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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26   **Short Title:** Neuronal Activity in Medulla Oblongata Nuclei of GERC

36

37   **Summary**

38   We investigated neuronal activity of the medulla oblongata during gastroesophageal

39   reflux-related cough (GERC).

40   A rat model of GERC was generated by perfusing HCl into lower esophagus and

41   inducing cough with citric acid. The HCl group rat was received HCl perfusion

42   without citric acid-induced cough. The saline control rat was perfused with saline

43   instead and cough was induced. Citric acid-induced cough rat was only induced by

44   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^{2+} signal

following intraperitoneal injection of $MnCl_2$.

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^{2+}

signal was also observed in most of these nuclei in model rats, compared with blank

group animals. The Mn^{2+} 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.

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

67　　　control cough. However, whether other neurons are activated during gastroesophageal

68　　　reflux-related cough (GERC) is unclear. Magnetic resonance imaging (MRI) is a new

69　　　technique and is widely used in neuroscience research. Manganese-enhanced MRI

70　　　(MEMRI), also called activity-induced manganese-dependent MRI (AIM-MRI), has

71　　　been employed to study different phenomena in various species. Mn^{2+} may enter the

72　　　neurons through calcium (Ca^{2+}) channels due to similarities between Mn^{2+} and Ca^{2+}.

73　　　More neuronal excitement results in more Mn^{2+} entry and accumulation, which can be

74　　　detected using MRI via differences in signal intensity (Aoki *et al.* 2002, Takeda 2003,

75　　　Silva *et al.* 2004). Mn^{2+} accumulation in medulla oblongata nuclei may reflect

76　　　neuronal excitation and thus implicate neurons that participate in the process of

77　　　GERC.

78

79　　　　　Previous studies have proved that intra-esophageal HCl perfusion could

80　　　cause airway hyperresponsiveness, airway inflammation and cough in

81　　　animals(Hamamoto *et al.* 1997, Kohrogi *et al.* 2001, Cheng *et al.* 2014). In this study,

82　　　a GERC rat model was generated by acid perfusion into the lower esophagus and by

83　　　inducing cough with citric acid, Neuronal activity was observed via Mn^{2+}

84　　　accumulation detected using MRI. We also examined the expression of Fos, a protein

85　　　marker of neuronal activity in the central nervous system (CNS), and compared the

86　　　localization of Fos versus Mn^{2+}.

87

88　　　**Methods**

Animals and GERC model generation

Male Sprague-Dawley rats (n = 60; body weight 300–350g; 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 (Figure 1).

Immunohistochemistry

In the five groups, immunohistochemistry was performed in all rats that

111   were not injected with MnCl$_2$. Animals were deeply anesthetized with urethane (1g/kg,

112   i.p.) and transcardially perfused with 0.3% phosphate buffered saline (PBS) followed

113   by 4% paraformaldehyde in PBS. The brainstems were removed, placed in 4%

114   paraformaldehyde at 4°C for 4 h, and then cryoprotected in 30% sucrose at 4°C

115   overnight. Tissues were rapidly frozen with optimal cutting temperature compound

116   and cut into 30-μm thick coronal sections (the total brainstem sections thickness is

117   2mm from rostral and caudal to obex) using a Leica freezing microtome. Brain

118   sections were incubated with 3% H$_2$O$_2$ for 15 min to block endogenous peroxidase

119   activity, washed with 0.3% PBS (3 × 5 min), incubated for 1 h at room temperature

120   with a blocking solution (10% goat serum), and subsequently incubated overnight

121   with a primary antibody (rabbit anti-Fos; 1:500; Santa Cruz). The tissue was washed

122   with 0.3% PBS (3 × 5 min), followed by incubation for 1 h at room temperature with

123   a biotinylated secondary antibody (goat anti-rabbit; 1:300; Abcam). After washing

124   with 0.3% PBS (3 × 5 min), sections were incubated for 30 min with

125   avidin/biotinylated horseradish peroxidase (HRP), then washed with 0.3% PBS (3 × 5

126   min), and reacted with DAB as a chromogen. Sections were observed using an

127   Olympus light microscope.

128

129   **MEMRI**

130   MEMRI was performed using a Bruker 7.0T micro-MR imaging system for

131   rat obex scanning (Figure 2). Animals were anesthetized with 4% isoflurane;

132   anesthesia was maintained using 1.5% isoflurane-oxygen/nitrogen (30:70) mixed gas

133 while simultaneously monitoring heart rate and respiratory status. Mn^{2+} signal

134 intensity changes were detected using rapid acquisition with relaxation enhancement

135 (RARE). T1W anatomical scans were acquired (individual scan time = 10 min 57 s

136 780 ms; TR = 571 ms; TE = 8.09 ms, FOV 3.00 cm × 3.00 cm; matrix 384 × 384; 12

137 slices; 1.0 mm slice thickness; 0.078 × 0.078 mm in-plane resolution).

138

Statistical analysis

140 According to rat brain in stereotaxic coordinates (Paxinos and Watson),

141 Fos-positive neurons stained by immunohistochemistry were observed in the obex

142 nuclei of the medulla oblongata, including nTS, DMV, Pa5, and IRt. Six brain

143 sections were randomly selected in each rat brainstem. Fos-positive neurons were

144 counted using Image-Pro Plus. Paravision 4.0 software was used for MEMRI to

145 measure the regions of interest (ROI) and background noise to calculate a

146 signal-to-noise ratio (SNR). Mn^{2+} signal changes among the blank group and the other

147 three groups were expressed as a pseudo-color value (pseudo-color value =

148 pixel-value difference × 0.001).

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

150 software was used for statistical analysis, including one-way analysis of variance

151 (ANOVA) (comparisons in multiple groups) and paired t test (comparisons between

152 the right and left brain areas in one group). $P < 0.05$ was considered statistically

153 significant.

154

155   **Results**

156   One rat that received a MnCl$_2$ injection died during the HCl model

157   preparation.

158

159   **Fos expression in medulla oblongata nuclei**

160   Fos-like immunoreactivity (Fos-li) was visualized as brown granules

161   following DAB staining. The greatest Fos-li was observed in the neuronal nuclei

162   (Figure 3). Fos-positive neurons were mainly distributed in the nTS (89.31 ± 9.04),

163   dorsal motor nucleus of the vagus (DMV; 61.83 ± 6.31), paratrigeminal nucleus (Pa5;

164   77.17 ± 9.01), and intermediate reticular nucleus (IRt; 54.94 ± 7.59) of the model rats

165   ($p < 0.05$ compared with each nucleus of the other four groups). Fos-positive neurons

166   in HCl group rats (nTS 75.47 ± 10.17, DMV 50.29 ± 5.27, Pa5 64.92 ± 8.83, IRt

167   48.26 ± 6.22) were more than the saline, cough and blank groups ($p < 0.05$). There

168   were no differences in the nuclei observed in the saline and cough groups (nTS, 22.28

169   ± 4.44 *versus* 15.58 ± 3.55; DMV, 15.61 ± 3.86 *versus* 13.14 ± 2.58; Pa5, 12.19 ±

170   2.20 *versus* 14.53 ± 3.26; and IRt, 14.94 ± 3.59 *versus* 15.94 ± 3.03; all $p > 0.05$).

171   Fos-li was rarely observed in the blank group rats. No differences were detected

172   between the right and left side nuclei in the five groups ($p > 0.05$).

173

174   **Mn^{2+} signal changes in medulla oblongata nuclei**

175   The Mn^{2+} signal was shown in the nTS, DMV, Pa5, and IRt (Figure 4). In

176   the model group rats, the Mn^{2+} signal in the nTS was much higher than that observed

177 in the other four groups ($p < 0.05$). The Mn^{2+} signal was similar between the right and

178 left nTS in all five groups ($p > 0.05$). The DMV of the model group had a higher Mn^{2+}

179 signal than that of the other four groups ($p < 0.05$). However, the signal of the right

180 DMV (0.58 ± 0.06) was higher than that of the left (0.23 ± 0.04; $p < 0.05$). Similar

181 results were observed in the Pa5 of the model group compared with the other four

182 groups ($p < 0.05$). However, the signal of the right Pa5 (1.63 ± 0.12) was lower than

183 that of the left (1.92 ± 0.19) ($p < 0.05$). The Mn^{2+} signal of both the right and left IRt

184 nuclei in saline was higher than those in the model, HCl and cough groups (all $p <$

185 0.05).

186

187 **Discussion**

188   Previous studies have suggested that GER-associated cough is mainly

189 related to neurogenic inflammation of airways, micro aspiration, and

190 esophageal-bronchi reflex(Hamamoto *et al.* 1997, Kohrogi *et al.* 2001, Kollarik and

191 Brozmanova 2009). The traditional view is that GERC is due to aspiration of gastric

192 contents to the larynx and trachea, however, most patients with GERC only showed

193 distal reflux, rather than proximal reflux, and the micro aspiration theory does not

194 explain the mechanism of GERC(Irwin *et al.* 2000). Due to the common histological

195 origin of the trachea and esophagus, esophageal-bronchi reflex may contribute to

196 GERC by inducing neurogenic inflammation of airways. Previous study has shown

197 that unilateral vagotomy alleviated neurogenic inflammation and neuronal

198 activities(Chen *et al.* 2017), which suggests that central nervous system may

199   participate in the process of GERC.

200   *C-fos* can be induced to express Fos protein, a marker of neuronal excitation,

201   in the cell nucleus after stimulation. We included the saline perfusion group and citric

202   acid-induced cough groups to exclude the possibility that surgical tube insertion and

203   liquid perfusion influenced Fos expression. The medulla oblongata is a basal center

204   related to respiration, digestion, and cardiovascular integration. In our study, we found

205   that Fos expression was increased in the model rats, more so than that observed

206   following saline stimulation or cough induced. Previous studies(Gestreau *et al.* 1997,

207   Ohi *et al.* 2005, Jakus *et al.* 2008) have confirmed the location of neurons related to

208   cough using *c-fos*. Jakus(Jakus *et al.* 2008) used Fos to locate the brainstem neurons

209   related to cough, and revealed that a large number of the medulla oblongata, pons, and

210   midbrain neural nuclei are involved in the regulation of coughing in cats. The central

211   terminals of cough receptors are a critical component to cough gating, and by

212   microinjection and dual-tracing studies, terminals which were localized in the medial

213   subnuclei of NTS were confirmed(Canning and Mori 2010).Our results indicated that

214   acid perfusion and induced cough resulted in excitation of a greater number of

215   neurons. As we reported previously(Chen *et al.* 2018), the active neurons in the

216   medulla may participate in the cough and airway inflammation related to the GER.

217   Cough-related neurons are mainly located in the nTS, a secondary sensory

218   center, which also regulates respiratory functions. Canning et al. (Canning and Mori

219   2010) found that neurons in the cnTS (a subnucleus of the nTS), the location of

220   central cough receptor terminals, were critical components involved in cough gating.

221   Suwanprathes et al. (Suwanprathes *et al.* 2003) used Fos to observe neuronal

222   excitation in the brain after a single episode of esophageal acid stimulation.

223   Fos expression was also observed in the Pa5, another sensory nucleus. The

224   Pa5 receives visceral sensation terminals from the airway and digestive tract via the

225   vagus and glossopharyngeal nerves (Altschuler *et al.* 1989, Hayakawa *et al.* 2001,

226   O'Neal and Zheng 2015), and is also referred to as an "extrasolitarial target"

227   (Menetrey *et al.* 1987). Mazzone et al. (McGovern *et al.* 2015) found dual projecting

228   pathways (Sol airway-specific projections and Pa5 airway-specific projections) from

229   the airway to the brain by virus tracing. These studies indicate that medulla oblongata

230   neurons receive airway and esophageal stimulation signal input, and the signal maybe

231   input to and integrated in higher center (Figure 5).

232   The nTS has fiber communications with the DMV and area postrema (AP),

233   and thus is called the dorsal vagal complex (DVC). The DMV directly receives vagal

234   sensory fiber projections, and innervates the airway and digestive tract via efferent

235   fibers. The DVC, together with the IRt, nucleus ambiguus, and ventrolateral medulla,

236   form the medullary visceral zone (MVZ). The MVZ plays a key role in visceral

237   functions. In our study, most of the aforementioned nuclei were excited, particularly

238   following dual stimulation (i.e., HCl perfusion into the lower esophagus and inhaled

239   citric acid).

240   Similar results were observed in the MEMRI study. MEMRI was used to

241   confirm the locations of excited neurons in addition to Fos expression. Mn^{2+} quickly

242   enters into neurons and is released slowly, reflecting neuronal excitation over a period

243   of time; in contrast Fos expression is time restricted. In this study, Mn^{2+} signals were

244   increased in the nuclei, with higher signals in most of the aforementioned nuclei of

245   the model rats compared with those in the saline and cough groups. The Mn^{2+} signals

246   in the IRt were not consistent with Fos expression. Fos is an important marker of

247   neuronal activation within the CNS, and also Fos protein expression may be induced

248   by various stimuli. In previous studies reported in the literatures, Fos could be

249   induced in half to one hour after stimuli, and time of peak expression is two hours.

250   Then, Fos expression would be decreased after two hours. The dual stimulation (HCl

251   and citric acid) in model group would induce more Fos expressions in the nuclei than

252   those in other groups. But Mn2+ differs from Fos protein, it would be accumulated in

253   the cell bodies. Intermediate reticular nucleus (IRt) is a medulla nucleus which is

254   involved in cardiovascular, respiratory, and digest functions. In this study, the cough

255   induced by citric acid (respiratory system) and perfusion stimulation (digest system)

256   would cause more Mn2+ accumulation in the IRt than that in NTS, DMV, and Pa5.

257   In our study, we have not investigated Fos expression or MEMRI in higher

258   brain areas. Toxic effects are a major drawback of using Mn^{2+} (Barbeau 1984,

259   Crossgrove and Zheng 2004, Dobson *et al.* 2004). Toxicity, including cardiac, renal,

260   and liver failure, is one of the main limitations to applying this approach in humans.

261   Indeed, MEMRI is now mainly applied in the animal study according to the existing

262   literature, and inappropriate to use in the human clinical study because of the toxic

263   effect of manganese. Instead, blood oxygenation level-dependent functional MRI

264   (BOLD-fMRI) is more suitable for human study. Coughing is a complex reflex that

265   involves the CNS and is regarded as a neuropathic disorder (Chung *et al.* 2013) that

266   may be regulated by circuits involving higher brain areas. Blood oxygenation

267   level-dependent functional MRI (BOLD-fMRI) was used to study cough-related

268   mechanisms. Mazzone and colleagues (Mazzone *et al.* 2007, Mazzone *et al.* 2011,

269   Farrell *et al.* 2012, Farrell *et al.* 2014) found that brain regions, such as the cortex and

270   gyrus cinguli, control the urge to cough, cough suppression, and voluntary cough.

271   Fos expression and MEMRI showed that medulla oblongata neurons were

272   excited in a HCl perfusion and citric acid-induced cough rat model, and nTS, DMV,

273   Pa5 and IRt neurons may be involved in the cough process and signal integrate.

274   Medulla oblongata neurons were activated following intra-esophageal HCl perfusion

275   and inhaled citric acid to induce coughing. These activated neurons may participate in

276   the cough process and cough signal input into higher brain areas. It is also suggested

277   that CNS neurons may be involved in postinfectious cough that responds poorly to

278   standard treatments. For further treatment of GER-associated cough, chronic

279   refractory cough, and even severe asthma, the CNS may serve as a therapeutic target,

280   and blocking the CNS to alleviate airway neurogenic inflammation may provide

281   insight for future drug development.

282   In conclusion, multiple medulla nuclei were excited in a rat model with HCl

283   perfusion and citric acid-induced cough, and nTS, DMV, Pa5 and IRt neurons maybe

284   involved in the GERC.

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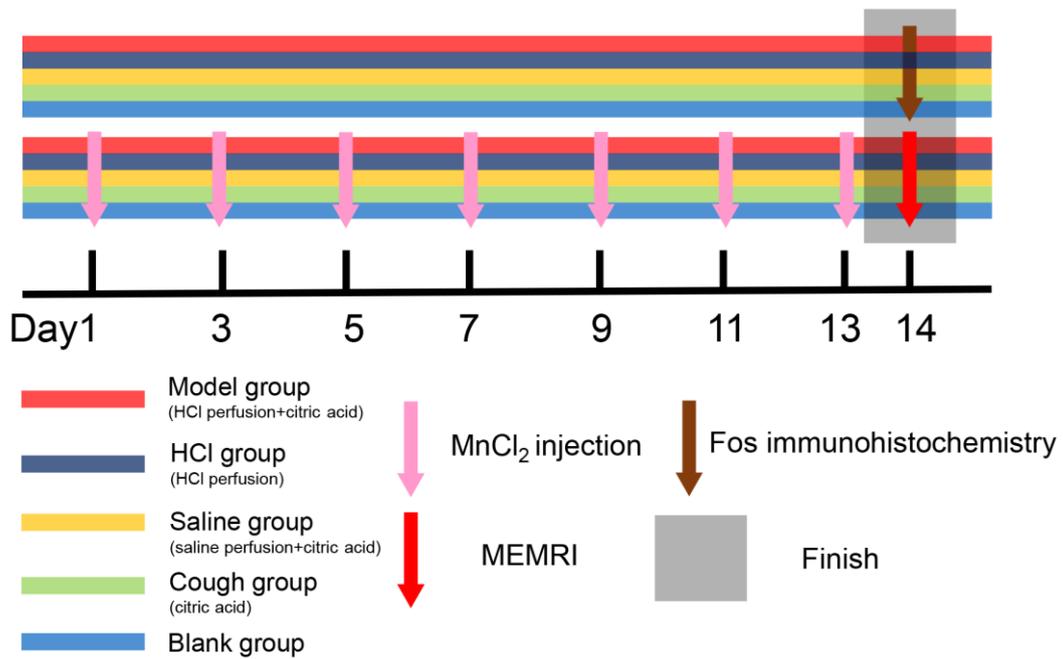
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Figure legends

Figure 1. The experimental procedure.

Six animals of each group were for immunohistochemistry and other six animals were
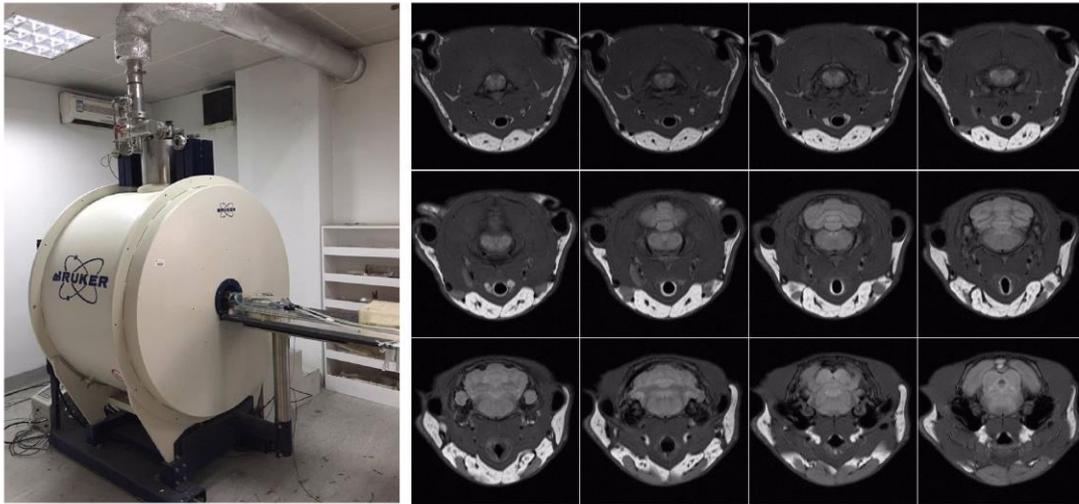
for MEMRI.

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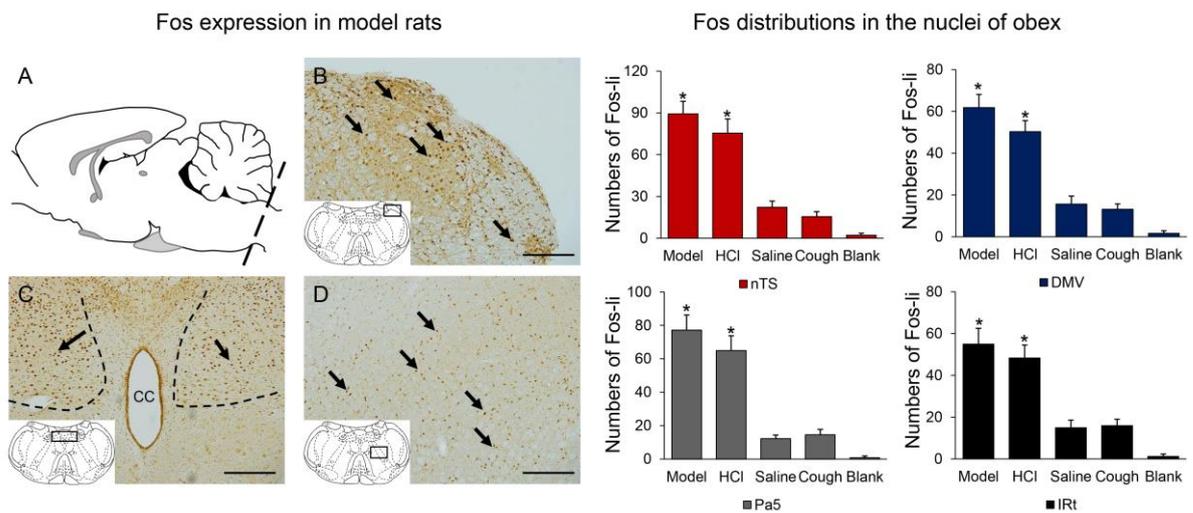
393 **obex images.**

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396 **Figure 3. Fos expression in the model rat and distribution in the nuclei of obex.**

397 A. a frozen brainstem section (black dotted line). B. Fos expression in Pa5. C. Fos

398 expression in the DVC (including nTS and DMV). D. Fos expression in the IRt. The

399 black arrow points Fos-li.

400 Fos-li mainly locating on nTS, DMV, Pa5, and IRt, was more than other four groups.

401 * $P < 0.05$. DAB staining. scale bar $= 50\mu m$
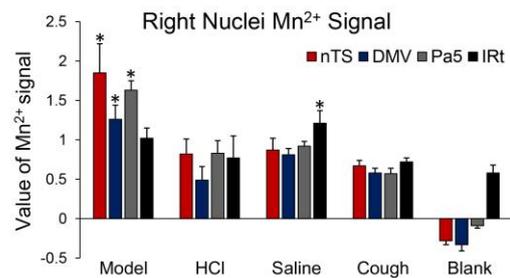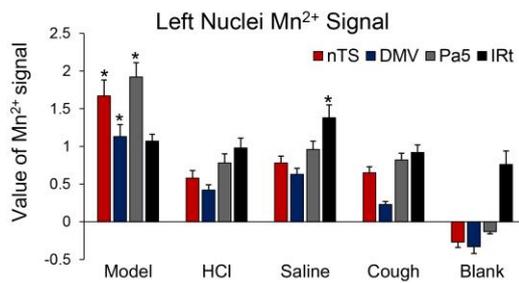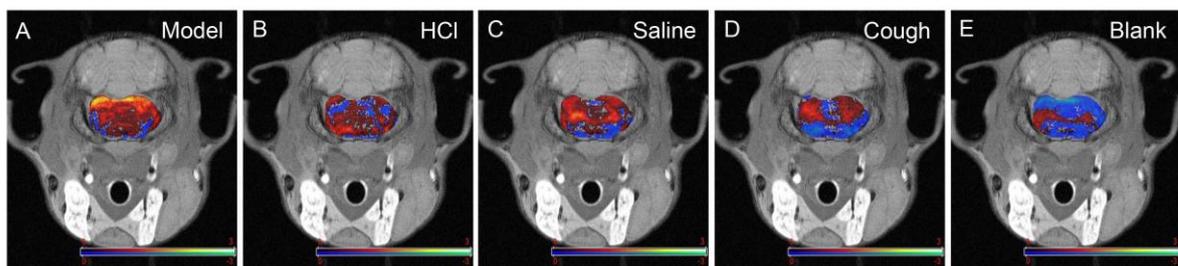

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Figure 4. MEMRI in the rat obex and Mn^{2+} signal changes in the nuclei.

The pictures A-E were model group, HCl group, saline group, cough group and blank

group, respectively. The pseudo-color value was from -3 to 3. Right and left nuclei

Mn^{2+} signal changes were shown respectively. * $P < 0.05$.

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Figure 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^{2+} also enters into

the activated neurons.

Medulla Oblongata

Pa5

nTS

DMV

CC

IRt

Vagus

Nodose
ganglion

Mn^{2+}
accumulation

Neuron Excitation

Fos expression

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