Phenomenon of non-associative learning in Hering-Breuer reflex simulated by electrical vagal stimulation in rabbits

WANG Gui-Min, SONG Gang*, ZHANG Heng
Institute of Physiology, School of Medicine, Shandong University, Jinan 250012, China

Abstract: The purpose of this study was to explore learning and memory in the Hering-Breuer (HB) reflex simulated by a 60-second-long electrical stimulation of vagus nerve. The responses of phrenic nerve discharge to electrical stimulation (10–100 Hz, 20–60 µA, pulse duration 0.3 ms, for 60 s) of the vagus nerve were observed in rabbits. The results showed that 60-second-long stimulation of vagus nerve produced classic HB reflex, which is composed of two components — lung inflation reflex that is the inhibition of inspiration, and lung deflation reflex that is the facilitation of inspiration. (1) High frequency stimulation (≥ 40 Hz, 60 s) of the central end of vagus nerve induced shortening of the inspiratory phase and lengthening of expiratory duration. The inhibitory effect on phrenic discharge was released gradually during sustained vagal stimulation, indicating the habituation of the inhibition. At the cessation of stimulation, the phrenic discharge showed transient post-stimulus rebound. Low frequency stimulation (<40 Hz, 60 s) of the central end of vagus nerve caused an increase in respiratory frequency (f) and shortening of expiratory duration. The excitatory effect on phrenic discharge was also released gradually during the vagal stimulation. The phrenic discharge returned to control level gradually after the removal of the vagal stimulus, indicating short-term potentiation (STP). (2) The habituation of HB reflex was inversely dependent on stimulus intensity and frequency. With an increase in the stimulus frequency or intensity, the degree of the habituation decreased. On the other hand, with the decrease of stimulation intensity and frequency, the degree of the habituation increased. These data indicate a phenomenon of non-associative learning in HB reflex simulated by vagal stimulation. Neural synaptic plasticity and accommodation may exist in the reflex control of respiration in rabbits.

Key words: learning; Hering-Breuer reflex; rabbits

Received 2005-01-31 Accepted 2005-05-09

*Corresponding author. Tel: +86-531-88382037; E-mail: gangsg@mit.edu
Learning and memory are basic functions of the nervous system. Habituation and sensitization are two common forms of nonassociative learning that exhibit in neuronal structures of vertebrate and invertebrate nervous systems [1]. Non-associative learning has been proved to be a behavioral correlate of the integrator evident in oculomotor control [2]. Many respiratory reflexes exhibit activity-dependent phasic adaptation upon sustained afferent stimulation [3]. Short-term potentiation (STP) is prevalent in the respiratory system and is exemplified by the carotid chemoreflex response to hypoxia or electrical stimulation of the carotid sinus nerve, which arises from the peripheral chemoreceptors in the carotid body [4,5]. It has been suggested that the induction of respiratory STP is due to the plasticity of neurotransmissions in neurons of the solitary tract nucleus in the dorsomedial medulla or phrenic motoneurons.

Studies that had been carried out on rats revealed evidence of habituation in the Hering-Breuer (HB) reflex in vivo. This habituation of the HB reflex had similar characteristics to the synaptic accommodation in NTS neurons [6] and was NMDA receptor-independent. It was accompanied by an NMDA receptor-dependent desensitization of a parallel pathway in the pontine “pneumotaxic center”, which represented a novel and demonstrable pairing of dual non-associative learning processed in the mammalian brain.

Classically, the HB reflex is considered to have two components: a lung inflation reflex that caused inhibition of inspiration, and a lung deflation reflex that caused excitation of inspiration. Generally, the slowly adapting pulmonary stretch receptor (SAR) and rapidly adapting pulmonary stretch receptor (RAR) are mainly respectively responsible for the two components of HB reflex. The expression of HB reflex shows significant species difference. For example, low-frequency and low-intensity stimulation of the vagus nerve caused an excitation of respiration in rabbits, but in rats, only inhibitory response was observed. Therefore, we speculate that the HB reflex of rabbits may exhibit different learning phenomenon due to both the excitatory and inhibitory effects on respiration by vagus nerve stimulation.

In this study, we simulated the rabbit lung inflation and deflation reflexes with electrical vagal stimulation and characterized the non-association learning expressed by them. Our data showed that there were two parallel mechanisms of learning and memory in the HB reflex that could be unique to rabbits.

1 MATERIALS AND METHODS

1.1 Animal preparation
Experiments were conducted on 6 adult rabbits (weighing 2.2~2.6 kg) of either sex. The rabbits were anesthetized with urethane (1.0 g/kg, i.v.). The adequacy of anesthesia was assessed periodically by monitoring changes in arterial blood pressure (BP) and respiratory rate. Tracheotomy was performed for artificial ventilation. All rabbits were paralyzed with pancuronium bromide (Sigma; initial dose 0.5 mg/kg, supplemented by 0.1 mg/kg·h⁻¹, i.v.) and ventilated with oxygen enriched medical air (oxygen concentration at 40%). The end-tidal CO₂ was monitored (OIR-7101, Nihon Konden) and kept at (4~5)%. Blood pressure was monitored through a femoral artery cannula and maintained at 90~113 mmHg by continuous infusion of 10% glucose in physiological saline through the femoral vein. Rectal temperature was kept at 37~38°C with a heating pad.

1.2 Stimulation and recording
Both vagus nerves were separated and severed. The proximal end of the right vagus was mounted on a bipolar silver hook electrode for stimulation. The left cervical phrenic nerve was separated and mounted on a similar electrode for recording. The phrenic discharge, stimulation signal, and blood pressure were recorded and analyzed with a computer using Biobench software (NI Corporation, USA).

In order to elicit the HB reflex, we defined the stimulation threshold as the lowest stimulus current that produced a detectable inhibitory or excitatory response in phrenic nerve discharge with a 5-second test stimulation (frequencies at 10 or 100 Hz, respectively). The stimulus current was then adjusted to 1.5 × threshold, usually at about 20~60 µA.

After 30 min’ stabilization period, the vagus nerve was stimulated (10~100 Hz, pulse duration 0.3 ms) for 60 s. Phrenic activity was recorded continuously during the vagal stimulation.

The average amplitude of inspiration (f Phr) was calculated by averaging the peak amplitudes of the integrated phrenic discharge of several consecutive respiratory cycles (the number of cycles is 3~5 with different respiratory frequency). The instant respiratory frequency (i f) was
calculated as 60/duration of one respiratory cycle (inspiratory time + expiratory time). The average respiratory frequency \( f_{av} \) was calculated by averaging the \( f \) of several consecutive respiratory cycles. The average expiratory time \( T_e \) and average inspiratory time \( T_i \) were calculated by averaging the expiratory time or inspiratory time of several consecutive respiratory cycles. All the data about \( f_{avg} \), \( f_{avg} \), and \( T_e \), \( T_i \) were from variable time windows that ranged from 1 s (at the onset and offset of vagal stimulation) to 5 s (in the control period), in accordance with the relative speed of response. The degree of habituation is defined as \( f_{avg}/f_c \), which is the ratio of \( f_{avg} \) of the last 5 s of stimulation) / \( f_{avg} \) before stimulation). The degree of post-stimulus rebound is defined as \( f_{avg}/f_c \), which is the ratio of \( f_{avg} \) of the first 5 s post-stimulation) / \( f_{avg} \) before stimulation). The degree of STP is also defined as the ratio of \( f_{avg} \) of the first 5 s post-stimulation) / \( f_{avg} \) before stimulation).

1.3 Statistic analysis

Results were presented as mean ± SD. Student’s t test was used to determine the difference. Statistical significance level was set at \( P<0.05 \).

2 RESULTS

2.1 Hering-Breuer reflex simulated by electrical stimulation of vagus nerve

Repetitive high frequency stimulation of the vagus nerve (40–100 Hz, including 40 Hz) caused a typical inhibition of phrenic discharge with shortening of \( T_i \) and lengthening of \( T_e \), as well as corresponding decrease in \( f \) similar to the lung inflation reflex (Fig. 1A and Fig.2). On the other hand, low frequency vagal stimulation (10–40 Hz, including 10 Hz) caused an increase in \( f \) and shortening of \( T_e \) similar to lung deflation reflex (Fig.1B and Fig.3). Changes in the phrenic discharge amplitude for both lung inflation and lung deflation reflexes were not as remarkable (no significant difference with the control) as those in \( f \), \( T_e \) and \( T_i \) (Fig. 1C). No significant changes were observed in blood pressure (Fig.1) and concentration of end-tidal \( CO_2 \) [varying within (4–5)%].

2.2 Phenomenon of non-associative learning in Hering-Breuer reflex

2.2.1 Adaptation and post-stimulus rebound of the lung inflation reflex

The respiratory rhythm recovered gradually upon repetitive high frequency stimulation of the vagus nerve. For example, during the stimulation period (80 Hz, 40 \( \mu A \), 60 s), the \( f_{avg} \) of the first 5 s was decreased by (68.81 ± 6.76)% and \( T_e \) increased by (79.51±4.99)%). But during the last 5 s of the simulation, the \( f_{avg} \) was decreased by (40.33±2.44)% and the \( T_e \) increased by (31.50±4.13)%). All those changes were statistically significant (\( n=6, P<0.01 \)). This indicates the habituation of the responses upon repetitive vagal stimulation.

At the cessation of stimulation, the \( f_{avg} \) showed a transient increase of (11.89±3.49)% (\( n=6, P<0.05 \) vs control) and the \( T_e \) decreased by (26.12±1.19)% (\( n=6, P<0.05 \) vs control), showing a post-stimulus rebound that occurred at the termination of vagal stimulation (Fig.2).

The degree of habituation showed inverse frequency and intensity dependency. For example, \( f_{avg}/f_c \) was 0.9012±0.0424 at 60 Hz (40 \( \mu A \), 60 s) of stimulation, and 0.5967±0.0301 at 80 Hz (40 \( \mu A \), 60 s), showing a significant decrease of \( f_{avg}/f_c \) (\( n=6, P<0.01 \)). Therefore, with the increase of stimulation frequency, there’s a significant decrease in the degree of habituation.

The post-stimulus rebound showed a tendency of increase with the increase in stimulation frequency, but not statistically significant when the frequency was increased from 60 Hz to 80 Hz (\( f_{avg}/f_c \) increased from 1.1012±0.0209 to 1.1189±0.0349; \( n=6, P=0.3116 \)) (Fig. 2.4).

2.2.2 Adaptation and short-term potentiation (STP) of the lung deflation reflex

The stimulation-simulated lung deflation reflex also showed adaptation. For example, the respiratory rhythm recovered toward the control level gradually during repetitive low frequency stimulation of the vagus nerve. During the stimulation period (10 Hz, 40 \( \mu A \), 60 s), the \( f_{avg} \) of the first 5 s was increased by (50.16±4.07)% and the \( T_e \) decreased by (49.28±2.90)%). But the \( f_{avg} \) of the last 5 s during the stimulation was increased by (33.63±3.19)% (\( n=6, P<0.01 \) vs \( f_{avg} \) of first 5 s), and the \( T_e \) increased only by (27.61±1.10)% (\( n=6, P<0.01 \) vs \( T_e \) of the first 5 s), indicating an adaptation or habituation of the response. While after stimulation, the \( f_{avg} \) of the first 5 s was increased by (12.98±4.39)% (\( n=6, P<0.05 \) vs control), the \( T_e \) decreased by (22.20±1.41)% (\( n=6, P<0.05 \) vs control), indicating a STP that occurred at the cessation of vagal stimulation (Fig. 3).

The habituation of lung deflation reflex showed inverse frequency and intensity dependency. For example, \( f_{avg}/f_c \) changed from 0.2523±0.0625 (40 \( \mu A \), 10 Hz, 60 s) to 0.4233±0.0814 (60 \( \mu A \), 10 Hz, 60 s), showing significant weakening (\( n=6, P<0.01 \)) of the habituation with the increase in the stimulus intensity. \( f_{avg}/f_c \) changed from 1.2115±0.0255 (40 \( \mu A \)) to 1.3271±0.0295 (60 \( \mu A \)), showing significant enhancement (\( n=6, P<0.01 \)) of the STP with the increase in the stimulus intensity (Fig. 3.4).
Fig. 1. Hering-Breuer reflex simulated by repetitive electrical stimulation of vagus nerve in rabbits. A: Lung inflation reflex induced by high frequency stimulation (80 Hz, 40 µA, 60 s). B: Lung deflation reflex induced by low frequency stimulation (20 Hz, 40 µA, 60 s). C: $\bar{f}$ Phr before and 60 s after vagal stimulation. Upper, integrated phrenic discharge; middle, original phrenic discharge; lower, arterial blood pressure; lower right, time marker for the original; inset at the top of B shows expanded views of phrenic discharges in the control period and at the onset and offset of the vagal stimulation; $f$ Phr, the amplitude of integrated phrenic activity (the spot shows mean response).
3 DISCUSSION

The current study gave a demonstrable example of non-associative learning of HB reflex in rabbits, showing activity-dependent habituation and post-stimulus rebound, as well as STP in vivo in the mammalian respiratory center, which are physiological bases of learning and memory.

Habituation and desensitization in the lung inflation reflex have been studied in rats [6], but lung deflation reflex was not addressed in that report. Most experiments were processed with high-frequency vagal stimulation, so few reports were about low-frequency stimulation to vagus nerve. But recently, it has been demonstrated that low-frequency vagal afferent stimulation caused inspiratory-promoting response in anaesthetized rabbits [7,8]. It was suggested that the neuronal mechanism underlying this frequency-dependent switching lies in the greatly varied effect of the summation of repeated postsynaptic potentials in the medullary respiratory neurons, depending on the stimulus frequency.

In this study, we found that low-frequency vagal stimulation can mimic the lung deflation reflex, causing the facilitation of respiration, while high-frequency stimulation can mimic lung inflation reflex, causing the inhibition of respiration. Different phrenic activity may represent that different frequency filters exist in the first pathway from the vagus nerve to the NTS, and the second pathway from NTS to the pons, and also in the pathway to the phrenic neuron.

In the present study, we used the relatively low stimulation intensity so mainly to activate the Aβ fibers that conduct signals from pulmonary stretch receptors, which is one of the complementary signaling pathways that contribute significantly to the regulation of the normal respiratory rhythm. Although non-associative learning is gener-
ally defined on the basis of whole-animal behavior irrespective of the specific afferent fibers\(^9\), the relatively low stimulation intensity used in this study precluded the activation of pulmonary C-fibers, which have significantly higher activation thresholds than A\(_p\) fibers in rats\(^{10,11}\). Thus, vagal stimulation in rabbits at low intensities with different frequencies in this study can elicit responses in phrenic discharge that were similar to the HB reflex\(^{12}\).

To examine whether the recovery of the phrenic activity in the HB reflex conformed to the classical definition of habituation, we employed some criteria that have been applied variously to the study of habituation in certain brain structures\(^{13}\). The habituation of the HB reflex and the post-stimulus rebound response observed in the present study confirmed previous reports of adaptations of the respiratory rhythm on sustained vagal stimulation or lung inflation\(^{14}\) in dogs and cats. Characteristics of the phrenic nerve discharge elicited by vagal stimulation were also found in the present study, which are habituation and post-stimulus rebound, as well as the STP of the HB reflex showed inverse frequency and intensity dependency. But mechanisms of the habituation, post-stimulus rebound and STP in HB reflex are still to be studied. We suggest that they are manifestations of habituation in corresponding signal pathways engendered by synaptic accommodation in the NTS or pons. Some studies indicated that the habituation was the result of synaptic adaptation within the NTS, and the post-stimulus rebound was related to pontine pneumotaxic center\(^{15}\). The post-stimulus rebound and STP persisted respectively in the lung inflation reflex and lung deflation reflex even after the vagal input was removed, as a result, they are not caused by the direct vagal inhibition of pontine respiratory-related neurons. On the other hand, pontine modulation of the NTS may also contribute to the habituation of HB reflex, since the descending projection from the pons could modulate neural transmission of certain peripheral afferents within the NTS\(^{16}\). Further studies are necessary to elucidate the cellular mechanisms of habituation and post-stimulus rebound in lung deflation reflex, habituation and STP in the lung inflation reflex, as well as the phenomenon of their activity-dependence.

Non-associative learning process in parallel signal pathways may have important functional significance in the regulation of respiration. In mammalian or invertebrate nervous systems, habituation and sensitization are usually associated with innocuous and noxious stimuli, respectively. Thus, the habituation (adaptation) of the lung inflation reflex may alleviate the animal from the inhibition caused by the sustained activation of pulmonary stretch receptors to resume respiration. The post-stimulus rebound and the STP in lung inflation and deflation reflex may be viewed as "back-up" systems that provide the fail-safe mechanism for respiratory rhythmogenesis, which is demonstrated in the other experiment in rats\(^{16}\).

REFERENCES