Original Article
RVLM-IML pathway may implicate controlling peripheral airways by melanocortinergic-sympathetic signaling: a transneuronal labeling study using pseudorabies virus

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Abstract: Pseudorabies virus (PRV)-614 was injected into the tracheal wall of male MC4R-GFP transgenic mice, resulting in retrograde infections in spinal cord and autonomic premotor areas of the brain including the rostroventrolateral medulla (RVLM). This polysynaptic pathway to the airway may form the substrate underlying the impact of IML and RVLM on airway function. The neurochemical phenotype of MC4R-GFP-positive neurons was identified using fluorescence immunocytochemical labeling. PRV-614/MC4R-GFP dual labeled neurons were detected in spinal IML and the rostral ventrolateral medulla (RVLM). These data demonstrate the RVLM-IML pathway of synaptically connected neurons extending to the airway through melanocortinergic-sympathetic signaling.

Introduction

It is well known that the rostral ventrolateral medulla (RVLM) is the primary region of the brainstem that regulates sympathetic outflow to vasomotor tone [1, 2]. Moon et al indicated that spinally projecting sympathoexcitatory neurons in the RVLM, synapse with sympathetic preganglionic neurons and regulate the activity of sympathetic nerves that control the adrenal medulla [3]. Considerable evidence indicates that two major neuronal subpopulations in the RVLM include pre-sympathetic (sympathoexcitatory) neurons, and expiratory neurons of the Bötzinger complex [4]. It was reported that neurons in the brainstem midline and ventrolateral medulla participate in the control of breathing [5]. Therefore, RVLM may implicate controlling breathing by sympathetic signaling.

It is demonstrated that sympathetic regulation is an important component of central melanocortin action [6-9], and sympathetic activity is tightly interconnected via central melanocortinergic pathways involving the melanocortin-4 receptor (MC4R) [10-13]. However, the exact neurosubstrate underlying the airway regulation of sympathetic function by the central melanocortin system is poorly understood. In the present study, we used transgenic recombinants of an attenuated PRV strain, PRV-614, expressing the red fluorescent protein (RFP) for direct visualization under fluorescence microscope [14-18]. The aim of this study was to provide morphologic evidence of the neuroanatomical circuitry between RVLM and peripheral airway in MC4R-green fluorescent protein (GFP) transgenic mice, by PRV-614-mediated transsynaptic retrograde tracer.

Materials and methods

Animals

The MC4R-GFP transgenic mice weighing between 25 g and 30 g (n = 5) were used for these experiments. Mice were genotyped as described before [19]. Mice were housed under controlled conditions (12 h alternating light-dark cycle, 21±1°C, ~60% relative humidity).
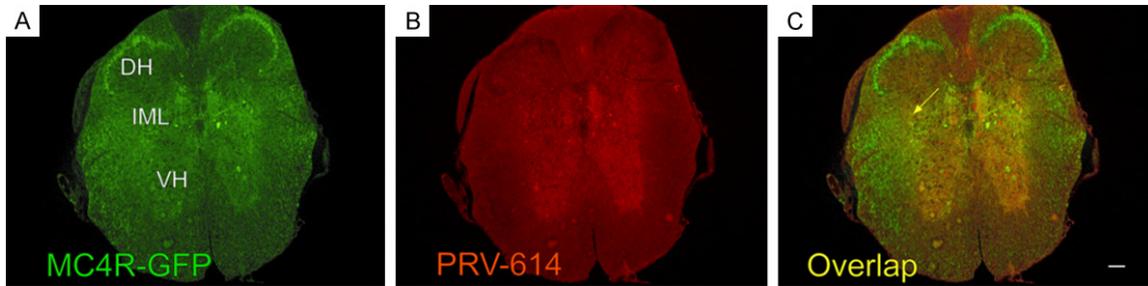
Figure 1. PRV-614/MC4R-GFP double-labeled neurons in the spinal cord. Images A-C were taken from the animal spinal cord after injections of PRV-614. A. Showed MC4R-GFP positive neurons in the spinal cord; B. Showed neurons infected with PRV-614, which send transsynaptic projections to the tracheal wall; C. Showed overlaid images of A plus B. Arrows (yellow) indicate double-labeled neurons. DH, Dorsal horn; IML, the intermediolateral cell column; VH, ventral horn. Scale bars, 50 μm.

Standard diet and water were provided *ad libitumm* unless noted otherwise. Experimental procedures and protocols were approved in advance by the Animal Care and Use Committee of Huazhong University of Science & Technology. Only male mice were used in this study.

Microinjection of virus into the tracheal wall

PRV-614 was generated by the Enquist laboratory at Princeton University and was made available through the Center for Neuroanatomy with Neurotropic Viruses (NIH P40 OD010996). PRV-614 was used as a retrograde transneuronal marker to identify the CNS neurons that innervate the tracheal wall. Experiments were performed on anesthetized mice (n = 5), as previously described [20, 21]. Injections were made carefully under magnification. Following C8 spinalectomy, the tracheobronchial tree was exposed and a four unilateral 0.5 μl injections of PRV-614 (2×10^8 = plaque forming units/ml) were made in the tracheal wall of C8 spinal cord with a Hamilton syringe and a 26-gauge needle. The needle was held in place for 2 min following each injection to promote uptake of PRV-614 at the injection site. The mice were carefully monitored and 1 ml/100 g body weight of sterile saline was given subcutaneously every 12 h.

Fluorescence immunohistochemistry and tissue analysis

Four days after the PRV-614 injection, the mice were anesthetized and perfused. Spinal cords and brainstem were removed and post-fixed for 2-3 h at 4°C. Tissues were sectioned into 30 μm coronal sections on a freezing-stages ledge microtome, and collected into 4 serially-ordered sets of sections. Sections were prepared for immunohistochemistry as described above [14, 22]. Immunofluorescence studies were carried out to determine expression of MC4R-GFP in IML and RVLM slices. Sections were pre-incubated in 0.01 M PBS and 2% normal donkey serum followed by incubation for 48 h with primary antibody to GFP in chicken (1:1000) in 0.01M PBS containing 0.5% Triton-X 100 at 4°C. Slices were then washed with 0.01 M PBS 3 times for 10 minutes and incubated with Alexafluor 488-conjugated anti-chicken IgG (1:1000) for 2 h; then they were washed several times at room temperature. After a final wash, sections were mounted onto slides and cover slipped with mounting media. Negative controls for all immunohistochemical reactions omitted the primary antibody. All negative controls lacked cellular staining.

To identify immunohistochemical co-localization of MC4R-GFP and tracheal wall-related neurons, an Olympus IX81 photomicroscope was used. Borders of the RVLM as defined in The Mouse Brain Atlas [23] were superimposed on each drawing. Double labeled neurons were merged by using Adobe Photoshop. These data were obtained from at least 4-5 sections.

Results

Distribution of pseudorabies virus-614 labeled neurons

Transsynaptically and retrogradely labeled PRV-614 immunoreactive neurons were distributed in the intermediolateral cell column (IML) and ventral horn (VH) of spinal cord (**Figure 1**) and throughout the medulla (**Figure 2**). Stained neurons were present in autonomic premotor
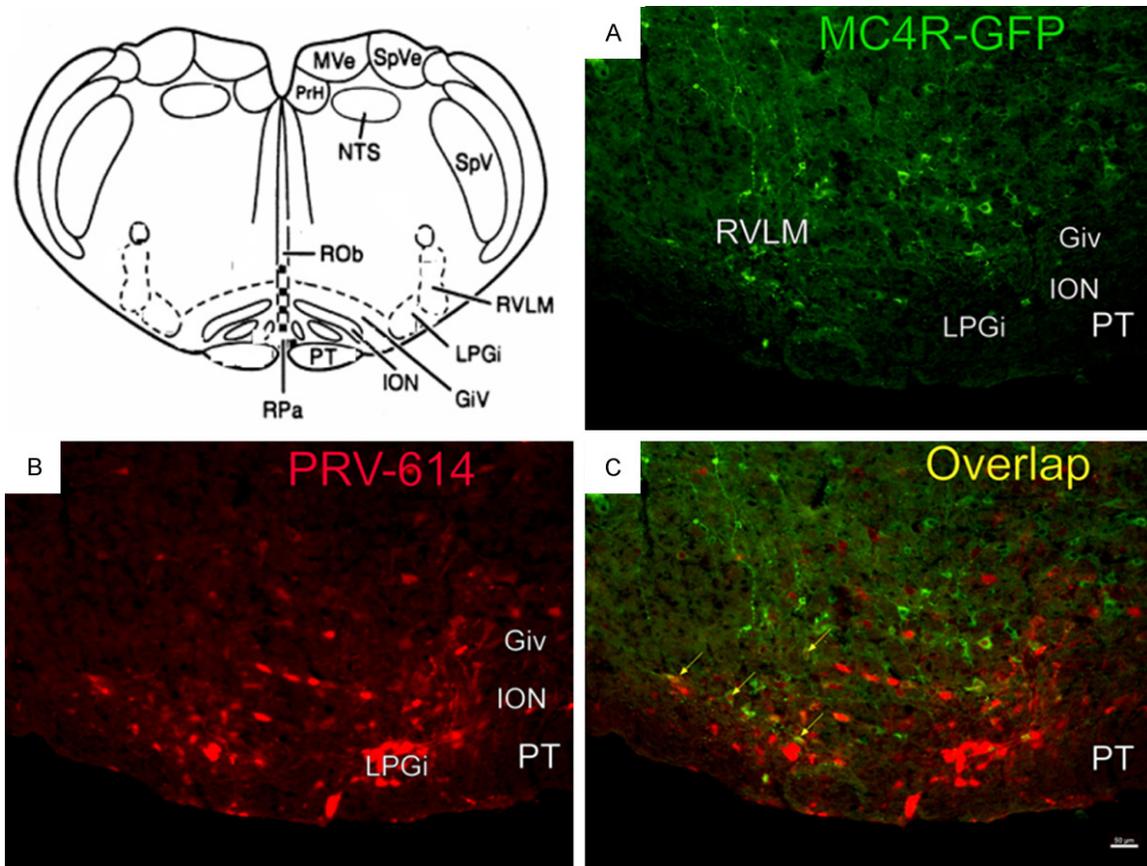
Figure 2. Sympathetic and melanocortinergic neurons in rostroventrolateral reticular nucleus (RVLM). We seek to map the melanocortin-sympathetic pathway between tracheal wall and RVLM region in MC4R-GFP transgenic mice by using retrograde tracing techniques of PRV-614. Drawings in the left-upper column indicate the locations of anatomical regions shown to the right and lower panel. Images (A-C) were taken from an animal that survived 6 d after injections of PRV-614. (A) Showed MC4R-GFP positive neurons in the STN; (B) Showed neurons infected with PRV-614, which send transsynaptic projections to the tracheal wall; (C) Showed overlaid images of A plus B. Arrows (yellow) indicate double-labeled neurons. GiV, Gigantocellular reticular nucleus, ventral part; ION, Inferior olivary nucleus; LPGi, Lateral paragigantocellular reticular nucleus; PT, Pyramidal tract; RVLM, Rostroventrolateral reticular nucleus; Scale bars, 100 μm. Some drawings were taken from S Hadziefendic (J Auton Nerv Syst 1999).

areas of the brain including the rostroventrolateral medulla (RVLM), the nucleus raphe pallidus, nucleus raphe obscurus, nucleus raphe magnus and gigantocellular reticular nucleus (Gi). The infection stage following inoculation of the peripheral airway with PRV-614 was consistent with previously published results using PRV Bartha [20, 21].

Distribution of PRV-614/MC4R-GFP double-labeled neurons

MC4R-GFP immunoreactive neurons were found in the IML of spinal cord (**Figure 1**) and throughout the caudal medulla, localized to the midline raphe and adjacent reticular formation (**Figure 2**). The greatest numbers of stained neurons were found in the RVLM, nucleus raphe magnus, raphe pallidus and raphe obscures. Most PRV-614/MC4R-GFP double-labeled neurons were located in sections through IML (**Figure 1C**) and RVLM (**Figure 2C**).

Discussion

We used transsynaptic retrograde transport of PRV-614 combined with immunoreactivity for MC4R to locate the premotor neurons innervating the peripheral airways. Two major findings have emerged from this investigation: (1) a large number of GFP-positive neurons were located in the IML and RVLM; (2) most of the GFP-positive neurons in the IML and RVLM were PRV-614-immunoreactive.
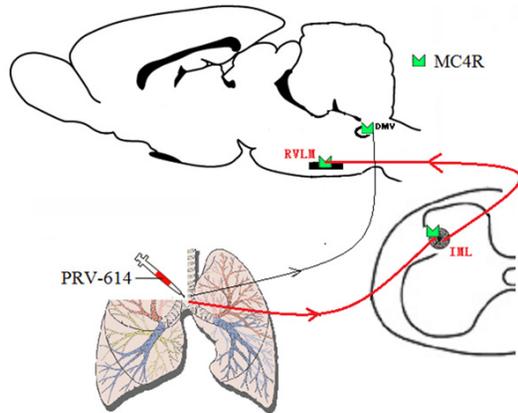
Figure 3. Summary diagram showed that the sympathetic pathway between the RVLM and the tracheal wall. Injection of PRV-614 into the tracheal wall resulted in retrograde infection of neurons in the DMV by parasympathetic pathway (shown in black line), and IML and RVLM by sympathetic pathway (shown in red line). PRV-614/MC4R-GFP dual-labeled neurons were detected in the IML, RVLM and DMV. DMV, dorsal motor nucleus of the vagus nerve; IML, intermediolateral column; MC4R, melanocortin-4 receptor; RVLM, rostroventrolateral reticular nucleus. Some drawings were taken from HB Xiang (Brain 2013).

The regions of RVLM reported by Kerman et al [24] and Lee et al [25] were collective structures consisting of the triangular region at the ventral surface of the brainstem ventral to nucleus ambiguous and lateral to the inferior olive. Gowen et al indicated that the RVLM was defined as the region of the reticular formation from the ventral surface of the brainstem, lateral to the inferior olivary nucleus but medial to the spinal trigeminal nucleus [1]. In this study, the RVLM areas we termed were consistent with the prior reported by Kerman et al [24] and Lee et al [25]. Previous evidence showed that the CNS inputs to the parasympathetic preganglionic cells that innervate peripheral airways originate mainly from regions of the lateral and the medial ventral medulla [26-28]. In this study, we provided evidence linking RVLM neurons to the control of airway function by sympathetic signaling (**Figure 3**), which were also in line with previous studies in which the RVLM provide drive to vasomotor sympathetic premotor neurons [26].

A large body of evidence has shown that MC4R is an important regulator of energy homeostasis and is broadly expressed in the CNS [9], so, we ascertained whether MC4R-GFP-positive neurons located in the IML and RVLM expressed airway-related mouse neurons. Our study showed that most of the GFP-positive neurons in the IML and RVLM were PRV-614-immunoreactive, suggesting that RVLM and IML may implicate controlling peripheral airways by melanocortinergic signaling (**Figure 3**).

In our knowledge, this is the first study in mice that has identified RVLM innervating MC4R-GFP positive sympathetic preganglionic neurons that regulate functions of peripheral airways, using the transsynaptic transmission of PRV-614 via sympathetic preganglionic neurons. Based on all these findings, we speculate that RVLM-IML pathway may play a major role in controlling peripheral airways by melanocortinergic- sympathetic signaling.

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Disclosure of conflict of interest

None.

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