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Research Report

Acupuncture-like stimulation at auricular point Heart evokes cardiovascular inhibition via activating the cardiac-related neurons in the nucleus tractus solitarius

Xin Yan Gao[a,b], Yan Hua Li[a], Kun Liu[a], Pei Jing Rong[a], Hui Ben[a], Liang Li[a], Bing Zhu[a,*], Shi Ping Zhang[b,**]

[a]Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences, 16 Nanxiaojie Street, Dongzhimennei, Beijing 100700, PR China

[b]School of Chinese Medicine, Hong Kong Baptist University, 7 Baptist University Road, Kowloon Tong, Kowloon, Hong Kong, PR China

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ABSTRACT

Fifty-eight male Sprague-Dawley rats used in the present study to investigate the role of baroreceptor sensitive neurons of the nucleus tractus solitarius (NTS) in the regulation of cardiovascular inhibition during acupuncture at the auricular point Heart, single unit recording was made in anesthetized Sprague-Dawley rats. A neuron was considered to be excited or inhibited by acupuncture stimulation if it displayed 15% more or less spikes s^{-1}, respectively. NTS neurons were classified into cardiac-related (CR) neurons and non-cardiac-related neurons based on whether their rhythmic discharges were synchronized with the R-waves and responding to sodium nitroprusside (NP; 20 μg/kg, i.v.) administration. Manual acupuncture was applied at the auricular point Heart and somatic acupuncture points ST36 and PC6. Acupuncture at auricular point Heart showed a more significant inhibitory effect on arterial pressure (−22.1±2.4 mm Hg; $P<0.001$) and heart rate (−12.7±1.7 bpm; $P<0.001$) than that at ST36 and PC6. Acupuncture at auricular point Heart also increased the level of response of CR neurons in the NTS (93.8%±26.0% increase in discharge rate; $P<0.01$). Systemic or local administration of atropine attenuated the cardiovascular inhibition and activation of CR neurons evoked by auricular acupuncture, but had no effect on the same responses evoked by somatic acupuncture. Inactivation of the NTS with local anesthetics also decreased the cardiovascular inhititory responses evoked by auricular acupuncture. Our results show that acupuncture at the auricular point Heart regulates cardiovascular function by activating baroreceptor sensitive neurons in the NTS in a similar manner as the baroreceptor reflex in cardiovascular inhibition.

* *Correspondence to:* Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences, 16 Nanxiaojie Street, Dongzhimennei, Beijing 100700, PR China. Fax: +86 10 64032682.

** *Correspondence to:* School of Chinese Medicine, Hong Kong Baptist University, 7 Baptist University Road, Kowloon Tong, Kowloon, Hong Kong, PR China. Fax: +852 34112461.

E-mail addresses: gaoxy@mail.cintcm.ac.cn (X.Y. Gao), liyanhua09@126.com (Y.H. Li), liukun0125@163.com (K. Liu), Rongpj@mail.cintcm.ac.cn (P.J. Rong), benh@mail.cintcm.ac.cn (H. Ben), liliang1160@139.com (L. Li), zhubing@mail.cintcm.ac.cn (B. Zhu), spzhang@hkbu.edu.hk (S.P. Zhang).

Abbreviations: NTS, nucleus tractus solitarius; AA, auricular acupuncture; CR, cardiac-related; AP, arterial pressure; MAP, mean arterial pressure; HR, heart rate; NP, sodium nitroprusside; CS, calamus scriptorius; i.v., intravenously

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1. Introduction

Previous studies have demonstrated that acupuncture can alleviate hypertension (Flachskampf et al., 2007; Zhang et al., 2009). Of the various types of acupuncture used in hypertension treatment, auricular acupuncture, in which acupuncture-like stimulation is applied to points located on the auricles (Nogier, 1983), is one of the most common acupuncture approaches. To date, the mechanism underlying the effect of auricular acupuncture on hypertension remains unclear, although the vagus nerve is suspected to be involved (Gao et al., 2008; La Marca et al., 2010; Tanaka and Mukaino, 1999).

The nucleus tractus solitarius (NTS), which receives afferent input from the head and several organs via cranial and spinal nerves, is an important neural substrate in the regulation of cardiovascular functions (Gamboa-Esteves et al., 2004; Jean, 1991). Somatosensory inputs originating from different parts of the body reach the NTS via distinct pathways (Meng and Lü, 1993). In particular, sensory neurons of the cranial nerves innervating the auricle send their central axons to form direct synapses with NTS neurons (Chien et al., 1996; Tekdemir et al., 1998), which differs from sensory inputs from primary afferent fibers of spinal nerves that reach the NTS via second order neurons in the dorsal horn of the spinal cord or trigeminal nucleus (Gamboa-Esteves et al., 2004; Menétrey and Basbaum, 1987; Potts et al., 2003) or via interneurons in other brain regions (Menétrey and DePommery, 1991; Yu et al., 2002). Chemically and mechanically sensitive neurons with endings located in the heart, carotid, and aortic bodies send their axons that enter the brainstem via cranial nerves to form synapses within the medial part of the NTS, which mediates the baroreceptor and chemoreceptor reflexes (Sun et al., 2005). The NTS is ideally placed as the pivotal site for regulation of cardiovascular function via somatic inputs produced by external stimuli, such as acupuncture (Ku and Zou, 1993). Thus, we proposed that auricular acupuncture might regulate cardiovascular function more directly and with greater strength because of the more direct afferent inputs from the auricle to the NTS, much like the powerful baroreceptor reflex.

Our previous studies demonstrated that the auricular point Heart can produce the strongest cardiovascular inhibition (Gao et al., 2008). In the present study, we again select this point to detect the effects of auricular acupuncture on cardiovascular responses and determine the role of some NTS neurons in mediating reflex responses evoked by auricular acupuncture. As vagal efferents have been implicated in reflex circuit of cardiovascular inhibition, we also tested whether cholinergic transmission plays a role in the regulation of auricular acupuncture on cardiovascular function.

2. Results

2.1. *Auricular acupuncture induces cardiovascular inhibition*

Baseline values of mean arterial pressure (MAP) and heart rate (HR) were measured for one-second periods just before acupuncture stimulation. Responses in the arterial pressure (AP) and HR were also measured for one-second periods with the most remarkable change during acupuncture stimulation. The baseline MAP and HR was 114.6±1.7 mm Hg and 412.6±3.0 bpm (n=12), respectively. As shown in Fig. 1A, MAP was significantly reduced by auricular acupuncture (AA) when compared to ST36 at which depressor response could be evoked (Chen and Ma, 2003; Ohsawa et al., 1995) (−22.1±2.4 mm Hg vs. −9.8±2.3 mm Hg; P<0.05, n=12, one-way ANOVA); Fig. 1B showed that AA also reduced HR significantly (−12.7±1.7 bpm; P<0.001, n=12), whereas no apparent changes in MAP and HR were induced by PC6. ST36 also did not induce significant change in HR. The cardiovascular responses to auricular acupuncture started within a few seconds and usually outlasted the 30 s stimulation by 30 s or longer (Fig. 1C).

2.2. *Auricular acupuncture increases NTS neuronal responses*

In the current study, a total of 68 neurons that had spontaneous background activity and responded to brief pinches of the ear were recorded from the NTS region (between 0.6 mm caudal and 0.4 mm rostral to the calamus scriptorius (CS)) of 38 animals (Fig. 2). Neurons with spontaneous activity related to the respiratory cycle were excluded. Based on the presence or absence of cardiac rhythmic activity, 34 neurons were classified as cardiac-related (CR), and the other 34, as non-CR neurons. The firing rate of most CR neurons was increased by sodium nitroprusside (NP) intravenously (i.v.) (Fig. 1D). Both CR and non-CR neurons were found along the region of recording, but CR neurons excited by auricular acupuncture located mostly at the level of the CS (Fig. 2) exhibited lower averaged spontaneous activities than non-CR neurons (9.2±1.2 spikes s^{-1} vs. 16.6±2.2 spikes s^{-1}; P<0.05, n=34 per group). The discharge rates of the CR neurons, associated with decreases in HR and AP, increased during the period of auricular acupuncture.

During auricular acupuncture (AA), 25 (74%) of the 34 CR neurons were excited, 7 (20%) showed no change, and 2 (6%) were inhibited. In contrast, of the 34 non-CR neurons, 18 (53%) were excited, 15 (44%) showed no change, and 1 (3%) was inhibited. Thus, in the pool of neurons sampled, more CR neurons than non-CR neurons responded to auricular acupuncture (P<0.05, Chi-square test). The magnitude of excitation was higher in CR neurons than that in non-CR neurons (CR: from 6.6±1.1 to 12.4±1.8 spikes s^{-1}, n=25; non-CR: from 11.2±2.2 to 15.1±2.7 spikes s^{-1}; P<0.05, n=18). Interestingly, during somatic acupuncture, 46% CR neurons and 26% non-CR neurons were excited by stimulation at ST36, while 48% of CR neurons and 43% of non-CR neurons were excited by stimulation at PC6. This indicates that more CR neurons respond to auricular acupuncture than to somatic acupuncture (P<0.05).

2.3. *Atropine blocks cardiovascular inhibition and response of NTS cardiac-related neurons*

To determine the role of cholinergic transmission in mediating auricular acupuncture, atropine sulfate (2 mg/kg; David Bull Laboratories, Australia) was injected i.v. After systemic muscarinic blockade, the baseline values of MAP and HR were 118.8±5.3 mm Hg and 413.3±9.2 bpm, respectively (n=12). As shown in Fig. 1A, atropine significantly reduced the depressor responses evoked by AA (−22.1±2.4 mm Hg pre-atropine vs. −8.2±1.9 mm Hg post-atropine; P<0.001, n=12) but did not significantly affect

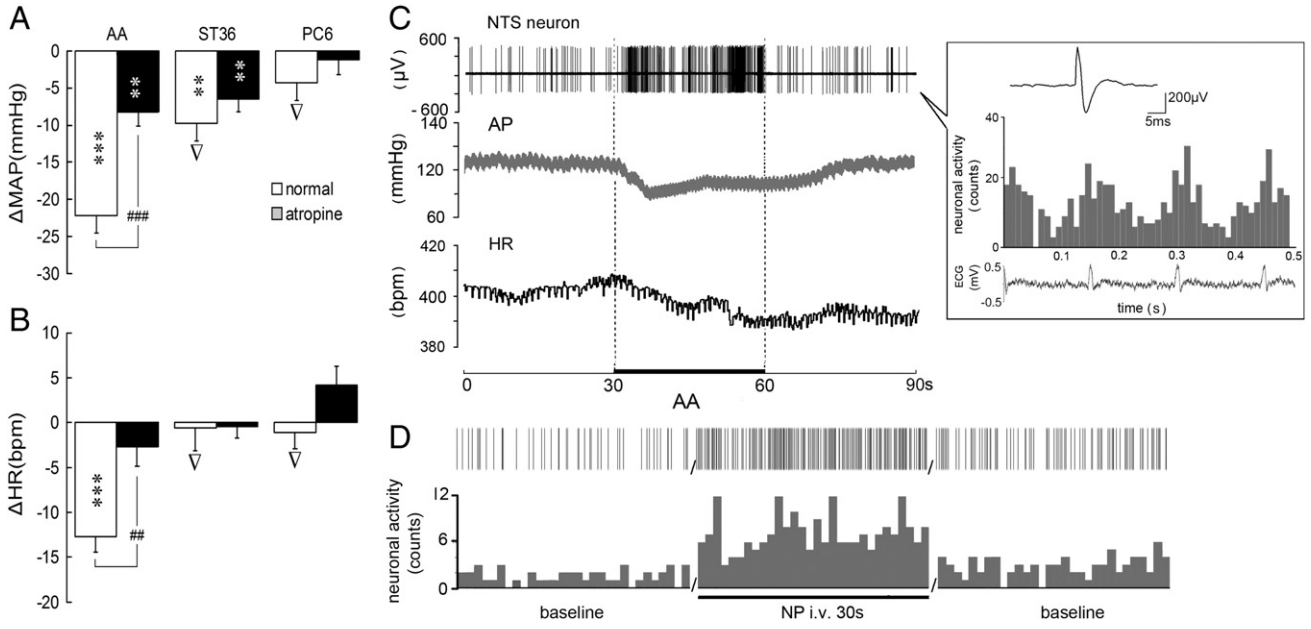

Fig. 1 – Pooled data from 12 experiments showing the effects of acupuncture stimulation at different points on (A) mean arterial pressure (MAP) and (B) heart rate (HR), and the antagonistic effects of atropine sulfate (2 mg kg^{-1}, i.v.). "*"s inside the columns indicate significant statistical differences between pre- and post-acupuncture stimulation ($^{**}P<0.01$; $^{***}P<0.001$; paired *t*-test). Δ indicates a significant change between group differences at different points pre-atropine ($P<0.05$; one-way ANOVA with LSD post-hoc test). "#"s outside the columns indicate significant differences between pre- and post-atropine ($^{\#\#}P<0.01$; $^{\#\#\#}P<0.001$; unpaired *t*-test). (C) Left: the effect of auricular acupuncture (AA) on the discharges from cardiac-related (CR) neurons of the NTS and on AP and HR; right: identification of the CR neuron. Top trace is the shape of a single action potential of the neuron, which was used for identification of single unit activity. Peristimulus time histogram in the middle shows the synchronization of neuronal discharges with the electrocardiogram (ECG) at the bottom. (D) Response of the CR neuron of NTS to sodium nitroprusside (NP) i.v.

depressor responses evoked by somatic acupuncture at ST36. Atropine also significantly reduced the bradycardiac response evoked by AA (−12.6±1.7 bpm pre-atropine vs. −2.7±2.2 bpm post-atropine; $P<0.01$, $n=12$) (Fig. 1B), suggesting that atropine inhibits the effect of AA on the cardiovascular response.

After systemic atropine administration, the baseline firing rates of CR and non-CR neurons were 7.3±1.2 spikes s^{-1} and 9.3±2.0 spikes s^{-1}, respectively. As shown in Fig. 3A and C, atropine significantly reduced the response of CR neurons evoked by AA (93.8%±26.0% increase rate pre-atropine vs. 32.8%±13.9 % increase rate post-atropine; $P<0.05$, $n=12$) but did not significantly affect the responses of CR neurons evoked by somatic acupuncture ($P>0.05$, $n=12$, for ST36 and PC6). For non-CR neurons, no difference was observed in their responsiveness to auricular or somatic acupuncture before and after atropine ($P>0.05$, $n=8$–10 neurons) (Fig. 3B).

To exclude the effect of systemic atropine administration on the vagus efferent nerve and further confirm whether NTS CR neurons play an important role in the regulation of the cardiovascular response by AA, we locally microinjected atropine methyl bromide (80 pmol/50 nl) into the NTS. Atropine microinjection induced a short time fluctuation but did not change baseline MAP (118.6±3.1 mm Hg pre-atropine vs. 109.4±4.1 mm Hg post-atropine;$n=11$) or HR (400.3±10.0 bpm pre-atropine vs. 395.6±11.9 bpm post-atropine;$n=11$). As shown in Fig. 4A, before atropine administration, MAP was significantly reduced by AA (118.6±3.1 mm Hg pre-AA vs. 100.7±3.6 mm Hg post-AA;$P<0.001$). However, after atropine microinjection, AA did not change MAP significantly (109.4±4.1 mm Hg pre-AA vs. 106.4±4.1 mm Hg post-AA; $P>0.05$, $n=11$). Fig. 4B shows that, before atropine administration, AA caused significant decrease in HR (400.3±10.0 bpm pre-AA vs. 389.7±10.8 bpm post-AA; $P<0.01$, $n=11$); after atropine microinjection, AA failed to change HR significantly (395.6±11.9 bpm pre-AA vs. 394.7±11.7 bpm post-AA; $P>0.05$, $n=11$). The above data suggest that AA can regulate the cardiovascular response through activation of CR neurons of the NTS, which is associated with the depressor reflex.

2.4. *Local anesthetic microinjection in the NTS abolishes cardiovascular inhibition*

An additional experiment, in which lignocaine was injected to the inside and outside of NTS locally, was conducted to detect the role of NTS in depressor reflex and responses evoked by acupuncture stimulation. Lignocaine microinjection (2%; 0.1 μl) into the medial NTS reduced the depressor reflex, such that the ratio of the net increase of HR to the net decrease of AP induced by NP i.v. changed from 0.59±0.04 beats mm Hg^{-1} before lignocaine microinjection to 0.33±0.03 beats mm Hg^{-1} after injection ($n=6$; $P<0.01$, paired *t*-test), indicating a diminished baroreceptor reflex. Meanwhile, the depressor and bradycardia responses evoked by AA were abolished (AA-induced AP change of pre-injection vs. that of post-injection: −17.9±3 mm Hg

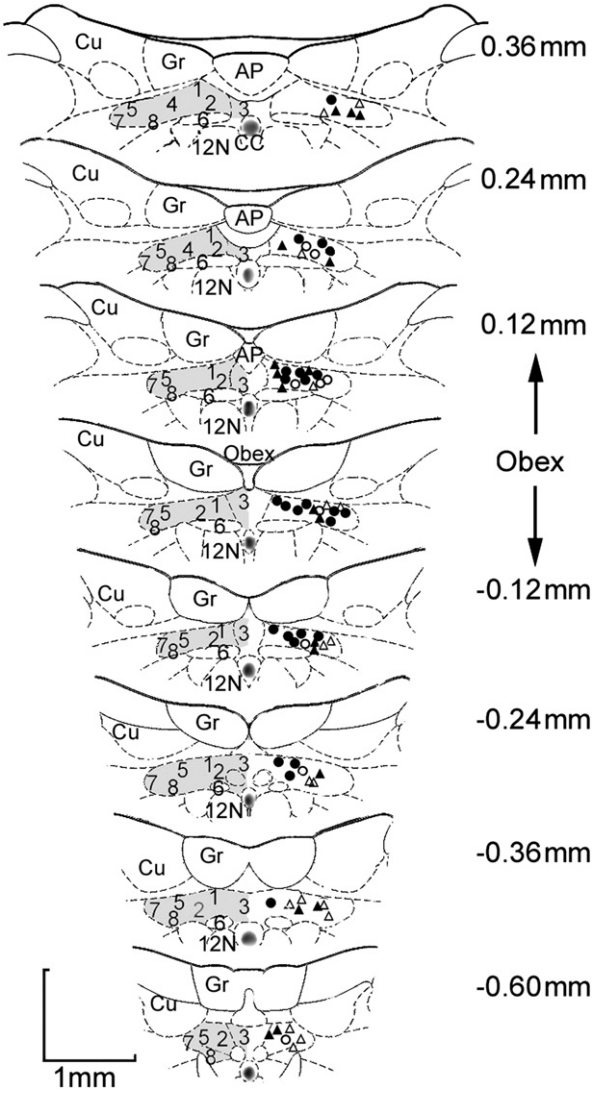

Fig. 2 – Drawings of coronal sections of rat medulla based on Paxinos and Watson (2005), showing the location of neurons studied by AA stimulation in the current experiment. The NTS is indicated by the shaded areas on the left hand side. • indicates CR neurons excited by AA; ○, CR neurons not excited by AA; ▲, non-CR neurons excited by AA; △, non-CR neurons not excited by AA. Cu indicates cuneate nucleus; Gr, gracile nucleus; CC, central canal; AP, area postrema; 1, dorsolateral NTS; 2, medial NTS; 3, commissural NTS; 4, intermediate NTS; 5, solitary tract; 6, dorsal motor nucleus of vagus; 7, ventrolateral NTS; 8, ventral NTS; 12N, hypoglossal nucleus.

vs. −4.7±2.2 mm Hg; $P<0.01$; Fig. 5A, D, and E) (AA-induced HR change of pre-injection vs. that of post-injection: −12.6±1.5 bpm vs. −2.8±0.6 bpm; $P<0.01$; Fig. 5B, D, and E), whereas the same dose of lignocaine microinjection outside of NTS (2 mm lateral to the midline, at the level of CS; Fig. 5C) did not significantly affect the depressor and bradycardia effect produced by AA (pre-AA: 110.3±3.1 mm Hg; post-AA: 89.9±2.2 mm Hg; $P<0.001$, $n=6$; Fig. 5A and F) (pre-AA: 386.9±6.5 bpm; post-AA: 379.0±5.4 bpm; $P<0.05$; Fig. 5B and F), suggesting that AA induces cardiovascular inhibition by the baroreceptor regulatory mechanism in the NTS.

3. Discussion

Although several groups have obtained the contradictory results, likely due to different points or protocols, regarding the effects of acupuncture on hypertension (Kraft and Coulon, 1999; Lee et al., 2009; Robinson et al., 2004), here, we demonstrate for the first time that AA can cause greater and more consistent reflex cardiovascular inhibition than somatic acupuncture. This response is related to greater inputs to the NTS from the auricular concha and to the specific role of the NTS in auricular acupuncture modulation of cardiovascular function. Our data clearly show that different patterns of cardiovascular responses can be evoked by the auricular point Heart, PC6, and ST36. These findings support the existence of point specificity, which has also been demonstrated in both experimental and clinical studies (Middlekauff et al., 2002; Tjen-A-Looi et al., 2004; Turgut et al., 2007).

In this study, we excluded neurons showing respiratory modulation and classified NTS neurons based on whether they displayed a cardiac rhythm and were excited by depressor reflex induced by NP. This allowed us to differentiate neurons that received baroreceptor inputs (Laubie and Schmitt, 1980; Nosaka et al., 1995) from those that did not. These barosensitive neurons are of particular interest because they are responsible for mediating baroreceptor reflex. Inactivation of these neurons has been shown to impair baroreceptor reflex (Colombari et al., 1994; Simms et al., 2006; Wang et al., 2007).

The predominant response of CR neurons to AA or even somatic acupuncture stimulation was excitatory. Direct excitation of CR neurons at the level of the CS, where most of the neurons in the present experiment were recorded, evokes depressor and bradycardic responses (Spencer and Talman, 1986). While the nature of the synaptic connections has not been determined for barosensitive neurons, we observed that CR neurons responding to NP i.v. show stronger activation coupled with stronger cardiovascular inhibition during auricular acupuncture than during somatic acupuncture. Moreover, there was much greater responsiveness of CR neurons than non-CR neurons during auricular acupuncture. These findings suggest that excitation of CR neurons is closely associated with cardiovascular inhibition during auricular acupuncture.

Previous studies suggest that cholinergic receptors in the NTS might be involved in the modulation of baroreceptor reflex. Microinjection of acetylcholine into the NTS elicits hypotension and bradycardic responses similar to those induced by stimulation of arterial baroreceptors, which could be inhibited by pretreatment with atropine (Criscione et al., 1983; Shihara et al., 1999). We found that systemic muscarinic receptor blockade attenuated the excitatory effect of auricular acupuncture on CR neurons but not on non-CR neurons and, simultaneously, partially abolished the cardiovascular responses evoked by AA. Local microinjection of atropine into the NTS also abolished the effect of AA. These results indicate that muscarinic receptors on CR neurons may be involved in mediating cardiovascular inhibition of AA. Thus, the above results suggest that NTS CR neurons are closely associated with reflex responses during auricular acupuncture.

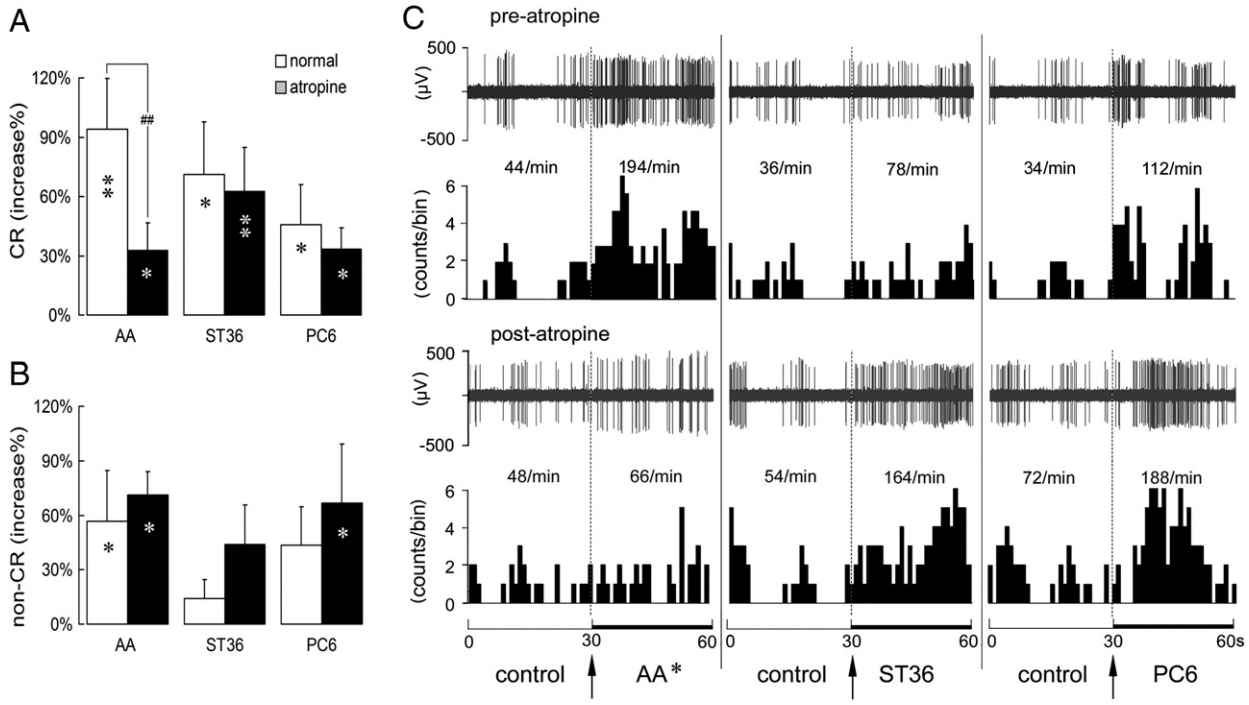

Fig. 3 – The effects of atropine on discharge rates of CR neurons and non-CR neurons in the NTS during acupuncture stimulation. (A) 12 CR neurons; (B) 10 non-CR neurons. "*"s inside the columns indicate significant differences between the baseline value and the value observed during acupuncture stimulation ($^{*}P<0.05$; $^{**}P<0.01$; paired t-test). "#"s outside the columns indicate significant differences in the responses to acupuncture stimulation pre- and post-atropine injection ($^{\#\#}P<0.01$; paired t-test). (C) Example showing the responses of a CR neuron to acupuncture stimulation at different points pre- and post-atropine sulfate injection in a urethane anesthetized rat. After atropine administration, the neuronal activity evoked by AA was reduced from 194 spikes per minute to 66 spikes per minute, but the activities evoked by stimulation of somatic points appear to have increased.

Auricular point Heart is located in the inferior concha of the auricle, which is mainly innervated by the V, VII, and X cranial nerves (Folan-Curran et al., 1994; Satomi and Takahashi, 1991). The distinct cranial nerve afferent innervation may be responsible for the powerful cardiovascular inhibition produced by auricular stimulation. Kumada et al. (1977) describe a trigeminal depressor response evoked by stimulation of the trigeminal nucleus, branches of the trigeminal or glossopharyngeal, and vagus nerves. However, the trigeminal depressor response was not altered by lesions of the NTS, which abolished the baroreceptor reflex and the depressor response evoked by aortic depressor nerve stimulation. The current study found that microinjection of lignocaine into the NTS attenuated baroreceptor reflex induced by NP i.v. and preferentially abolished the cardiovascular inhibitory effects of AA. However, the injection of lignocaine into the 2 mm lateral to midline (outside of NTS) at CS level did not diminish the inhibitory effect of AA on AP and HR. Thus, the cardiovascular inhibition induced by auricular acupuncture is clearly different from the trigeminal depressor response. On the other hand, the cardiovascular inhibition of AA bears striking similarities to arterial baroreceptor reflex evoked by stimulation of receptors in the carotid sinus and aortic arch or by direct stimulation of aortic depressor nerve.

Although the exact afferent nerves responsible for cardiovascular inhibition has not been determined in the current study, the depressor and bradycardic effects of AA seem to be the effect of vagal excitation. Firstly, vagal nerve anatomically innervates the heart and plays an important role in cardiovascular regulation. Secondly, our data show that the depressor effect was greatly attenuated and bradycardia was abolished by systemic muscarinic blockade. In addition, our previous study also demonstrates that vagal afferent nerves that innervate the ear are likely to be involved in the cardiovascular response (Gao et al., 2008). Therefore, the reflex cardiovascular inhibitory pathway of AA may also be similar to that involved in baroreceptor activation. The auricular cardiovascular inhibitory pathway exploits the baroreceptor regulatory mechanism to induce cardiovascular inhibition and is distinct from the depressor pathways of somatic acupuncture. In further studies, the use of heart rate variability as an additional parameter may enable new insights in assessing the changes in autonomic tone that influences cardiovascular function, which has been successfully used by others to analyze the effect of acupuncture (Litscher, 2007).

In summary, our findings suggest that the same neurons in the NTS may be involved in both baroreceptor responses and cardiovascular inhibition induced by auricular acupuncture. Auricular acupuncture evokes cardiovascular inhibition through activating baroreceptor-related neurons in the NTS, a novel mechanism of auricular acupuncture in regulation of cardiovascular function that is similar to the baroreceptor reflex, which is different from that of somatic acupuncture. This specific mechanism appears to underlie the powerful and consistent cardiovascular inhibitory effects of auricular

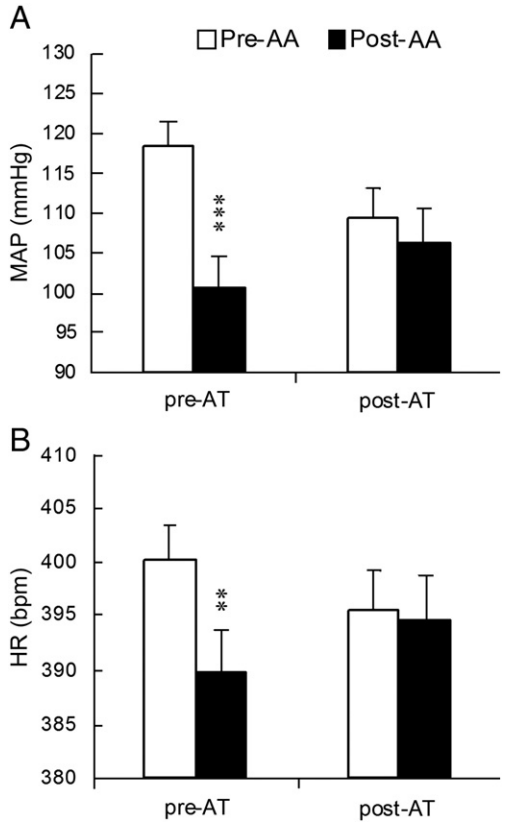

Fig. 4 – The effect of atropine microinjection into NTS on cardiovascular response induced by acupuncture. (A) MAP reduction induced by AA was abolished by atropine microinjection into NTS. (B) Atropine microinjection into NTS blocked bradycardia induced by AA. "*" shows the significant differences in MAP and HR between pre-AA stimulation and post-AA stimulation ($P<0.01$; ***$P<0.001$; $n=11$; paired t-test). Pre-AT indicates pre-atropine; post-AT, post-atropine.**

acupuncture observed in anesthetized rats. Our results may have important implications in the clinical practice of auricular acupuncture.

4. Experimental procedures

4.1. *Animal preparation*

Experiments were carried out in accordance with the Guide for Care and Use of Laboratory Animals issued by NIH and the procedures were approved by the institutional review board of the Hong Kong Baptist University and the Institutional Animal Care and Use Committee of the China Academy of Chinese Medical Sciences. Fifty-eight male Sprague–Dawley rats, with weight ranging from 300 to 400 g, were kept in an animal house maintained at 21±2 °C and 12 h light–dark cycle with free access to food and water. The animals were initially anesthetized with an intraperitoneal injection of pentobarbital sodium 200 mg/ml (50 mg/kg; Alfasan, Holland; $n=20$) or 10% urethane (1.0 g/kg; Sigma-Aldrich, USA; $n=38$). The left common carotid artery was cannulated with a polyethylene catheter filled with physiological saline containing heparin (200 IU/ml; LEO, Denmark) for recording of AP via a blood pressure transducer and amplifier (ADInstruments, Australia). A catheter was placed into the left jugular vein for fluid supplement and drug delivery. The depth of anesthesia was monitored by the changes in AP and additional anesthetics (either pentobarbital sodium 15 mg/kg/h or urethane 0.3 g/kg) were given if the animal showed large fluctuations in baseline AP, increased heart rate (HR), or a withdrawal response to pinch of the paw. After tracheal cannulation, animals breathed spontaneously and their core temperature was maintained at 37.0±0.5 °C by a feedback-controlled electric blanket (FHC, USA); needle electrodes were placed bilaterally at the upper arm and the left hind limb for electrocardiogram (ECG) recording. The animals were sacrificed after investigation by overdose of anesthetics.

4.2. *Single unit recording*

Animals were fixed on a stereotaxic frame (Stoelting, USA), and the dorsal surface of the caudal brainstem was exposed and covered with warm liquid paraffin. The calamus scriptorius (CS) was used as a mark to position the recording electrode in the medial nucleus tractus solitarius (NTS) (Simms et al., 2006) (Fig. 2). The glass recording electrode with a resistance of 10–15 MΩ was filled with 0.2% pontamine sky blue in 0.5 M sodium acetate and was stereotaxically placed 0.2–0.8 mm lateral to the midline, between 0.3 mm rostral and 0.6 mm caudal to the CS, and advanced to a depth of 0.4–0.8 mm from the dorsal surface of the medulla (Deuchars et al., 2000; Kalia, 1981). Extracellular discharges were amplified, and single unit activities were monitored on a dual beam oscilloscope. The action potentials and firing frequencies of neurons, as well as AP and HR, were recorded by a computer acquisition system (ADInstruments, Australia). When spontaneous discharges of a single NTS neuron were detected, only those neurons which showed responses induced by stimulating the ipsilateral auricle of the rat with a pair of forceps were studied further. The discharge rate of each NTS neuron was determined by counting the spikes for 30 s either immediately before or during the acupuncture stimulation and then dividing them by 30 to obtain the number of spikes per second (spikes s^{-1}). A neuron was considered to be excited or inhibited by acupuncture stimulation if it displayed 15% more or less spikes s^{-1}, respectively. Using the R-waves of the ECG records, off-line analysis of spontaneous discharge patterns of NTS neurons was carried out by constructing peristimulus time histograms (PSTHs; 100–200 sweeps of 500 ms duration).

NTS neurons were classified into cardiac-related (CR) neurons if they displayed rhythmic discharges that were synchronized with the R-waves (Laubie and Schmitt, 1980; Nosaka et al., 1995) or non-CR neurons if there was no rhythmic discharge synchronized with the R-waves. To verify whether these CR-related neurons were associated with baroreceptor reflex, sodium nitroprusside (NP; 20 μg/kg; Sigma-Aldrich, USA) was administered. If the firing rate of CR neurons increased, these CR neurons were considered to be depressor neurons.

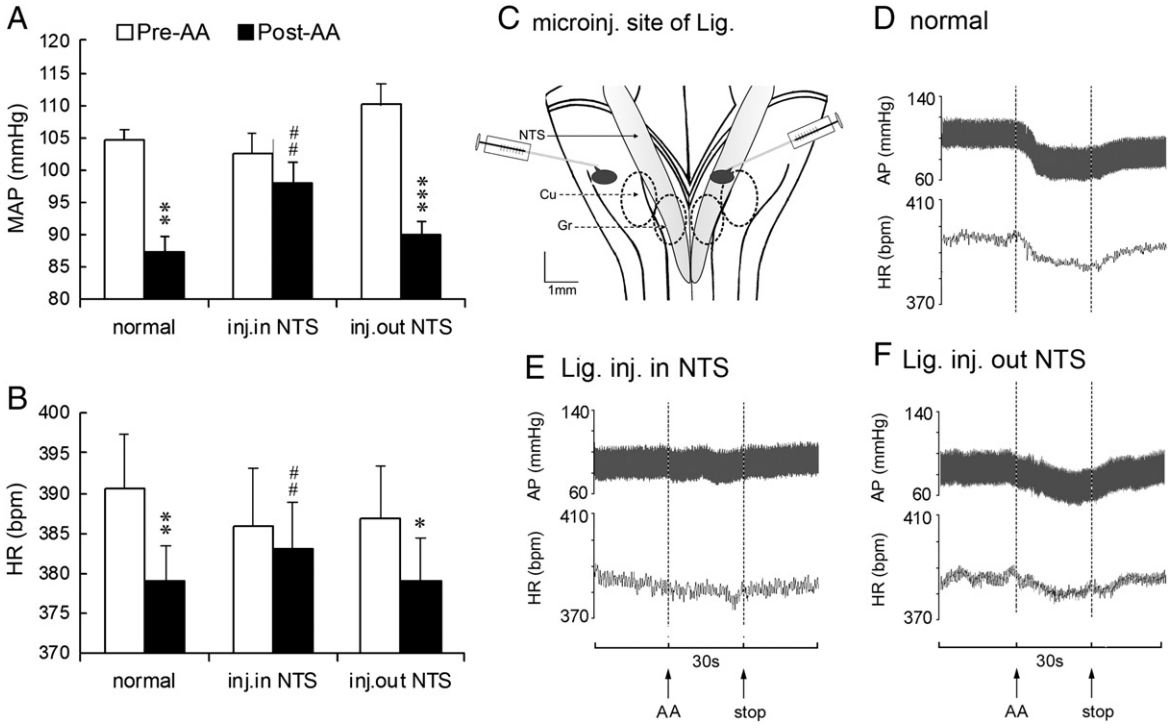

Fig. 5 – The responses evoked by AA under normal condition and after microinjection of 0.1 μl lignocaine (2%) into the NTS in pentobarbital anesthetized animals. Effect of acupuncture on (A) MAP and (B) HR under normal and lignocaine microinjection inside and outside of NTS ($n=12$). "*"s indicate significant differences between the baseline value and the value observed during acupuncture stimulation ($^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; paired t-test). "#"s indicate significant differences between changes of MAP and HR to AA after microinjection of lignocaine inside and outside of NTS and normal ($^{\#\#}P<0.01$; paired t-test). (C) Schematic diagram indicates the microinjection site of lignocaine into and out of NTS. D, E, F are Computer chart records showing a typical experiment in a pentobarbital anesthetized animal, in which the responses evoked by AA under normal condition (D) and after bilateral microinjections of 0.1 μl lignocaine (2%) into the NTS (E), as well as bilateral microinjections of the same dose of lignocaine outside of NTS, 2 mm lateral to the midline at the level of calamus scriptorius (F). Inj. and microinj. indicate microinjection; Lig., lignocaine; Cu, cuneate nucleus; Gr, gracile nucleus.

4.3. *Histological verification of recording sites*

At the end of each experiment, the neuronal recording site in the brainstem was marked by passing a negative current of 20 μA for 20 min through the glass electrode for iontophoretic deposition of pontamine sky blue. After fixing in vivo with 2% paraformaldehyde and 1% glutaraldehyde in 0.1 M PBS (pH 7.4), the brain stem was sectioned at 30 μm, and the sections were stained with 0.3% neutral red. The marked recording site was located by microscopic examination, and other recording sites were identified with reference to the marked recording site. Only those data with histological confirmation were accepted for statistical analysis.

4.4. *Acupuncture-like stimulation*

Auricular point Heart and control points including PC6 (Neiguan) and ST36 (Zusanli), as the homotopic and heterotopic point separately which also regulate cardiovascular functions, were determined by anatomical marks, based on the description in textbooks and previous reports (Cheng, 1996; Gao et al., 2008; Tjen-A-Looi et al., 2004). Briefly, PC6 is located proximal to the accessory carpel pad of the forelimb between ligaments of the flexor carpi radialis and palmaris longus. ST36 is located on the anterolateral side of the hindlimb near the anterior crest of the tibia below the knee under the tibialis anterior muscle. The auricular point Heart is located at the inferior concha. Manual acupuncture was used for stimulation in place of electroacupuncture that can produce stimulus artifacts that interfere with recordings of extracellular action potentials. Acupuncture needles (length, 13 mm; diameter, 0.2 mm; Hwato, PR China) were inserted perpendicularly by a depth of 2 mm into the auricular point (Heart) and 4–5 mm into somatic points, ipsilateral to the site of neuronal recording. When single unit activity was well isolated from background activity and the minute to minute fluctuations of AP and HR were less than 5%, acupuncture stimulation was applied by twisting the needle back and forth about once every second for 30 s. The order of point stimulation was randomly arranged.

4.5. *Data analysis*

Data were shown as mean±SEM. Comparison between means was analyzed by t-test for pre- and post-stimulation comparisons or one-way ANOVA with LSD post-hoc test for

testing the effects at different points. *P*-values <0.05 were considered significant.

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