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Original Article

Neuroanatomical autonomic substrates of brainstem-gut circuitry identified using transsynaptic tract-tracing with pseudorabies virus recombinants

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Abstract: To investigate autonomic substrates of brainstem-gut circuitry identified using trans-synaptic tracing with pseudorabies virus (PRV)-152, a strain that expresses enhanced green fluorescent protein, and PRV-614, a strain that expresses enhanced red fluorescent protein, injecting into the rat rectum wall. 3-7 days after PRV-152 injection, spinal cord and brainstem were removed and sectioned, and processed for PRV-152 visualization using immunofluorescence labeling against PRV-152. 6 days after PRV-614 injection, brainstem was sectioned and the neurochemical phenotype of PRV-614-positive neurons was identified using double immunocytochemical labeling against PRV-614 and TPH. We observed that the largest number of PRV-152- or PRV-614-positive neurons was located in the gigantocellular reticular nucleus (Gi), lateral paragigantocellular (LPGi), rostral ventrolateral reticular nucleus (RVL), solitary tract nucleus (Sol), locus coeruleus (LC), raphe magnus nucleus (RMg), subcoeruleus nucleus (SubCD). Double-labeled PRV-614/tryptophan hydroxylase (TPH) neurons were concentrated in the RMg, LPGi and Sol. These brainstem neurons are candidates for relaying autonomic command signals to the gut. The autonomic substrate of brainstem-gut circuitry likely plays an important role in mediating different aspects of stress behaviors.

Keywords: Brainstem-gut circuitry, transsynaptic tract-tracing, pseudorabies virus

Introduction

Knowledge on the neural circuit bases of brainstem-gut crosstalk can help us to explain many mechanisms associated with the bidirectional communication system between the central nervous system and the gastrointestinal tract, e.g., enteropathy and neurological function or certain psychiatric conditions [1-4]. Chronic rectal pain syndromes are therapeutically challenging because both physical and drug therapy management often are ineffective by pain physicians [5]. There is growing evidence that the neuronal connections to the rectum are important for studying the pathogenesis and treatment of digestive symptoms and chronic rectal syndrome [6-13]. The recent observation provided the interaction between irritable bowel syndrome and cortico-basal ganglia-thalamocortical loops [7, 14]. A major challenge in the cerebral neuronal connections to the rectum is to unravel the complex matrix of functional connections that characterize neuroanatomical loops or circuits within the central nervous system [15].

Retrograde transneuronal transport of pseudorabies virus (PRV) has proven to be especially useful to unravel multi-synaptic pathways within autonomic circuits [16-28]. There is general agreement that a self-amplifying virus tracer PRV-152, a strain that expresses enhanced green fluorescent protein (GFP), and PRV-614, a strain that expresses enhanced red fluorescent protein (RFP), have been successfully used as transneuronal tracers in the central nervous system [17, 20-22, 29-42]. Previous physiological investigations have suggested the existence of the brain-gut axis that coordinates the crosstalk of enteric and central nervous system [43-45]. Traditionally these brain-gut crosstalks have been postulated to be mainly involved in

Table 1. distribution of PRV-152-labeled neurons following injection into the rectum wall

Areas	PRV-152-positive neurons				
	d3	d4	d5	d6	d7
Spinal cord	+/-	+	+	+	
RPa	+/-	+	+	++	+
RVMM (RMg+LPGi)	+/-	+	++	+++	++
RVL		+	++	+++	+
A5		+	+	+	+
Sol		+	+	++	+/-
DMV		+/-	+	+	+/-
Amb		+/-	+	+	+/-
LC		+	++	+++	+
SubCD		+	++	+++	+
Gi		+/-	++	++	+/-
Icp		+/-	+	+	+/-
LPN		+	+++	+++	+
PRN		++	++	++	+/-
PAG, ventrolateral		++	++	+++	+
PAG, dorsal		+	++	++	+

The number of PRV-152-positive neurons was qualitatively estimated in the whole brains of mice. Semi-quantitative estimates of the signals are indicated as follows: +++ (high: more than 20 PRV-152-positive neurons per brain section); ++ (moderate: between 10 and 20 PRV-152-positive neurons per brain section); + (low: less than 10 PRV-152-positive neurons per brain section); +/- (low, inconsistent staining across the animals: less than 5 PRV-614-positive neurons per brain section in most animals but with no staining observed in some animals); and - (No PRV-152-positive neurons). Amb: the nucleus ambiguous, LC: locus coeruleus. Gi: the gigantocellular reticular nucleus, LPGi: lateral paragigantocellular, RVL: rostral ventrolateral reticular nucleus, Sol: solitary tract nucleus, RMg: raphe magnus nucleus, SubCD: subcoeruleus nucleus, RPa: raphe pallidus nucleus, Icp: inferior cerebellar peduncle, PAG: periaqueductal gray, LPN: lateral parabrachial nucleus, PRN: Pontine reticular nucleus.

neuropeptide and neurotransmitter, e.g. brain-gut peptide, 5-HT, etc. There are no data about brainstem modulating the rectal function. The aim of this study was to identify the autonomic substrates of brainstem-gut circuitry identified using trans-synaptic tracing with pseudorabies virus recombinants PRV-152 and PRV-614.

Material and methods

Animal maintenance and care

Adult male Sprague Dawley rats (200-250 g body weight) were maintained in a standard 12-h light, 12-h dark cycle with ad libitum access to food and water. After PRV-152 or PRV-614 injection, they were housed individually. 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 local Animal Care and Use Committee.

Virus injections, perfusion and tissue preparation

After rats were anesthetized with a mixture of ketamine and xylazine, the anterior wall of the rectum at a distance of 2 mm from the anal verge was exposed for injection under direct vision. 2 μl injections of PRV-152 (PRV-152 injection group, n=16) or PRV-614 (PRV-614 injection group, n=3) was injected into the rectum wall (0.5 μl per injection at 4 injection sites per rat) using a 30-gauge needle connected to a Hamilton syringe (10 μl) under microscopic guidance [35, 36, 46-48]. After each injection site was swabbed with a cotton-tip applicator to minimize nonspecific viral spread [48].

In PRV-152 group, the time course of infection was empirically determined by carefully observing the pat tern of infect ion at exactly 3 d (n=3), 4 d (n=3), 5 d (n=4), 6 d (n=3) and 7 d (n=3) survival times. After a survival time of 3-7 d (PRV-152 group), or after survival for 6 days (PRV-614 group), the animals were deeply anesthetized and euthanized with an injection of sodium pentobarbital [49, 50], and perfused through the heart with 100 ml of 0.9% saline solution, followed by 200 ml of 4% (W/V) paraformaldehyde made in 0.1 mol/L sodium phosphate buffer (pH=7.4). After the brain tissues were embedded in optimal cutting temperature (OCT) compound, series of transverse sections were cut at 30 μm-thick using a freezing microtome as groups of 4 sections per sample. As a note, this study focused on transverse sections from the spinal cord and brainstem region.

Fluorescence immunohistochemistry and tissue analysis

PRV-152-infected neurons express the green fluorescent protein and PRV-614-infected neurons express the red fluorescent protein for direct visualization under fluorescence microscope. A band pass filter for Alexa 488 (excitation of 500 nm, emission of 535 nm) was used to identify cells infected by PRV-152. The red

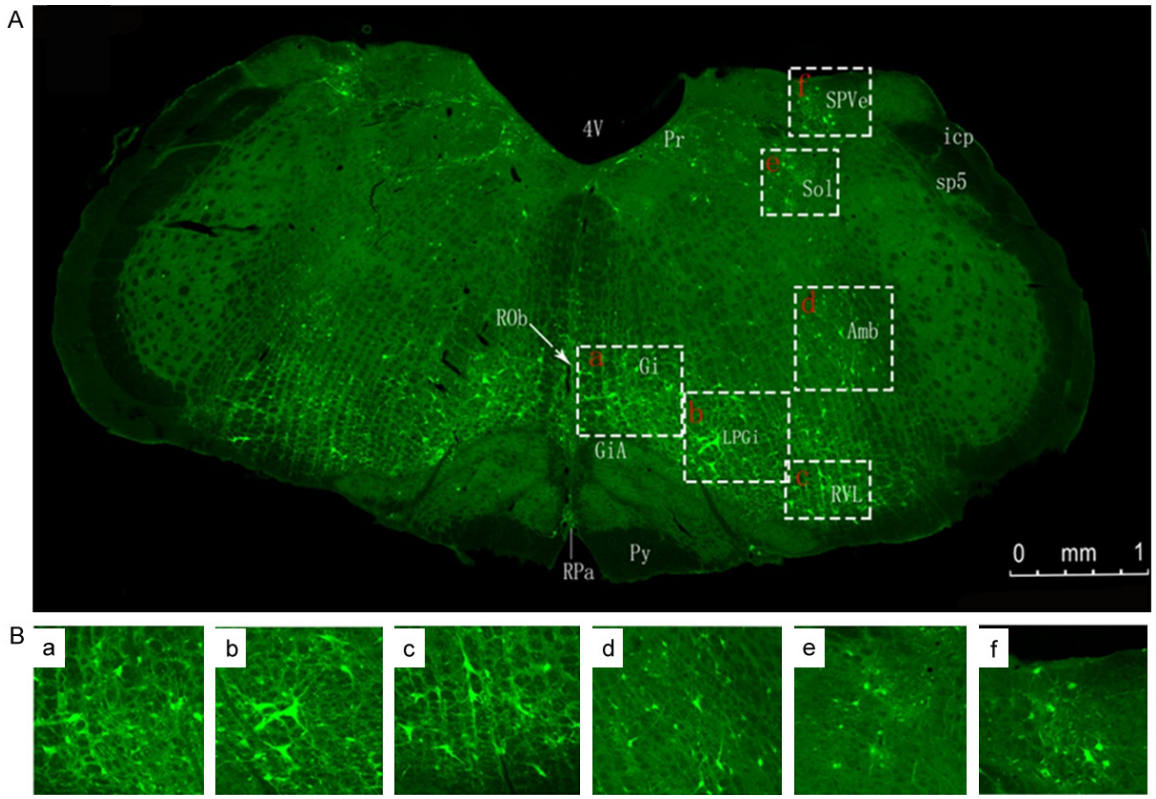

Figure 1. Micrographs of transverse sections from the medulla oblongata 6 days after PRV-152 injection. (A) Coronal medullary section (Bregma -11.64 mm). (Ba-Bf) High-magnification, split-channel images of the corresponding Gi, LPGi, RVL, Amb, Sol and SPVe area, respectively, shown in (A), illustrating a cluster of virally labeled (green) neurons. 4 V, 4th ventricle. Amb, ambiguus nucleus. Gi, gigantocellular reticular nucleus. GiA, gigantocellular reticular, alpha. Icp, inferior cerebellar peduncle. LPGi, lateral paragigantocellular. Py, pyramidal tract. pPr, prepositus nucleus. Rob, raphe obscurus nucleus. RPa, raphe pallidus nucleus. RVL, rostral ventrolateral reticular nucleus. Sol, solitary tract nucleus. SPVe, SpVe spinal vestibular nucleus. Scale bar 1 mm for (A).

fluorescence of CY3 was used to identify cells infected by PRV-614, whereas the green fluorescence of Alexa Fluor 488 was used to identify neurons containing 5-HT, and the blue fluorescence of Alexa Fluor 350 was used to identify TPH-containing neurons. Images were overlaid using Adobe Photoshop, and double-labeled neurons were presented as yellow or pink. The neuroanatomical nomenclature is defined from the rat atlases of Paxinos and Franklin [51]. Photographs were taken with a fluorescence microscope Olympus IX81 (Olympus, Tokyo, Japan) and the resulting TIFF files were imported into the software (Version 10, ACD Systems, Inc.).

Results

Temporal pattern of multisynaptic projections after PRV-152 injection into the rectum wall

Animals used in this study (n=15) were arranged into five groups based on their survival times (**Table 1**): earliest survival group (survival time=3 days, n=3), short survival group (4 days, n=3), intermediate survival group (5 days, n=3), late survival group (6 days, n=3) and latest survival group (7 days, n=3). Initial analysis focused on qualitative characterization of areas that contained PRV-152-infected neurons.

Injection of PRV-152 into the rectum wall resulted in the uptake, replication, and transsynaptic passage of the virus through circumscribed groups of neurons. The central distribution of PRV-152-immunolabeled neurons generally increased with extended post-injection survival times, although the presence and extent of central PRV-614 labeling varied among individual cases within each survival time group (**Table 1**). At survival times of 3-7 d, no cellular damage or lysis, which could result in the release of PRV-152 into the extracellular space and the nonspecific spread of the virus, was detected in infected neurons, except in the spinal cord at the longest survival times (>6 d).

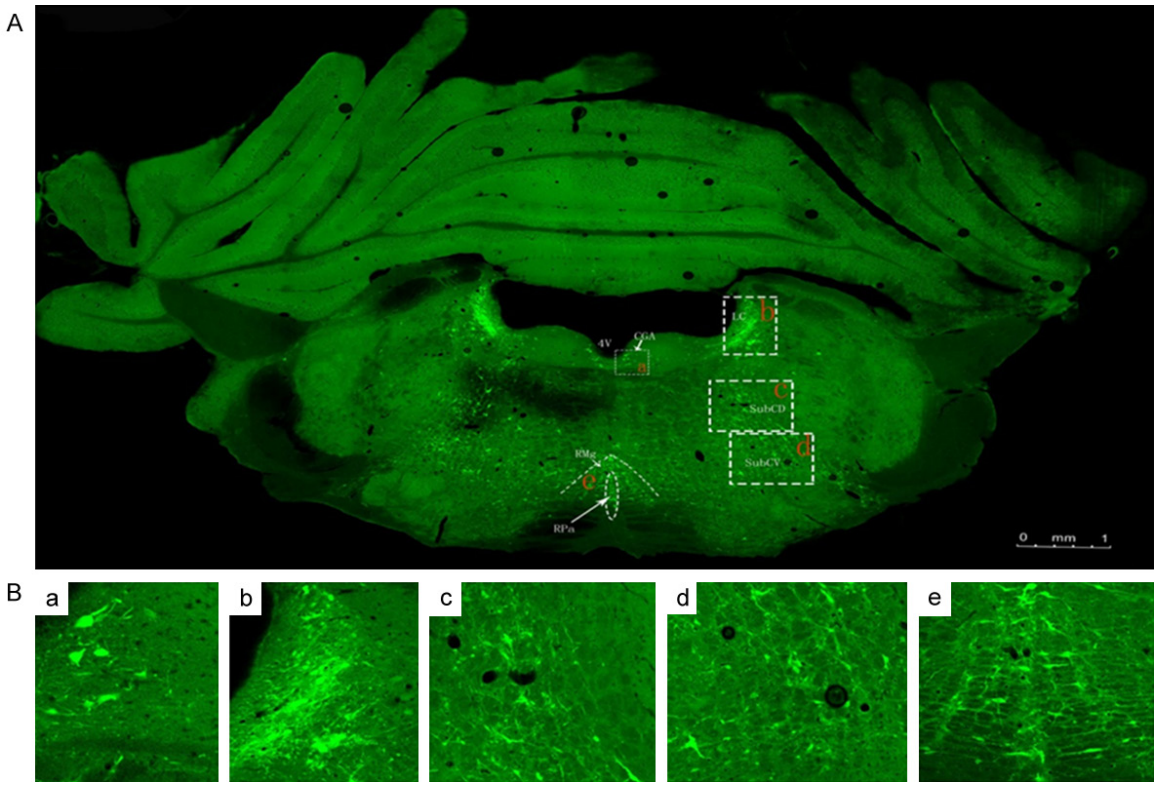

Figure 2. Micrographs of transverse sections from the locus coeruleus 6 days after PRV-152 injection. (A) Coronal medullary section (Bregma -9.84 mm). (Ba-Be) High-magnification, split-channel images of the corresponding CGA, LC, SubCD, SubCV and RMg area, respectively, shown in (A), illustrating a cluster of virally labeled (green) neurons. 4 V, 4th ventricle. CGA, central gray, alpha part. LC, locus coeruleus. RMg raphe magnus nucleus. RPa, raphe pallidus nucleus. SubCD, subcoeruleus nucleus, dorsal part. SubCV subcoeruleus nucleus, ventral part. Scale bar 1 mm for (A).

6 days after PRV-152 injection into the rat rectum wall, transsynaptically and retrogradely labeled PRV-152-immunoreactive (green) neurons were distributed throughout the medulla (**Figures 1** and **2**). The greatest number of stained neurons was found in the gigantocellular reticular nucleus (Gi), lateral paragigantocellular (LPGi), rostral ventrolateral reticular nucleus (RVL), solitary tract nucleus (Sol), locus coeruleus (LC), raphe magnus nucleus (RMg), subcoeruleus nucleus (SubCD). Labeling in the raphe pallidus nucleus (RPa), ambiguus nucleus (Amb) and inferior cerebellar peduncle (Icp) was less intense (**Figure 1Bd** and **1Bf**).

Infection of serotonergic neurons 6 days after PRV-614 injection into the rectum wall

6 days after PRV-614 injection into the rectum wall, PRV-614-labeled neurons were distributed throughout the brainstem (**Table 2**). The greatest number of PRV-614-immunoreactive stained neurons was found in the RMg, LPGi, RVL, LC, SubCD, and PAG region (**Table 2**). We defined the rostral ventromedial medulla region as including the RMg and LPGi. Most TPH-positive cells were found in the Rpa, RVM, RVL, LC and PAG, whereas fewer TPH-positive neurons found in the RMg, Sol DMV, and PRN (**Table 2**). Double-labeled PRV-614-/TPH-positive neurons were mainly located in the RVM area, and 66.7% of the virally infected neurons in the RVM were also TPH-immunoreactive (**Table 2**).

Discussion

The current study exploited the functions of PRV-152 and PRV-614 from the rectum wall as trans-synaptic tracing agents that are capable to infect the brainstem neuron. Consensus exists on the importance of the brain-gut axis in affecting the clinical outcome after gastrointestinal disorder [1, 52]. There is growing evidence that the neuronal networks have a well-established role in coordinating the crosstalk of br-

Table 2. Quantitative analysis of double-labeled PRV-614-/TPH positive neurons 6 days after PRV-614 injection

Areas	PRV-614-positive neurons	TPH-positive neurons	% PRV-614-positive neurons co-expressing TPH	% TPH-positive neurons co-expressing PRV-614
RPa	++	+	25	50
RVMM	+++	++	36.4	66.7
RVL	+++	+	28.6	37.5
A5	+	+/-	*	*
Sol	++	+	9.1	16.7
DMV	+	+/-	*	*
Amb	+	+/-	*	*
LC	+++	+	12	42.9
SubCD	+++	+	18.2	66.7
Gi	++	++	35.7	45.5
LPN	+++	+/-	*	*
PRN	++	+/-	*	*
PAG	+++	+	7.4	25

The number of positive neurons per region from a 1:4 subset of sections: +/-, 1-5, but not in all mice; +, 6-10; ++, 11-20; +++, >20; *, some co-expression was observed, but the percentages were not calculated unless all mice showed co-expression. Amb: the nucleus ambiguous, LC: locus coeruleus. Gi: the gigantocellular reticular nucleus, LPGi: lateral paragigantocellular, RVL: rostral ventrolateral reticular nucleus, Sol: solitary tract nucleus, RMg: raphe magnus nucleus, SubCD: subcoeruleus nucleus, RPa: raphe pallidus nucleus, PAG: periaqueductal gray, LPN: lateral parabrachial nucleus, PRN: Pontine reticular nucleus.

ain and gut. Our observations suggest that several areas may participate in the integration of brainstem-gut circuitry. It is known that the spread of the PRV infection is an indicator of neuronal interconnectivity [53-56]. Many areas labeled, e.g., RVM, Sol, LC and PAG, have an important role in the regulation of autonomic nervous system. The data presented showed a broad central representation of autonomic efferent neurons involved in rectum control. To our knowledge, this is the first description of CNS structures directly involved in rectal neuronal control.

It is known that the Sol have pivotal roles in the interpretation and relaying of peripheral information via sensory vagal afferent fibers [57-59]. We found many PRV-152-labeled neurons in the Sol of the dorsal vagal complex (DVC) within the brainstem, and these results supported previous tracing and neurophysiological investigations showing the Sol is well known for its role in viscerosensory processing [58], suggesting that signals from the rectum such as rectal distension are crucial in transmitting information via vagal afferents to the Sol in the caudal brainstem.

The rostral ventrolateral medulla (RVL) primarily regulates the autonomic nervous system [60, 61]. We also reported here the characterization of the polysynaptic connectivity from the rectum to PRV-152-labeled populations of neurons in RVL by using viral tracing system. Consistent with data from rats [10, 50, 53, 60], these neurons in RVL regions targeted the rectum involved in autonomic regulation.

It is demonstrated that tryptophan hydroxylase (TPH) is the rate-limiting enzyme in the synthesis of 5-HT in the CNS and has been used in the past as a measure of serotonin synthesis [62-67]. 5-HT-expressing neurons in the medulla oblongata are thought to form a “medullary 5-HT system” that regulates energy balance and potentially integrates autonomic functions, according to the physiological level [68]. There is a major projection of spinal serotonergic axons originating from an anatomically discrete group of 5-HT-expressing neurons in the medulla that constitutes the descending 5-HT pathway to the spinal cord [69, 70]. We demonstrated the presence of PRV-614/TPH dual-labeled neurons in the RVL, and RVM, suggesting that these TPH-positive neurons can project directly or indirectly to the rectum. Many anatomical studies have demonstrated that all TPH-immunoreactive neurons within the caudal raphe nuclei are also immunoreactive for

glutaminase, the synthesizing enzyme for glutamate [71, 72]. These TPH-/glutamate-immunopositive caudal raphe neurons project to many different regions of the neuroaxis, including the spinal cord [73, 74], and regulate autonomic outflow to the rectum via the sympathetic and parasympathetic preganglionic neuron. Together, the data from these studies in vivo and in vitro suggest that a subset of these medullary projection neurons transmits information about rectum-related internal stimuli and modulates the activity of the rectum by dual glutamatergic and serotonergic mechanisms.

Conclusion

It's confirmed the ability of PRV-152 and PRV-614 to retrogradely infect chains of trans-synaptically linked neurons and examined the locations of the brainstem neurons that innervate the rectum.

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Disclosure of conflict of interest

None.

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