sham animals.

Immunofluorescence Double-Staining

In the cNTS, there were no significant differences in the number of c-Fos positive cells, co-labeled c-Fos and GlyT2 cells and the percentage of c-Fos positive cells co-labeled with GlyT2 between experimental and sham groups (Figure 3, Figure 4).

In the iNTS (Figure 5), there were no significant differences in the number of c-Fos positive cells and co-labeled c-Fos and Gly T2 cells between these two groups (Figure 6A, Figure 6B). The percentage of c-Fos positive cells co-labeled with GlyT2 showed significant differences between two groups ($p < 0.01$) (Figure 6C). The percentage of c-Fos positive cells co-labeled with GlyT2 was significantly greater in the experimental group than the sham group ($p < 0.01$).

Discussion

Breathing Pattern

The load compensation responses in conscious animals were found to be different from those in anesthetized animals. In anesthetized animals, external and transient tracheal occlusions resulted in prolongation of T_i , T_e and T_{tot} which is reflex load compensation mediated by the brainstem (Tsai and Davenport, 2014). However, conscious animals behaviorally prolonged T_e in response to ITTO without changing T_i . Compared to the prolongation of T_i , the prolongation of T_e had more contribution to the increase of T_{tot} . During occlusions, conscious rats did not respond to ITTO in the beginning of the occlusions (O1 and O2), but behaviorally increased their expiration only by O3 and then returned to normal breathing immediately after the removal of occlusions demonstrating that the load compensation response in conscious animals might be affected by consciousness and behavior. These results are different from previous studies (Calabrese et al., 1998; Forster et al., 1994; Hutt et al., 1991; Phillipson, 1974; Watts et al., 1997) that have only used single breath loads during one phase of breathing (inspiration or expiration) or one entire breath. However, in the present study, consecutive sustained respiratory loads to both inspiration and expiration were presented in conscious animals for up to 2–3 sec that could cause an aversive sensation. If an aversive sensation was elicited with the ITTO then the conscious animals may hold their breaths during late phase of ITTO rather than try to breathe against the loads, i.e. behavior load compensation. In addition, ITTO was used to elicit load compensation responses in this study whereas previous studies used external respiratory loads to generate load compensation (Calabrese et al., 1998; Forster et al., 1994; Hutt et al., 1991; Phillipson, 1974; Watts et al., 1997). In fact, ITTO is a more appropriate method to simulate patients with respiratory obstructive diseases compared to external respiratory load since it reduces airflow intrinsically without changing lung compliance.

Immunofluorescence Double-Staining

ITTO elicited the activation of glycinergic neurons in the iNTS but not in the cNTS in conscious animals. This is in contrast with the results in anesthetized animals with external and transient tracheal occlusions in which glycinergic neurons were activated in both rostral and caudal parts of NTS (Tsai and Davenport, 2014). These results demonstrated that NTS plays a reduced role for the neurogenesis of load compensation in conscious animal compared to anesthetized animals. Furthermore, ITTO-activated glycinergic neurons were concentrated in the medial subnucleus of solitary tract (SolM), ventral subnucleus of solitary tract (SolV) and ventrolateral subnucleus of solitary tract (SolVL). Glycinergic neurons activated in the SolM and SolV is consistent with the previous studies that ETTO activated glycinergic neurons in the same subdivisions of NTS in anesthetized animals (Tsai and Davenport, 2014). The activation of glycinergic neurons in the SolVL was only seen in conscious animals but not in anesthetized animals. ISolM is related to respiratory reflex, including termination of inspiration and prolongation of expiration (Bonham et al., 1993; Bonham and McCrimmon, 1990). However, the SolVL is not critical for production of these respiratory reflexes since lesion of this area still leaves these reflexes intact (McCrimmon et al., 1987). In addition, previous studies have shown that neurons in the SolM mainly project to the ventrolateral medulla, parafacial region and rVRG and only few SolM neurons send

projections to the BötC and pre-BötC (Alheid et al., 2011). The projections from SolVL to the ventral respiratory column are more broadly distributed compared to other subdivisions of NTS (Alheid et al., 2011). It has been previously reported (Pate and Davenport, 2013) that expression of cFOS in the NTS is specific to load compensation and not the result of surgical preparation, animal handling or occluder cuff activation without airway occlusion. Therefore, the activated glycinergic neurons in the SolM in both conscious and anesthetized animals may be the primary neural area responsible for the generation of load compensation responses. Additionally, the different distribution of projections from the SolM and SolVL might mediate difference mechanisms to modulate breathing pattern.

In the present study, activated glycinergic neurons in the SolM and SolV may be vagally mediated p-cells. P-cells are the second order neurons in the NTS activated by PSRs in the lungs and airways and send projection to the ventral respiratory column and pons for the modulation of breathing pattern. It has been demonstrated that two-thirds of p-cells in the NTS use GABA and glycine as neurotransmitters (Ezure and Tanaka, 2004). Although we saw the elevated expression of glycinergic neurons in the SolM and SolV in conscious animals as we found in anesthetized animals, the patterns of load compensation responses are different between conscious and anesthetized states suggesting that other mechanisms play important roles in the behavioral load compensation. SolVL is a candidate brain region for the neurogenesis of load compensation in conscious animals since glycinergic neurons in the SolVL are only activated in conscious animals. As noted previously, the SolVL is not necessary for reflex load compensation. Several studies have demonstrated that the SolVL receives projections from cortical and subcortical structures, such as subcortical cortex (van der Kooy et al., 1984) and the central nucleus of amygdala (Danielsen et al., 1989). The activated glycinergic neurons in the SolVL may be activated by these higher cortical and subcortical structures for behaviorally control of breathing pattern.

To our knowledge, this is the first study to investigate the importance of glycinergic neurons in the NTS for the neurogenesis of load compensation responses with sustained respiratory loads in conscious animals. Based on these results, cortical and subcortical processing of respiratory load sensation overrides brainstem reflexes for conscious modification of breathing pattern.

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Highlights

1. ITTO elicited respiratory load compensation responses in conscious rats.
2. ITTO prolonged T_e and T_{tot} but not T_e and EMGdia.
3. ITTO activated glycinergic neurons in the iNTS but not in the cNTS in conscious rats.

Figure 1. ITTO behavior modulation of breathing pattern in conscious animals. Physiological results during control (C3-C1), occluded (O1-O3) and recovery (R1-R3) breaths. Determination of T_i and T_e are shown in the trace of $\int EMG_{dia}$. Shadow area represents the period of tracheal occlusions.

Figure 2.

Effect of ITTO in conscious animals. Normalized breath timing values during control (C3, C2, C1), occluded (O1, O2, O3) and recovery (R1, R2, R3). The relationship between (A) T_i , (B) T_e , (C) T_{tot} and (D) $\int EMG_{dia}$ amplitude and breath number. (B) The relationship between T_e and breath number. (C) The relationship between T_{tot} and breath number. (D) The * indicates a significant difference, $p < 0.05$.

Figure 3.

The effect of ITTO on the activation of inhibitory glycinergic neurons in the cNTS in conscious animals. Immunofluorescence double staining of c-Fos (red) and GlyT2 (green) in the cNTS (Bregma -13.8 mm) in sham (B-G) and experimental groups (H-M). The dashed box area in part A represents the area of rat brain atlas corresponding to the region in B-D and H-J. E-G and K-M represent the dashed area in B-D and H-J, respectively. Arrows represent immunoactive cells. B-D and H-J: scale bars, 100 μ m; E-G and K-M: scale bars, 50 μ m. 10: dorsal motor nucleus of vagus; 12: hypoglossal nucleus; Amb: ambiguus

nucleus; AP: area postrema; Cu: cuneate nucleus; cu: cuneate fasciculus; CVL: caudoventrolateral reticular nucleus; CVRG: caudoventral respiratory group; ECu: external cuneate nucleus; gr: gracile fasciculus; Py: pyramidal tract; RVL: rostroventrolateral reticular nucleus; sol: solitary tract; SolC: nucleus of solitary tract, commissural; SolG: nucleus of solitary tract, gelatinous; SolI: nucleus of solitary tract, interstitial; SolDM: nucleus of solitary tract, dorsomedial; SolIM: nucleus of solitary, intermediate; SolM, nucleus of solitary tract, medial; SolVL: nucleus of solitary tract, ventrolateral; IOD: inferior olive, dorsal nucleus; RVRG: rostral ventral respiratory group; TS: solitary tract.

Figure 4. Immunofluorescence double-staining of c-Fos and GlyT2 in the cNTS in conscious animals. (A) c-Fos labeled cell number (B) co-labeled c-Fos and GlyT2 cell number (C) the percentage of c-Fos positive cells co-labeled with GlyT2.

Figure 5.

ITTO activated inhibitory glycinergic neurons in the iNTS in conscious animals. Immunofluorescence double-staining of c-Fos (red) and GlyT2 (green) in the iNTS (Bregma -13.2 mm) in sham (B–G) and experimental groups (H–M). The dashed line in part A represents the area of rat brain atlas corresponding to the region in B–D and H–J. E–G and K–M represent the dashed area in B–D and H–J, respectively. Arrows represent immunoactive cells. B–D and H–J: scale bars, 100 μm; E–G and K–M: scale bars, 50 μm

4V: fourth ventricle; C2: C2 adrenaline cells; chp: choroid plexus; PSol: parasolitary nucleus; SolCe: nucleus of solitary tract, central.

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Figure 6. Immunofluorescence double-staining of c-Fos and GlyT2 in the iNTS in conscious animals. (A) c-Fos labeled cell number (B) co-labeled c-Fos and GlyT2 cell number (C) the percentage of c-Fos positive cells co-labeled with GlyT2. The ** indicates a significant difference, $p < 0.01$.