

# Neuronal Activity of the Medulla Oblongata Revealed by Manganese-Enhanced Magnetic Resonance Imaging in a Rat Model of Gastroesophageal Reflux-Related Cough

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## Summary

We investigated neuronal activity of the medulla oblongata during gastroesophageal reflux-related cough (GERC). A rat model of GERC was generated by perfusing HCl into lower esophagus and inducing cough with citric acid. The HCl group rat was received HCl perfusion without citric acid-induced cough. The saline control rat was perfused with saline instead and cough was induced. Citric acid-induced cough rat was only induced by citric acid. Blank group rats were fed normally. Fos expressions were observed in medulla oblongata nuclei using immunohistochemistry. Manganese-enhanced magnetic resonance imaging (MEMRI) was performed to detect the Mn<sup>2+</sup> signal following intraperitoneal injection of MnCl<sub>2</sub>. HCl perfusion and citric acid-induced cough caused Fos expressions in the nucleus of solitary tract (nTS), dorsal motor nucleus of the vagus (DMV), paratrigeminal nucleus (Pa5), and intermediate reticular nucleus (IRt), which was higher than HCl group, saline control group, citric acid-induced cough group, and blank group. A high Mn<sup>2+</sup> signal was also observed in most of these nuclei in model rats, compared with blank group animals. The Mn<sup>2+</sup> signal was also higher in the HCl, saline and citric acid-induced cough group animals, compared with blank group animals. The study showed medulla oblongata neurons were excited in a HCl perfusion and citric acid-induced cough rat model, and nTS, DMV,

Pa5 and IRt neurons maybe involved in the cough process and signal integrate.

## Key words

Gastroesophageal reflux-related cough • Manganese-enhanced magnetic resonance imaging• c-fos • Nucleus of solitary tract • Dorsal motor nucleus of the vagus

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## Introduction

Chronic cough is the most common symptom of respiratory outpatients, while gastroesophageal reflux (GER) is one of the most common causes of chronic cough (Irwin *et al.* 1993, Harding and Richter 1997, Lai *et al.* 2013). Neurons in the medulla oblongata, such as those in the nucleus of the solitary tract (nTS), may

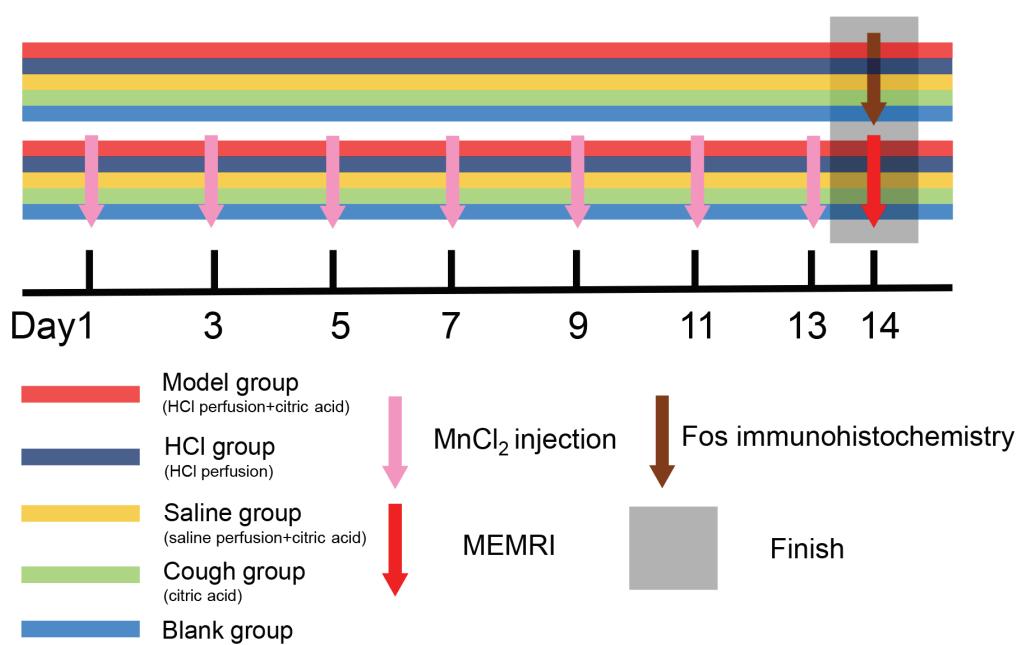
control cough. However, whether other neurons are activated during gastroesophageal reflux-related cough (GERC) is unclear. Magnetic resonance imaging (MRI) is a new technique and is widely used in neuroscience research. Manganese-enhanced MRI (MEMRI), also called activity-induced manganese-dependent MRI (AIM-MRI), has been employed to study different phenomena in various species.  $Mn^{2+}$  may enter the neurons through calcium ( $Ca^{2+}$ ) channels due to similarities between  $Mn^{2+}$  and  $Ca^{2+}$ . More neuronal excitement results in more  $Mn^{2+}$  entry and accumulation, which can be detected using MRI *via* differences in signal intensity (Aoki *et al.* 2002, Takeda 2003, Silva *et al.* 2004).  $Mn^{2+}$  accumulation in medulla oblongata nuclei may reflect neuronal excitation and thus implicate neurons that participate in the process of GERC.

Previous studies have proved that intrasophageal HCl perfusion could cause airway hyperresponsiveness, airway inflammation and cough in animals (Hamamoto *et al.* 1997, Kohrogi *et al.* 2001, Cheng *et al.* 2014). In this study, a GERC rat model was generated by acid perfusion into the lower esophagus and by inducing cough with citric acid. Neuronal activity was observed *via*  $Mn^{2+}$  accumulation detected using MRI. We also examined the expression of Fos, a protein marker of neuronal activity in the central nervous system (CNS), and compared the localization of Fos versus  $Mn^{2+}$ .

## Methods

### Animals and GERC model generation

Male Sprague-Dawley rats ( $n=60$ ; body weight 300–350 g; obtained from the Experimental Animal Center of Jiangsu Province) were divided into five groups: model (HCl perfusion + citric acid-induced cough) group, HCl (HCl perfusion) group, saline (saline perfusion + citric acid-induced cough) group, cough (only citric acid-induced cough) group, and blank group (each group,  $n=12$ ). All animal experimental protocols were approved by Southeast University (permission number 2014062002) and performed in accordance with the guidelines of ‘Animal Care and Use’ laid down by The Animal Research Committee of Southeast University. According to our previous method (Liu *et al.* 2013), the model group rats that received acid perfusion were anesthetized with ketamine hydrochloride (50 mg/kg, intraperitoneally [i.p.]). Then, 0.1 mol/l HCl (including 0.5 % pepsin) was perfused into the lower esophagus (8 drops/min, 20 min/session) *via* a stomach tube once a day for 14 consecutive days. Rats in the HCl group were only perfused with HCl, and without citric acid treatment. Rats in the saline group were perfused with saline. Cough in the model, saline, and cough groups was induced by citric acid treatment (0.8 mol/l) for 5 min once a day for 14 consecutive days. Blank group rats were fed normally.  $MnCl_2$  (0.12 mol/l, 0.45 g/kg, i.p.) was injected into 6 random rats in each group (including the blank group) on Days 1, 3, 7, 5, 9, 11, and 13 (Fig. 1).



**Fig. 1.** The experimental procedure. Six animals of each group were for immunohistochemistry and other six animals were for MEMRI.

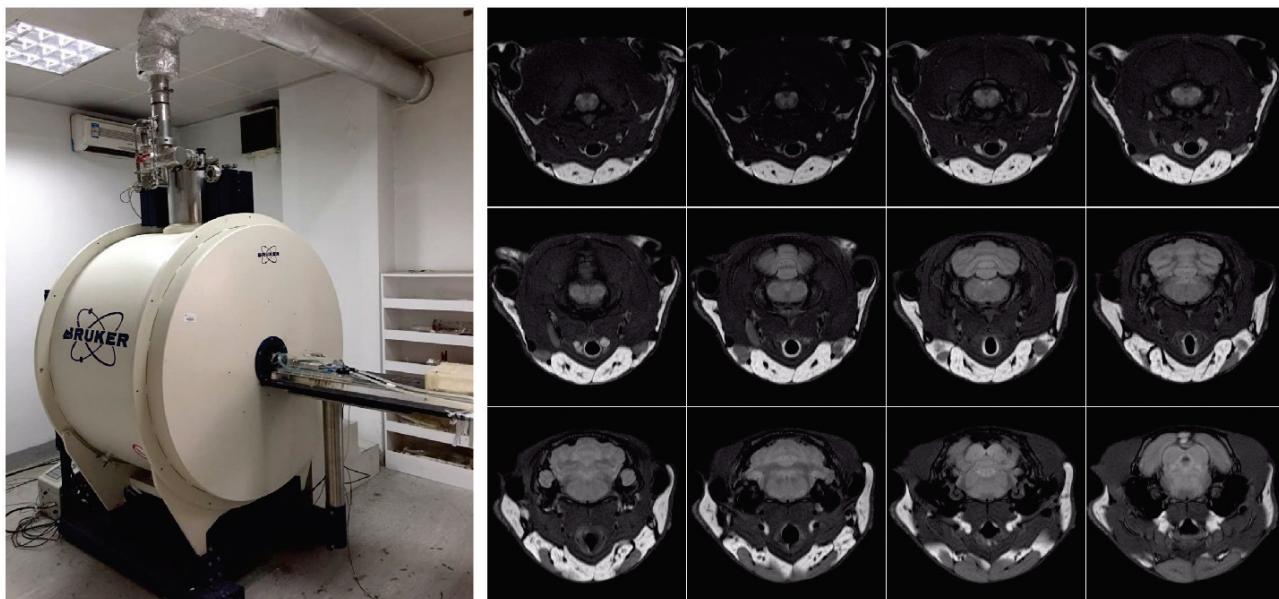
### Immunohistochemistry

In the five groups, immunohistochemistry was performed in all rats that were not injected with MnCl<sub>2</sub>. Animals were deeply anesthetized with urethane (1 g/kg, i.p.) and transcardially perfused with 0.3 % phosphate buffered saline (PBS) followed by 4 % paraformaldehyde in PBS. The brainstems were removed, placed in 4 % paraformaldehyde at 4 °C for 4 h, and then cryoprotected in 30 % sucrose at 4 °C overnight. Tissues were rapidly frozen with optimal cutting temperature compound and cut into 30-μm thick coronal sections (the total brainstem sections thickness is 2 mm from rostral and caudal to obex) using a Leica freezing microtome. Brain sections were incubated with 3 % H<sub>2</sub>O<sub>2</sub> for 15 min to block endogenous peroxidase activity, washed with 0.3 % PBS (3 × 5 min), incubated for 1 h at room temperature with a blocking solution (10 % goat serum), and subsequently incubated overnight with a primary antibody (rabbit anti-Fos; 1:500; Santa Cruz). The tissue was washed with 0.3 % PBS (3 × 5 min), followed by incubation for 1 h at room temperature with a biotinylated secondary antibody

(goat anti-rabbit; 1:300; Abcam). After washing with 0.3 % PBS (3 × 5 min), sections were incubated for 30 min with avidin/biotinylated horseradish peroxidase (HRP), then washed with 0.3 % PBS (3 × 5 min), and reacted with DAB as a chromogen. Sections were observed using an Olympus light microscope.

### MEMRI

MEMRI was performed using a Bruker 7.0T micro-MR imaging system for rat obex scanning (Fig. 2). Animals were anesthetized with 4 % isoflurane; anesthesia was maintained using 1.5 % isoflurane-oxygen/nitrogen (30:70) mixed gas while simultaneously monitoring heart rate and respiratory status. Mn<sup>2+</sup> signal intensity changes were detected using rapid acquisition with relaxation enhancement (RARE). T1W anatomical scans were acquired (individual scan time=10 min 57 s 780 ms; TR=571 ms; TE=8.09 ms, FOV 3.00 cm × 3.00 cm; matrix 384 × 384; 12 slices; 1.0 mm slice thickness; 0.078 × 0.078 mm in-plane resolution).



**Fig. 2.** Bruker 7.0T micro-MR imaging system and rat medulla oblongata obex images.

### Statistical analysis

According to rat brain in stereotaxic coordinates (Paxinos and Watson 2007), Fos-positive neurons stained by immunohistochemistry were observed in the obex nuclei of the medulla oblongata, including nTS, DMV, Pa5, and IRt. Six brain sections were randomly selected in each rat brainstem. Fos-positive neurons were counted using Image-Pro Plus. Paravision 4.0 software was used

for MEMRI to measure the regions of interest (ROI) and background noise to calculate a signal-to-noise ratio (SNR). Mn<sup>2+</sup> signal changes among the blank group and the other three groups were expressed as a pseudo-color value (pseudo-color value=pixel-value difference × 0.001).

Data were expressed as mean ± standard deviation ( $\bar{x} \pm SD$ ). The SPSS 17.0 software was used for

statistical analysis, including one-way analysis of variance (ANOVA) (comparisons in multiple groups) and paired t test (comparisons between the right and left brain areas in one group).  $p<0.05$  was considered statistically significant.

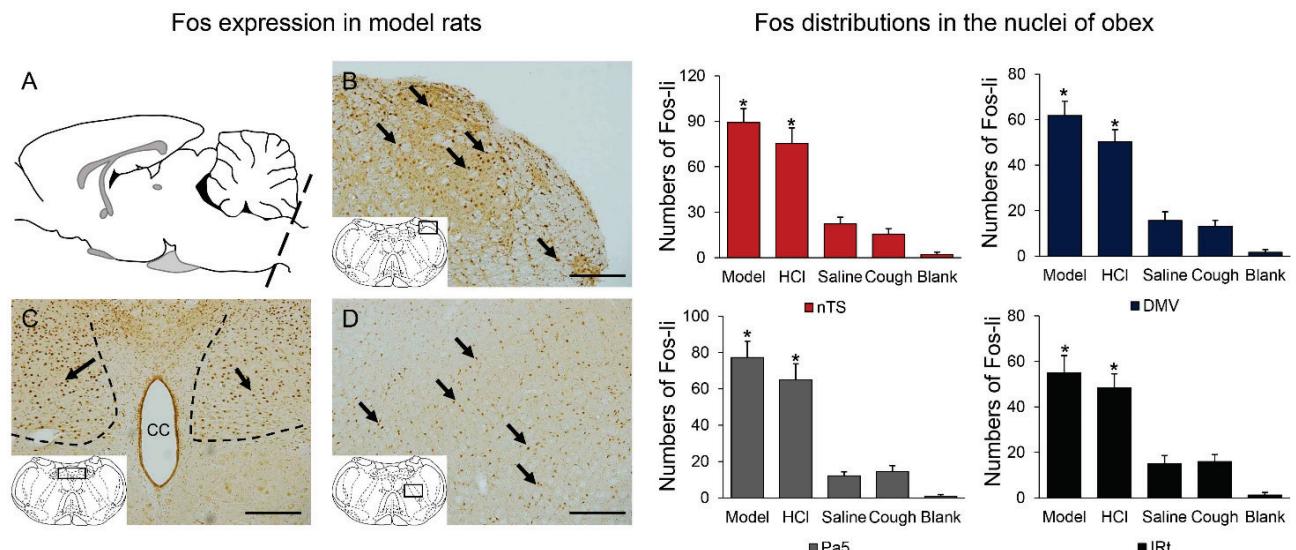
## Results

One rat that received a  $MnCl_2$  injection died during the HCl model preparation.

### Fos expression in medulla oblongata nuclei

Fos-like immunoreactivity (Fos-li) was visualized as brown granules following DAB staining. The greatest Fos-li was observed in the neuronal nuclei (Fig. 3). Fos-positive neurons were mainly distributed in

the nTS ( $89.31\pm9.04$ ), dorsal motor nucleus of the vagus (DMV;  $61.83\pm6.31$ ), paratrigeminal nucleus (Pa5;  $77.17\pm9.01$ ), and intermediate reticular nucleus (IRt;  $54.94\pm7.59$ ) of the model rats ( $p<0.05$  compared with each nucleus of the other four groups). Fos-positive neurons in HCl group rats (nTS  $75.47\pm10.17$ , DMV  $50.29\pm5.27$ , Pa5  $64.92\pm8.83$ , IRt  $48.26\pm6.22$ ) were more than the saline, cough and blank groups ( $p<0.05$ ). There were no differences in the nuclei observed in the saline and cough groups (nTS,  $22.28\pm4.44$  vs.  $15.58\pm3.55$ ; DMV,  $15.61\pm3.86$  vs.  $13.14\pm2.58$ ; Pa5,  $12.19\pm2.20$  vs.  $14.53\pm3.26$ ; and IRt,  $14.94\pm3.59$  vs.  $15.94\pm3.03$ ; all  $p>0.05$ ). Fos-li was rarely observed in the blank group rats. No differences were detected between the right and left side nuclei in the five groups ( $p>0.05$ ).



**Fig. 3.** Fos expression in the model rat and distribution in the nuclei of obex. **(A)** a frozen brainstem section (black dotted line). **(B)** Fos expression in Pa5. **(C)** Fos expression in the DVC (including nTS and DMV). **(D)** Fos expression in the IRt. The black arrow points Fos-li. Fos-li mainly locating on nTS, DMV, Pa5, and IRt, was more than other four groups. \*  $p<0.05$ . DAB staining. Scale bar=50  $\mu$ m.

### $Mn^{2+}$ signal changes in medulla oblongata nuclei

The  $Mn^{2+}$  signal was shown in the nTS, DMV, Pa5, and IRt (Fig. 4). In the model group rats, the  $Mn^{2+}$  signal in the nTS was much higher than that observed in the other four groups ( $p<0.05$ ). The  $Mn^{2+}$  signal was similar between the right and left nTS in all five groups ( $p>0.05$ ). The DMV of the model group had a higher  $Mn^{2+}$  signal than that of the other four groups ( $p<0.05$ ). However, the signal of the right DMV ( $0.58\pm0.06$ ) was higher than that of the left ( $0.23\pm0.04$ ;  $p<0.05$ ). Similar results were observed in the Pa5 of the model group compared with the other four groups ( $p<0.05$ ). However, the signal of the right Pa5

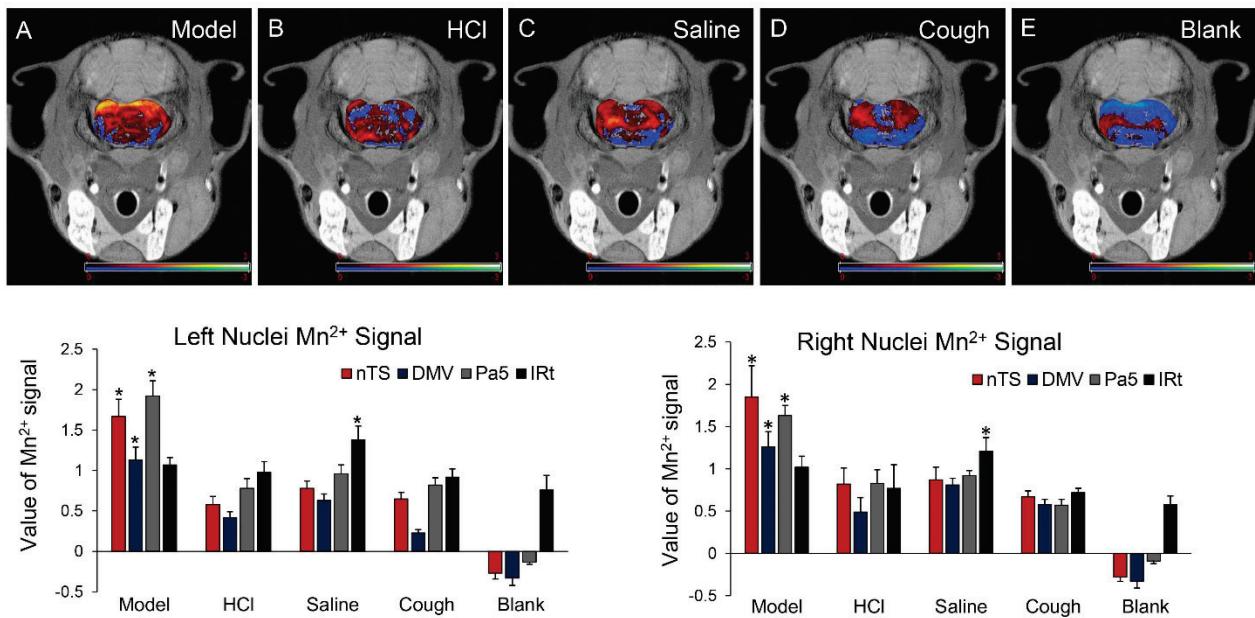
( $1.63\pm0.12$ ) was lower than that of the left ( $1.92\pm0.19$ ) ( $p<0.05$ ). The  $Mn^{2+}$  signal of both the right and left IRt nuclei in saline was higher than those in the model, HCl and cough groups (all  $p<0.05$ ).

## Discussion

Previous studies have suggested that GER-associated cough is mainly related to neurogenic inflammation of airways, micro aspiration, and esophageal-bronchi reflex (Hamamoto *et al.* 1997, Kohrogi *et al.* 2001, Kollarik and Brozmanova 2009). The traditional view is that GERC is due to aspiration of

gastric contents to the larynx and trachea, however, most patients with GERC only showed distal reflux, rather than proximal reflux, and the micro aspiration theory does not explain the mechanism of GERC (Irwin *et al.* 2000). Due to the common histological origin of the trachea and esophagus, esophageal-bronchi reflex may

contribute to GERC by inducing neurogenic inflammation of airways. Previous study has shown that unilateral vagotomy alleviated neurogenic inflammation and neuronal activities (Chen *et al.* 2017), which suggests that central nervous system may participate in the process of GERC.



**Fig. 4.** MEMRI in the rat obex and Mn<sup>2+</sup> signal changes in the nuclei. The pictures were (A) model group, (B) HCl group, (C) saline group, (D) cough group and (E) blank group, respectively. The pseudo-color value was from -3 to 3. Right and left nuclei Mn<sup>2+</sup> signal changes were shown respectively. \*  $p < 0.05$ .

*C-fos* can be induced to express Fos protein, a marker of neuronal excitation, in the cell nucleus after stimulation. We included the saline perfusion group and citric acid-induced cough groups to exclude the possibility that surgical tube insertion and liquid perfusion influenced Fos expression. The medulla oblongata is a basal center related to respiration, digestion, and cardiovascular integration. In our study, we found that Fos expression was increased in the model rats, more so than that observed following saline stimulation or cough induced. Previous studies (Gestreau *et al.* 1997, Ohi *et al.* 2005, Jakus *et al.* 2008) have confirmed the location of neurons related to cough using *c-fos*. Jakus *et al.* (2008) used Fos to locate the brainstem neurons related to cough, and revealed that a large number of the medulla oblongata, pons, and midbrain neural nuclei are involved in the regulation of coughing in cats. The central terminals of cough receptors are a critical component to cough gating, and by microinjection and dual-tracing studies, terminals, which were localized in the medial subnuclei of NTS, were

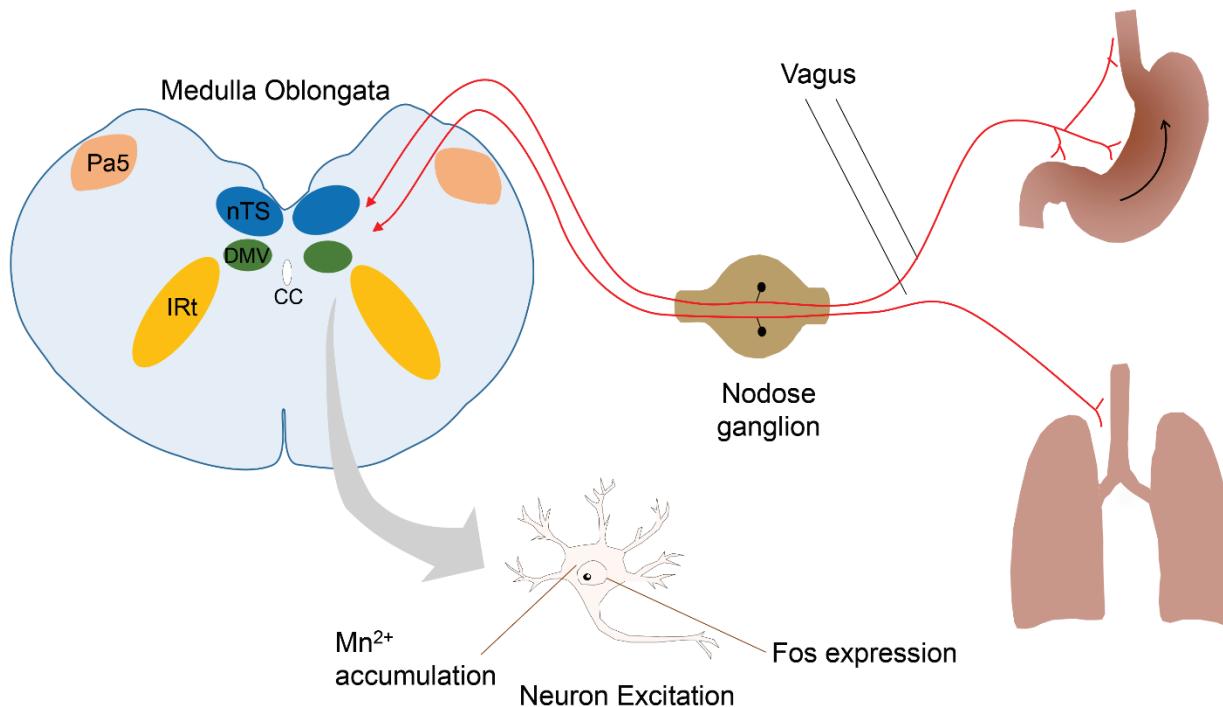
confirmed (Canning and Mori 2010). Our results indicated that acid perfusion and induced cough resulted in excitation of a greater number of neurons. As we reported previously (Chen *et al.* 2018), the active neurons in the medulla may participate in the cough and airway inflammation related to the GER.

Cough-related neurons are mainly located in the nTS, a secondary sensory center, which also regulates respiratory functions. Canning and Mori (2010) found that neurons in the cnTS (a subnucleus of the nTS), the location of central cough receptor terminals, were critical components involved in cough gating. Suwanprathes *et al.* (2003) used Fos to observe neuronal excitation in the brain after a single episode of esophageal acid stimulation.

Fos expression was also observed in the Pa5, another sensory nucleus. The Pa5 receives visceral sensation terminals from the airway and digestive tract via the vagus and glossopharyngeal nerves (Altschuler *et al.* 1989, Hayakawa *et al.* 2001, O'Neal and Zheng 2015), and is also referred to as an "extrasolitary target"

(Menetrey *et al.* 1987). McGovern *et al.* (2015) found dual projecting pathways (Sol airway-specific projections and Pa5 airway-specific projections) from the airway to the brain by virus tracing. These studies indicate that

medulla oblongata neurons receive airway and esophageal stimulation signal input, and the signal maybe input to and integrated in higher center (Fig. 5).



**Fig. 5.** Neuronal excitation following afferent signals from the periphery. Sensory nuclei of the medulla oblongata receive stimulatory input *via* the vagal nerves, thereby activating the neurons, which then express Fos protein. Mn<sup>2+</sup> also enters into the activated neurons.

The nTS has fiber communications with the DMV and area postrema (AP), and thus is called the dorsal vagal complex (DVC). The DMV directly receives vagal sensory fiber projections, and innervates the airway and digestive tract *via* efferent fibers. The DVC, together with the IRt, nucleus ambiguus, and ventrolateral medulla, form the medullary visceral zone (MVZ). The MVZ plays a key role in visceral functions. In our study, most of the aforementioned nuclei were excited, particularly following dual stimulation (i.e. HCl perfusion into the lower esophagus and inhaled citric acid).

Similar results were observed in the MEMRI study. MEMRI was used to confirm the locations of excited neurons in addition to Fos expression. Mn<sup>2+</sup> quickly enters into neurons and is released slowly, reflecting neuronal excitation over a period of time; in contrast Fos expression is time restricted. In this study, Mn<sup>2+</sup> signals were increased in the nuclei, with higher signals in most of the aforementioned nuclei of the model rats compared with those in the saline and cough groups. The Mn<sup>2+</sup> signals in the IRt were not consistent with Fos

expression. Fos is an important marker of neuronal activation within the CNS, and also Fos protein expression may be induced by various stimuli. In previous studies reported in the literatures, Fos could be induced in half to one hour after stimuli, and time of peak expression is two hours. Then, Fos expression would be decreased after two hours. The dual stimulation (HCl and citric acid) in model group would induce more Fos expressions in the nuclei than those in other groups. But Mn<sup>2+</sup> differs from Fos protein, it would be accumulated in the cell bodies. Intermediate reticular nucleus (IRt) is a medulla nucleus which is involved in cardiovascular, respiratory, and digest functions. In this study, the cough induced by citric acid (respiratory system) and perfusion stimulation (digest system) would cause more Mn<sup>2+</sup> accumulation in the IRt than that in NTS, DMV, and Pa5.

In our study, we have not investigated Fos expression or MEMRI in higher brain areas. Toxic effects are a major drawback of using Mn<sup>2+</sup> (Barbeau 1984, Crossgrove and Zheng 2004, Dobson *et al.* 2004).

Toxicity, including cardiac, renal, and liver failure, is one of the main limitations to applying this approach in humans. Indeed, MEMRI is now mainly applied in the animal study according to the existing literature, and inappropriate to use in the human clinical study because of the toxic effect of manganese. Instead, blood oxygenation level-dependent functional MRI (BOLD-fMRI) is more suitable for human study. Coughing is a complex reflex that involves the CNS and is regarded as a neuropathic disorder (Chung *et al.* 2013) that may be regulated by circuits involving higher brain areas. Blood oxygenation level-dependent functional MRI (BOLD-fMRI) was used to study cough-related mechanisms. Mazzone and colleagues (Mazzone *et al.* 2007, Mazzone *et al.* 2011, Farrell *et al.* 2012, Farrell *et al.* 2014) found that brain regions, such as the cortex and gyrus cinguli, control the urge to cough, cough suppression, and voluntary cough.

Fos expression and MEMRI showed that medulla oblongata neurons were excited in a HCl perfusion and citric acid-induced cough rat model, and nTS, DMV, Pa5 and IRt neurons may be involved in the cough process and signal integrate. Medulla oblongata neurons were activated following intra-esophageal HCl perfusion and inhaled citric acid to induce coughing. These activated neurons may participate in the cough process and cough signal input into higher brain areas. It

is also suggested that CNS neurons may be involved in postinfectious cough that responds poorly to standard treatments. For further treatment of GER-associated cough, chronic refractory cough, and even severe asthma, the CNS may serve as a therapeutic target, and blocking the CNS to alleviate airway neurogenic inflammation may provide insight for future drug development.

In conclusion, multiple medulla nuclei were excited in a rat model with HCl perfusion and citric acid-induced cough, and nTS, DMV, Pa5 and IRt neurons maybe involved in the GERC.

## Conflict of Interest

There is no conflict of interest.

## Acknowledgements

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