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Original Article

Identification of neuroanatomic circuits from spinal cord to stomach in mouse: retrograde transneuronal viral tracing study

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Abstract: To determine the spinal innervation and neuronal connections is important for studying gastric carbohydrate metabolism and motor responses. Neurons involved in the efferent control of the stomach were identified following visualization of pseudorabies virus (PRV)-614 retrograde tracing. PRV-614 was injected into the ventral stomach wall in 13 adult C57BL/6J strain male mice. On the fifth day postinjection, animals were humanely sacrificed, and spinal cords were removed and sectioned, and processed for PRV visualization. The virus injected into the ventral stomach wall was specifically transported to the thoracic spinal cord. At 5 d after injection of the PRV-614, stomach enlargement and tissue edema were found, and PRV-614 positive cells were found in the intermediolateral cell column, the intercalates nucleus or the central autonomic nucleus of spinal cord segments T3 to L1, and major PRV-614 labeled cells were focused in the T6-10 segment. Our results revealed neuroanatomical circuits between stomach and the spinal intermediolateral cell column neurons.

keywords: Stomach, spinal cord, autonomic nervous system, pseudorabies virus, transsynaptic tracing

Introduction

The sympathetic nervous system of the stomach is the important neural mediator in the control of gastric secretory and motor responses [1-3]. Although information about the gastric sympathetic preganglionic neurons (second order motor neurons) within the spinal cord has been found on the rat by pseudorabies virus (PRV) [4], which is a neurotropic α-herpes virus used for the anatomical tracing of synaptically linked neural circuits after peripheral or central inoculation [5-7], and strain H129 of herpes simplex virus-1 [8], it isn't clear in this regard of the mouse. Previous studies have proved the high and specific value of PRV tracing for describing neuroanatomical pathways to study central neural networks, including the central control of the stomach [4, 9], however, little is known about the characterization of spinal cord segments dominating the stomach. Recently, our laboratory has used PRV to gain useful information about neural circuits associated with the sympathetic nervous systems [10-17]. 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. The aim of this study was to provide morphological evidence of the neuroanatomical circuitry between spinal cord and stomach in the mouse, by PRV-614 mediated transsynaptic retrograde tracier.

Materials and methods

Animals

Adult male C57BL/6J strain mice weighing between 25 and 30 g, maintained in a standard 12-h light, 12-h dark cycle with *ad libitum* access to food and water. All animal treatments and procedures conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee

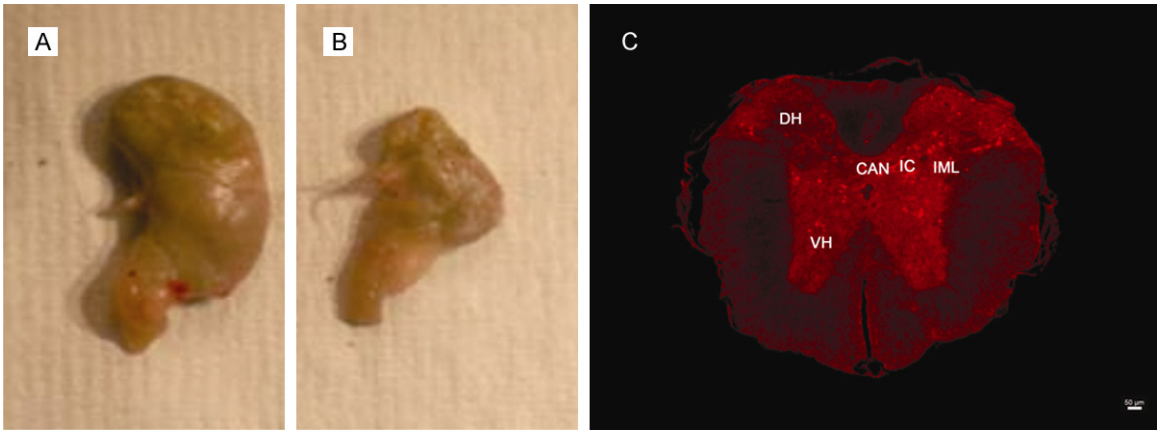

Figure 1. Stomach and thoracic spinal cord at 5 d post-injection of the ventral stomach wall. A: Enlarged stomach and tissue edema in experiment group (survival times = 5 days). B: No change in the stomach morphology in control group. C: Transverse section of T9. Red fluorescence conjugated to the viral vector (PRV-614 infected neuron) shows obvious labeling of ipsilateral IML, the intercalates nucleus (IC) and central autonomic nucleus (CAN). Scale bar 50 μm for C. DH, Dorsal horn; VH, ventral horn.

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PRV-614 injection

The PRV-614 was generously donated by Dr. Lynn Enquist (Princeton University, Princeton, NJ, USA). The final titer was 2×10^8 plaque-forming units/ml for PRV-614. Aliquots (20 μl) of the virus were kept in the freezer (-80°C), and on each experimental day, an aliquot was thawed and kept on ice until injected. PRV-614 in excess was inactivated with alcohol and discarded.

Mice were anesthetized with isoflurane (1.5-2%) and the surgery was performed aseptically. A small incision in the skin was performed in order to extricate the stomach. The PRV-614 was injected with a 30-gauge needle connected to a Hamilton syringe (10 μl) inserted into the muscle layers of the ventral stomach wall (2×10^8 pfu/ml in a total of 1 μl per injection at three injection sites) under microscopic guidance. After every injection, the needle was kept in situ for 2 minutes in order to limit the spread of PRV-614. After withdrawal of the needle, pressure was applied to the injection site using a cotton tip to prevent any eventual leakage of the inoculum. The wounds were sutured with sterile surgical silk. The time course of infection was empirically determined by carefully observing the pattern of infection at exactly 3 d (n = 4), 4 d (n = 4) and 5-d (n = 5) survival times. Otherwise, three mice were injected with 0.9% saline into the ventral stomach wall (1 μl per injection at three injection sites) as control group (survival time = 5 days).

Fluorescence immunohistochemistry and tissue analysis

The animals were then killed under deep anesthesia with ketamine hydrochloride and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde-borate fixative (pH 9.5). Exploration of the stomach was done. Spinal cords were removed via dorsal laminectomy and postfixed for 2 h in 4% paraformaldehyde-borate and overnight in a 30% sucrose solution at 4°C. Postfixed spinal cords were sliced into 30 μm coronal sections on a freezing-stage sledge microtome, and collected into four serially ordered sets of sections.

PRV-614 infected neurons express the red fluorescent protein for direct visualization under fluorescence microscope using a technique described previously [11, 18]. The PRV-614-IR neurons were counted under the 20 × objective of a fluorescence microscope on both sides on all sections in each series. The number of neurons expressing PRV-614 per section was assessed for each animal.

Results

After transcardial perfusion, and harvesting of the entire stomach was explored, showing an

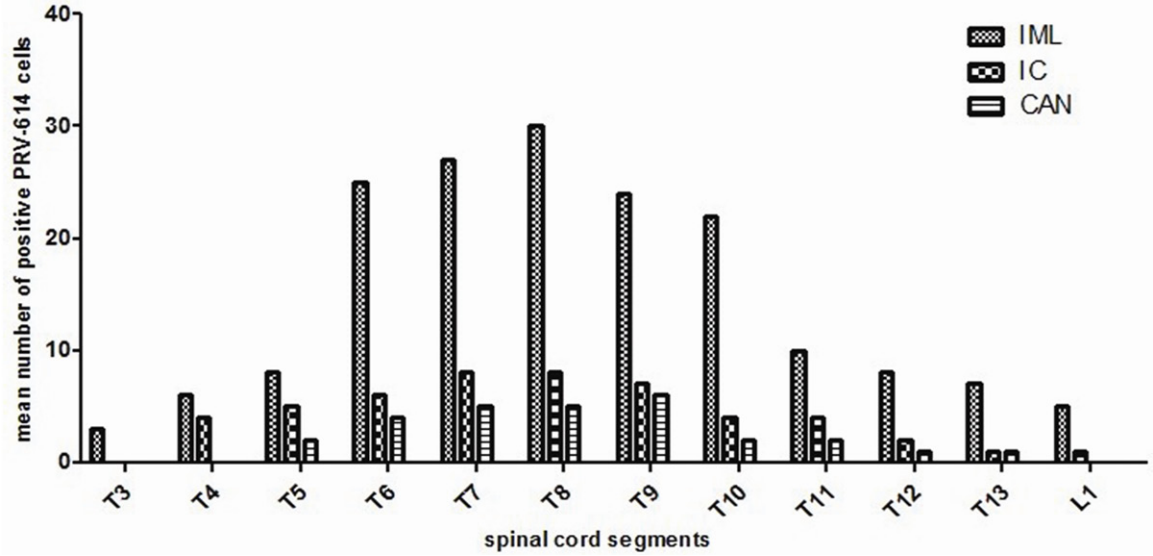

Figure 2. Levels of PRV-614 positive labeling 5 d after PRV-614 injection into the ventral stomach wall. IML, intermediolateral cell column. IC, nucleus intercalates spinalis. CAN, central autonomic nucleus.

enlarged stomach in 9 animals in experiment group (n = 13). In 4 animals that were sacrificed after 3 d, no significant changes in stomach morphology were found. In the remaining 4 animals (survival times = 4 days) signs of stomach enlargement were evident. In all animals sacrificed after 5 d, stomach enlargement and tissue edema were found (**Figure 1A**). In control group (survival times = 5 days), no changes in the stomach morphology were found (**Figure 1B**).

PRV-614 positive neurons were observed in bilateral sympathetic regions of the thoracic and upper lumbar spinal cord in all mice after stomach wall inoculation. Neural infection in the spinal cord always was more prominent on the intermediolateral cell column (IML), where sympathetic preganglionic neurons that project to the ventral stomach wall are located [19]. At the earlier survival time (3-4 d) after injection of the PRV-614, infection was restricted in the bilateral IML. As the infection progressed, some infected neurons were also observed between the intercalates nucleus (IC) and the central autonomic nucleus (CAN) (**Figure 1C**), and PRV-614-infecting cells were most heavily concentrated in IML and were distributed sparsely in IC and CAN. At 5 d, PRV-614 positive cells were found in IML, IC and CAN of thoracic spinal cord segments T3 to L1, and major PRV-614 labeled cells were focused in the T6-10 segment (**Figure 2**).

Discussion

In this study, we report the distribution of PRV-614-positive neuron in the autonomic centers of the spinal cord. Three major findings have emerged from this investigation: 1) stomach enlargement and tissue edema were found 5 days after PRV-614 inoculation; 2) the vast majority of neurons infected by retrograde transneuronal transport of PRV-614 from the stomach expressed red reporters, and 3) neurons that participate neural regulation of the stomach exist in spinal regions but were mainly concentrated in the IML of T6-10.

PRV-614 injected into the ventral stomach wall was taken up by enteric neurons and also directly by sympathetic axon terminals in celiac ganglion [9], then was retrogradely transported to the spinal cord. The spinal labeling reported in the study of Rinaman et al. after PRV-Bartha injection into the rat stomach [4] and our findings are very similar. The main same point between the two studies was that PRV-positive neurons were observed bilaterally in IML. However, the main difference between the two studies was that they did not observe the spinal cord segments of most PRV infected neurons of the IML. We found PRV-614 labeled neurons in IML of spinal cord segments T4 to L1, and most infected cells were focused in T6-10. Our data were also consistent with the prior demonstrations that gastric spinal sympathetic afferents

terminate in the mid-thoracic to upper lumbar spinal cord [20, 21].

Autonomic nerve activity controls the functions of the stomach by regulating of information from the stomach to the brain [22, 23]. A considerable amount of literature had demonstrated that sympathetic nervous system played an important role in the regulation of gastric function [23, 24]. It was reported that the mechanisms of stimulatory effect of the sympathetic trunk on gastric motor activity, and gastric contractions were augmented by preganglionic serotoninergic fibers related synaptically to serotoninergic neurons [25]. Our data further provided the morphological evidence of the neuroanatomical circuitry between stomach and the spinal IML in the mouse.

In this study, 5 days after PRV-614 inoculation, animals showed an enlarged stomach enlargement and tissue edema. This phenomenon may be hypothesized as the immune response of PRV-614 infected gastric neuron and the activation of a neighboring uninfected neuron [26], suggesting that the activated neuron released inflammatory mediators which caused vasodilatation and increased vasopermeability with subsequent tissue edema. This was also in line with previous study in which PRV infected cells may influence neighboring cells and cause sympathetic activation [27].

In conclusion, our results revealed neuroanatomical circuits between stomach and the spinal intermediolateral cell column neurons.

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Disclosure of conflict of interest

None.

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