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# Effects of 5-HT<sub>1A</sub> of Nucleus of Solitary Tract in the Regulation of Swallowing Activities evoked by Electroacupuncture at DU16 and RN23 acupoints

Ping Li <sup>1, a</sup>, Hui You <sup>2, b</sup>

<sup>1</sup>College of Economics and Management, Yibin University, Yibin 644000, China;

<sup>2</sup>Chinese Medicine Hospital Affiliated to Southwest Medical University, Luzhou 646000, and China

<sup>a</sup>1461284559@qq.com, <sup>b</sup>123248708@qq.com

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## Abstract

To understand the neuropharmacological mechanism of 5-HT<sub>1A</sub> in nucleus of solitary tract (NTS) during swallowing activities elicited by electroacupuncture(EA), we examined the 5-HT<sub>1A</sub> positive expression in nucleus of Solitary Tract of anesthetic rats after 20 minutes' EA at Fengfu(DU16) and Lianquan(RN23) acupoints. Meanwhile, swallows evoked by EA DU16 and RN23 acupoints in 60s before and after injecting 5-HT<sub>1A</sub> receptor antagonist WAY-100635 into the NTS were documented by recording electromyographic (EMG) activity of the mylohyoid muscle and by visualizing laryngeal elevation. The results showed that EA at DU16 and RN23 points could evoke the swallowing response while increased the 5-HT<sub>1A</sub> positive expression in the NTS of anesthetic rats, and the discharge of mylohyoid decreased after injecting 10 nmol WAY-100635 into the NTS. We therefore conclude that 5-HT<sub>1A</sub> in the NTS played a role involved in EA regulating swallowing function.

## Keywords

Nucleus of solitary tract; Electro-acupuncture; Swallowing; 5-HT<sub>1A</sub>.

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## 1. Introduction

Acupuncture has been treated as an effective complementary and alternative therapy in clinic and widely applied to treat various kinds of diseases such as stroke, facial paralysis and lumbago. Large amount of clinical practices have demonstrated that acupuncture at DU16 and RN23 acupoints have obvious effects on swallowing disorders especially swallowing dysfunctions after stroke. However, the inner mechanism of acupuncture regulating deglutition disorders has not been clarified yet.

Swallowing activity, which is divided into oral, pharyngeal and esophageal stages, is a complex motor sequence involving the tongue, pharynx, larynx, and esophagus[1]. In most species, the onset of oropharyngeal stage of swallowing starts with the contraction of the mylohyoid muscle, which is considered to be the first muscle to become active in swallowing. It has been clearly established that the sequential and rhythmic patterns of swallowing are formed and organized by a central pattern generator(CPG) which located in the medulla oblongata[2]. The pioneer study has amply confirmed that the dorsal medulla within the NTS which contained a great variety of neurotransmitters including acetylcholine, biogenic amines, neuropeptides and aminos acids, receives the information of afferent of cranial nerve fibers from the swallowing tract and of higher centers and forms part of the neuronal network that generated swallowing[3]. Meanwhile, NTS plays a vital role in the neural substrate of swallowing, and its reflex initiation can be caused by stimulation of peripheral regions such as glossopharyngeal(IX) nerve and the branch of the vagus nerve—superior laryngeal nerve(SLN)[4]. In addition, NTS is the significant bulbar center of acupuncture modulate various visceral functions[5-7], and EA exerts excitatory effect on NTS neurons on normal rats[8].

A number of experiments for the study of 5-HT as neurotransmitters in swallowing disorders were proposed. NTS contains a high level of 5-hydroxytryptamine (5-HT) and the existence of 5-HT in nerve terminals and fibers has been particularly well demonstrated[9-11]. The effects of serotonergic agents were surveyed in the rat with microinjection of 5-HT, on reflex swallowing elicited by long repetitive stimulation of SLN. These researches [12-14] have shown that 5-HT and its various mimetics exert a powerful excitatory effect on both reflex and automatic swallowing by micropneumophoretic ejection of 5-HT into NTS. However, other studies[9, 15] corroborated an inhibitory role of 5-HT in swallowing by the same way and indicated that this inhibitory effect was dose-related. Manaker S[16] confirmed that high concentration of 5-HT<sub>1A</sub> receptors in the central and intermediate subnuclei of NTS where IX and X nerve afferents from the pharynx, larynx, esophagus converged, suggested a role for these receptors in medullary reflex pathways subserving deglutition. Thus, in order to verify the serotonergic receptor-mediation involved in reflex swallowing, a 5-HT<sub>1A</sub> receptor antagonist was chosen.

In present study, the effects of 5-HT<sub>1A</sub> in the NTS on regulating pharyngeal stage of swallowing activities evoked by EA at DU16 and RN23 acupoints was investigated.

## 2. Material and methods

Adult Sprague-Dawley rats (weighing between 200 to 350g) of both sexes were employed, throughout this experiment. Animals were maintained under controlled light cycle(12/12h) and temperature ( $25 \pm 1$  °C), with free access to food and water. All procedures in the present experiment were performed in accordance with the guidelines of Guangdong Provincial Hospital of Chinese Medicine for the Care and Use of Research Animals. This experiment consisted of two parts.

### 2.1 Experiment One

30 animals were randomly divided into three groups: the EA group, the sham operated group and the blank group. There were ten rats in each group. After anesthetized by an intraperitoneal injection of 10% Chloral hydrate (0.35ml/100g), the rats of sham operated group and EA group were both given neck surgery operation and bipolar stainless needle electrodes insulated except at the tip were inserted into the mylohyoid muscle. Then the EA group was performed EA stimulation at DU16 and RN23 acupoints with parameters of a frequency of 5Hz and an intensity of 1 mA to elicit swallowing activities. Two stainless acupuncture needles were subcutaneously inserted into the DU16 and RN23 acupoints 8mm and 12mm separately, and left for 20 min. The laryngeal elevation was also visualized during the EA process while the electromyographic (EMG) activity of rats' mylohyoid muscle was recorded by means of needle electrodes. The sham operated group was given no electroacupuncture. The rats of blank group were given neither surgery nor EA. Then, the rats of three group were transcardially perfused with 200 ml of 0.9% physiological saline followed by 400ml of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB). The brainstem was removed and left in the same fixative for 6-8 h after the perfusion had been completed and then cryoprotected overnight in 20% sucrose in 0.1 M PB for 24h at 4 °C. Forty  $\mu$ m frozen tranverse sections were obtained at -20 °C by a freezing microtome (Thermo, Germany) and immunostained for 5-HT<sub>1A</sub> expression.

The medulla sections were incubated free floating with a rabbit monoclonal antibody (Millipore), diluted 1:100 and 1:1000 respectively in PB containing 0.3% Triton X-100 plus 5% of normal goat serum. Incubation with the primary antibody was conducted overnight at room temperature. After three washes for 5 min in PB (pH 7.4), the sections were incubated with biotinylated goat anti-rabbit sera (Santa Cruz, CA) at a dilution of 1:100 for 1 h at room temperature. Following the washing step, the sections were incubated with the avidin-biotin-peroxidase complex (Santa Cruz, CA) for 1 h. Finally, the sections were developed in 0.05% diaminobenzidine (DAB) 0.01% solution of hydrogen peroxide in PB and intensification with 0.05M Tris-HCl buffer (pH 7.6) for 3-5 min. The sections were mounted on gelatin-and chromoalumen-coated slides, dehydrated, cleared and coverslipped with neutral gum. As a control, primary antisera were replaced by normal rabbit serum, and there was no immunoreactivity.

Every fourth section within the NTS region from 2.0mm caudal to obex to 1.0mm rostral to obex was selected from each brainstem. Nuclear boundaries were identified according to stereotaxic rat brain atlas[17].The number of 5-HT1A positive cells in the NTS was counted under a light microscope and digital images were collected. A quantitative analysis of the immunolabeled material was performed with NIH Image J. Data were presented as the mean±standard error of the mean (SEM). Statistical analyses of data were generated using GraphPad Prism, version 4.02 (GraphPad Software Inc., San Diego, CA, USA). The data were analyzed using the Student's t-test and one-way ANOVA followed by Bonferroni's test. In all cases,  $P < 0.05$  was considered statistically significant.

## 2.2 Experiment Two

40 animals were randomly divided into four groups: the antagonist 1  $\mu$ l group, antagonist 0.1 $\mu$ l group, physiological saline 1  $\mu$ l group and physiological saline 0.1  $\mu$ l group. There were ten rats in each group.

The anaesthetized rats were fixed on a stereotaxic frame (MK-8003,China).Part of the occipital bone was removed to expose the dorsal surface of the medulla. Under the guidance of a stereotaxic apparatus, the multi-barreled micropipette (tip diameter 20-30  $\mu$ m) was inserted into the NTS(0.5mm rostral to the calamus scriptorius, 0.5 mm lateral to the middling, and 0.8 mm below the dorsal surface of the medulla). The micropipettes were filled with 5-HT1A receptor antagonist WAY-100635 (Sigma) or physiological saline. WAY-100635 was dissolved in ultrapure water and adjusted to 10 mM. After the discharges of mylohyoid of each group elicited by EA DU16 and RN23 acupoints in 60s were recorded, above drugs were administered into the NTS in a volume of 1  $\mu$ l or 0.1  $\mu$ l which delivered approximately 100s and 10s, respectively. Then the DU16 and RN23 acupoints were given EA 60s again in order to trigger swallows, laryngeal elevation was visualized and the discharges of mylohyoid were recorded simultaneously. In the end, the injection points were marked with 0.5 M Pontamine Sky Blue.

## 3. Results

### 3.1 Effects of EA at DU16 and RN23 acupoints on swallowing activities

EA at DU16 and RN23 acupoints could induce the swallowing activities of anaesthetized rats. The laryngeal elevation and the EMG activity of rats' mylohyoid muscle were observed during the EA process (Fig. 1).

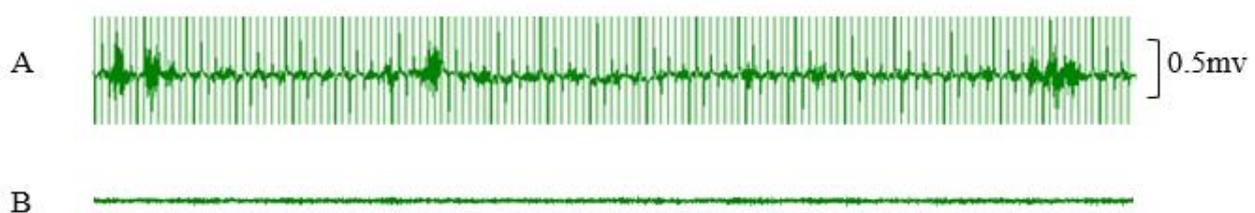


Fig. 1. Discharges of the mylohyoid muscle. (A) EA at DU16 and RN23 group; (B) sham operated group.

### 3.2 Effects of EA at DU16 and RN23 acupoints on 5-HT1A expression in the NTS

5-HT1A labeled mainly roundish and oval nuclei of the neurons, and the intensity of the reaction products ranged from light to brown. EA stimulation induced markedly the expression of 5-HT1A in the NTS especially in the central, medial and intermediate subnucleus of NTS. And the number of 5-HT1AR neurons of EA group in the NTS was significantly higher than that in other two group ( $31.00 \pm 10.69$  versus  $23.60 \pm 9.12$  and  $6.70 \pm 7.32$ ,  $P < 0.05$ , ANOVA) (Fig. 2)

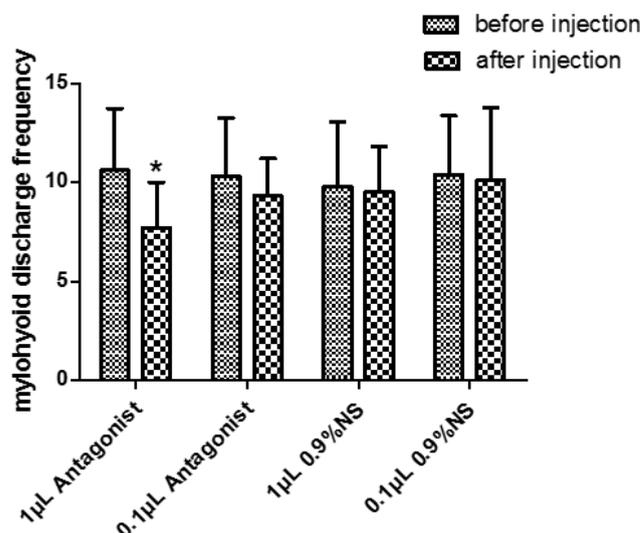
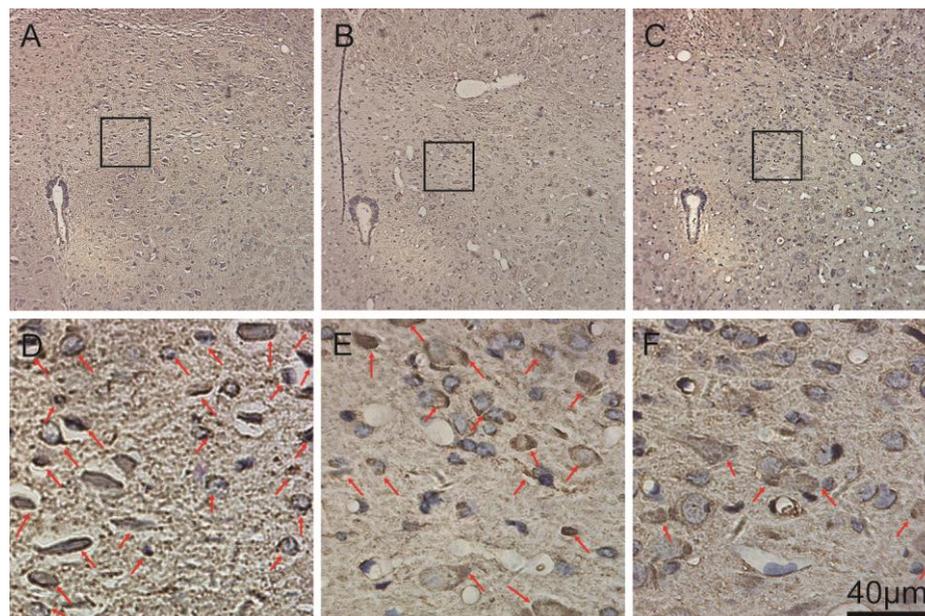


Fig.2 5-HT1A positive expression numbers in the NTS at the level of the obex in each group. (A) and (D) EA at DU16 and RN23 group; (B) and (E) sham operated group; (C) and (F) blank group. \*P<0.05 versus the sham operated group and blank group.

### 3.3 Swallowing effect of 5-HT1A in the NTS.

Microinjections of WAY-100635, an antagonist of 5-HT1A and physiological saline were performed into the NTS in doses of 1 0.1 µl and 1 µl, respectively. The injection of 1 µl (10nmol ) WAY-100635 induced a marked decrease of the number of swallows elicited by EA at DU16 and RN23 by examination of the mylohyoid EMG discharge (P < 0.05) (Figs. 3). This inhibitory effect was observed after the end of the injection. However, there was no change in the number of discharges of mylohyoid with the injection of 1 µl control injection of physiological solution (P>0.05), and no change of swallowing with the EMG discharge could be obtained after microinjections of WAY-100635 (1 nmol) and physiological saline performed 0.1 µl at the same site (P > 0.05) (Figs. 4). The discharge differences of mylohyoid muscle before and after injection of 1 µl antagonist group showed significant difference as compared with 1 µl physiological group, 0.1 µl antagonist group and 0.1µl physiological group (P < 0.05) (Figs. 5).

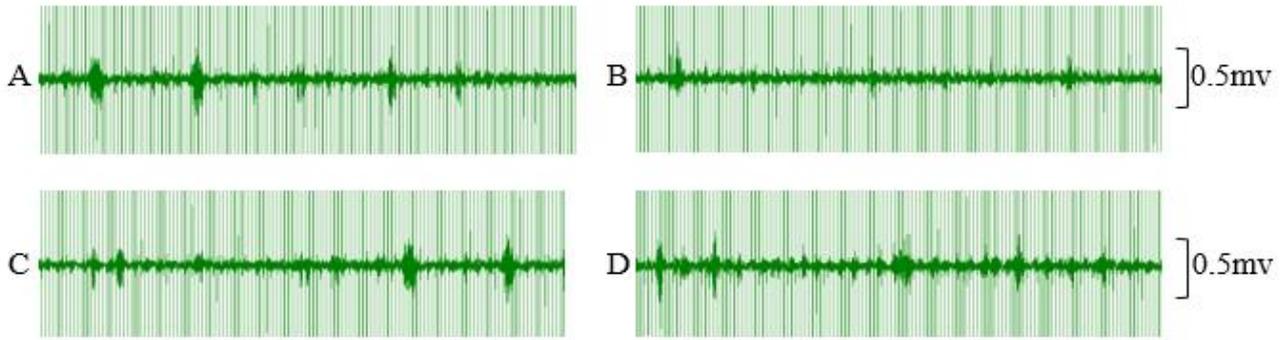


Fig.3 Discharges of the mylohyoid muscle. (A) 1 µl antagonist group before microinjection; (B) 1 µl antagonist group after microinjection; (C) 1 µl NS group before microinjection; (D) 1 µl NS group after microinjection.

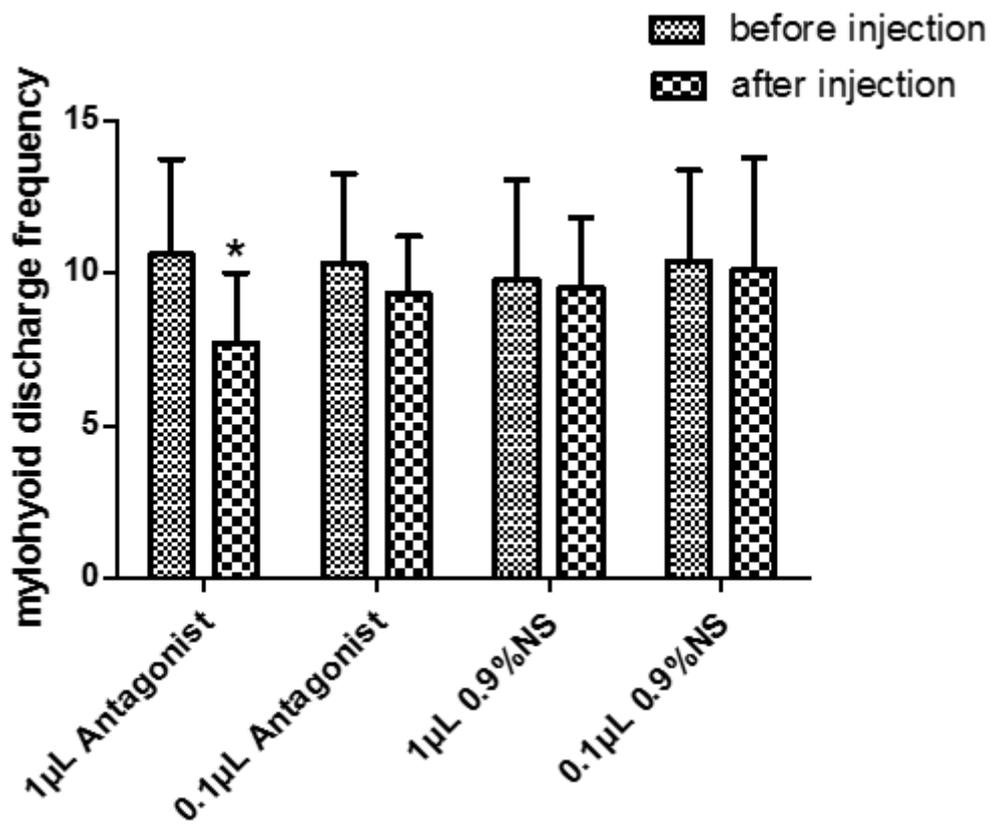


Fig. 4 Changes of the frequency of mylohyoid muscle discharge. \*P<0.05 versus before injection.

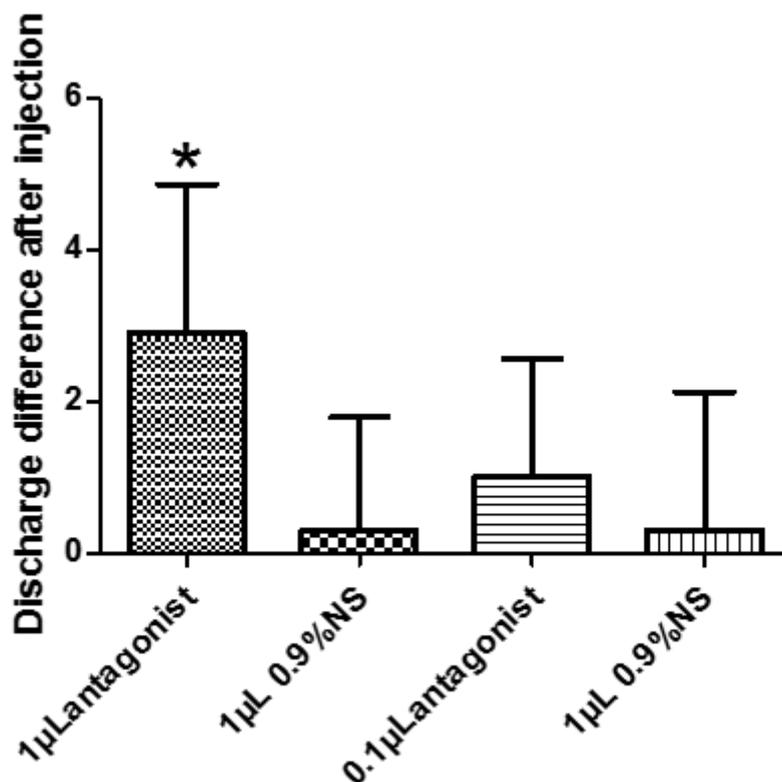


Fig. 5 Discharge difference of mylohyoid muscle before and after injection. \* $P < 0.05$  versus 0.1µl antagonist group, 1µl NS group and 0.1µl NS group.



Fig. 6. The injection points. CC refers to central canal. The bilateral arrows point to the NTS, and the right arrow represents injection position.

#### 4. Discussion

As is known to all, there are many issues in deglutition disorders benefited from animal model research such as surface electrical stimulation of the skin overlying the submental musculature in dysphagia and peripheral electrical stimulation of the superior laryngeal nerve (SLN) is widely used to induce pharyngeal swallow in the past animal experiments [1, 18-20]. In the present study, EA at DU16 and RN23 acupoints could also evoke swallows with laryngeal elevation and discharge of the mylohyoid muscle. As a vast amount of clinical results have demonstrated the effectiveness of acupuncture on deglutition disorders caused by stroke or other diseases [21-24], this experiment had shown the effect of EA at DU16 and RN23 acupoints on eliciting swallowing activities in terms of electrophysiological method.

DU16 acupoint is positioned in the Du Meridian (Governor vessel) and often used to relieve apoplexy and agitation disorders [25]. Clinical application regularity had shown that DU16 acupoint is mainly used for local problems in the human body [26]. As located at the depression between the occiput and

the atlas, on the dorsal midline, DU16 acupoint is close to the medulla of swallowing center. RN23 acupoint is positioned in the Ren Meridian (Conception vessel) and frequently used alone or combined other acupoints to cure different swallowing dysfunction[27-29]. Moreover, our previous experiments confirmed that RN23 and DU16 had given more effects of activating the swallowing neurons of NTS than PC6 and ST36 acupoints and of eliciting swallowing than PC6, suggesting that the choice of acupuncture points to regulate swallowing function is significant. Therefore, we suggested that EA at DU16 and RN23 acupoints played a role in facilitating swallowing function. However, the optimal acupuncture acupoints, EA stimulation frequency, amplitude are unknown and needed to be studied further.

Previous studies[9, 30]have demonstrated that 5-HT exerts a facilitatory effect on both reflex and automatic swallowing and implicated NTS as the principal locus of action. Whether the 5-HT exerts this excitatory effect on swallowing through its subtype 5-HT1A is not clear. This study demonstrated that EA at DU16 and RN23 acupoints induced significantly the expression of 5-HT1A in the NTS. Most of them were mainly distributed in the central, medial and intermediate subnucleus of NTS. Thor KB[31] corroborated that 5-HT1A sites were densely distributed in the NTS with the highest densities localized to the interstitial subnucleus and the central subnucleus that were associated with the coordination of swallowing, respiration, and cardiovascular function. Moreover, Yuki[32]examined the effect of 5-HT on hyperpolarization-activated current in neurons of the NTS and demonstrated that 5-HT1A receptors might regulate hyperpolarization-activated current channel activity in caudal and medial NTS. Although different types of 5-HT receptor might be involved in exerting the excitatory effect in swallowing, this experiment provides further evidence regarding the 5-HT1A receptor as one of the subtype of 5-HT mediating swallowing. As to the precise subnuclei of NTS where high concentration of 5-HT1A receptors located in were controversial, our experimental result is consistent with that of Manaker S[16] using autoradiographic techniques. Thus, we suggested that 5-HT1A could play a role in medullary reflex pathways subserving deglutition.

As described above, the injection of 1  $\mu$ l WAY-100635 induced a marked decrease of the number of swallows elicited by EA at DU16 and RN23 by examination of the mylohyoid EMG discharge. The reason that injection with 1  $\mu$ l 5-HT1A antagonist decreased the numbers of discharge of mylohyoid whereas the 0.1  $\mu$ l 5-HT1A antagonist at equal concentration has no effect on that of mylohyoid probably caused by the NTS had been blocked completed or not. Moreover, there are experiments[33] implemented with bilateral and multiple focal microinjections, but whether bilateral injection of 5-HT1A antagonist into the NTS has more powerful inhibitory effect on swallowing induced by EA is uncertain. Thus, additional experiments with different concentration and doses of 5-HT1A, multipoint injection in the NTS and other acupoints selection are needed to further clarify the role of 5-HT1A in the NTS following EA. Anyhow, as the present investigation may hold a direct clue to the mechanism underlying the excitatory effect of 5-HT1A on reflex swallowing in the rats, we concluded that 5-HT1A in the NTS facilitated the swallowing activity induced by EA at DU16 and RN23 acupoints.

In summary, the present data demonstrated that EA at DU16 and RN23 acupoints could evoke swallows and the primary effect of 5-HT1A in the NTS is a facilitation of pharyngeal stage of swallowing activity induced by EA.

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