

Original Article

Retrograde tracing of medial vestibular nuclei connections to the kidney in mice

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Abstract: Vestibular nuclei have been identified as a uniform multifunctional structure in order to maintain physiological homeostasis, including the participation of renal sympathetic activity. In this study, the medial vestibular nuclei (MVe) of 10 adult male C57BL/6J strain mice were mapped retrograde using injections of pseudorabies virus (PRV)-614. The virus, injected into the kidney, was specifically transported to the medial vestibular nuclei (MVe). We used a fluorescence immunohistochemistry to characterize the chemical neuroanatomical substrate of MVe innervating the kidney in the mouse. At five days after PRV-614 injection in the kidney, PRV-614 infected neurons were retrogradely labeled in MVeMC and MVePC; PRV-614/tyrosine hydroxylase (TH) double-labeled neurons located predominantly in MVeMC and not in MVePC, whereas PRV-614/tryptophan hydroxylase (TPH) neurons were not localized in MVeMC and MVePC. Our results revealed direct neuroanatomical evidence to identify catecholaminergic projections from the MVeMC to the kidney, suggesting that medial vestibulo-renal pathway may be catecholaminergic.

Keywords: Kidney, medial vestibular nuclei, retrograde tracing, pseudorabies virus

Introduction

Vestibular nuclei have been identified as a uniform multifunctional structure in order to maintain physiological homeostasis [1], including the participation of renal sympathetic activity [2]. A considerable amount of literature has demonstrated that the medial vestibular nuclei (MVe) play an important role in the sympathetic control of arterial blood pressure [3-5]. It has been shown that renal sympathetic innervations, which involve in the maintenance of fluid homeostasis, have a crucial role in kidney disease and hypertension [6-8]. Research on animal models as well as human subjects has demonstrated that the vestibular system contributes to regulating the distribution of blood in the body through effects on the sympathetic nervous system [9]. Previous studies demonstrated that responses of a particular sympathetic nerve to vestibular stimulation depend on the type of tissue the nerve innervates as well as its anatomic location [10]. These data suppose that MVe may involve in the mainte-

nance of renal vascular motor tone for balancing the volume and composition of body fluids.

Retrograde tracing techniques of pseudorabies virus (PRV) had become a very powerful tool to characterize multisynaptic neuroanatomic circuits from kidney to the CNS [11-14]. As far as we know, previous study had not involved the PRV-infected neurons in the medial vestibular nuclei after injection of PRV into the rat kidney. The medial vestibular nuclei divide into magnocellular (MVeMC) and parvicellular subfields (MVePC) [15]. In the present study, we used transgenic recombinants of a PRV-Bartha derivative, PRV-614, expressing a novel monomeric red fluorescent protein (mRFP1) under control of the cytomegalovirus immediate early promoter, for direct visualization under fluorescence microscope [14, 16-21], for retrograde-specific transneuronal tracing of multisynaptic pathways innervating the kidney. The aim of the present study was to provide morphological evidence of the neuroanatomical circuitry between MVe (MVeMC and MVePC) and kidney, by PRV-614

mediated transsynaptic retrograde tracing study [11, 12, 19, 21, 22].

Materials and methods

Animal care and use

Adult male C57BL/6J strain mice (25-30 g body weight) maintained in a standard 12-h light, 12-h dark cycle with *ad libitum* access to food and water. They were housed, two per cage, for at least 3 days before any manipulation. All experiments were approved by the Institutional Animal Care and Use Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology University.

PRV-614

PRV-614, a gift from Lynn W. Enquist (Princeton University, Princeton, NJ), is a specific transsynaptic retrograde tracer from axon terminals to the cell nucleus, where replication occurs. Viral stocks were aliquoted into 100- μ l volumes in 1-ml vials and stored at -80°C. On each experimental day, an aliquot was thawed and kept on ice until immediately before injections.

Surgical procedures, PRV injection and post-surgical care

To provide a description of the pattern and temporal progression of viral infection resulting from injection of PRV-614 into the kidney, virus was injected into the left kidneys of mice. First, the mice were anesthetized with isoflurane inhalation via a nose cone. A surgical plane of anesthesia was obtained such that spontaneous movement and withdrawal reflexes to foot pinch were absent. All surgical procedures were performed using aseptic techniques.

Under full anesthesia with isoflurane, the skin overlying the kidney was incised by an abdomen midline laparotomy to expose the upper pole of the kidney. 10 mice received a series of injections with PRV-614 into the upper pole of the visualized left kidney (2×10^8 pfu/ml in a total of 1 μ l per injection at five injection sites per kidney) using a 10-gauge needle connected to a Hamilton syringe (10 μ l) under microscopic guidance. After the final injection, the kidney surface was rinsed twice with sterile saline-soaked swabs, blotted dry, and then returned to the abdominal cavity. The abdominal muscle

incision was closed with silk sutures, and the skin incision was closed with stainless steel wound clips. The time course of infection was empirically determined by carefully observing the pattern of infection at exactly 3-d (n = 3), 4-d (n = 3) and 5-d (n = 4) survival times. The mice were provided with analgesia with an intramuscular injection of a mixture of ketamine (10 mg/kg) and ketoprofen (3 mg/kg) just before the surgery and every 12 h subsequently during a postsurgical period of 72 h. Animals were euthanized either before or when they showed apparent traits of illness or distress.

Perfusion and fluorescence immunohistochemistry

After a survival time of 3-5 d, mice were deeply anesthetized with an overdose of urethane and perfused with 0.9% saline, followed by 4% paraformaldehyde-borate fixative (pH 9.5) via the left ventricle. Brains were removed, postfixed in 4% paraformaldehyde-borate overnight at room temperature, and in a 30% sucrose solution for 2 d at 4°C. Postfixed brains were blocked, sliced into 30 μ m coronal sections on a freezing-stage sledge microtome, and collected into four serially ordered sets of sections. Tissue sections were stored at 4°C in cryoprotectant until they were processed for immunohistochemistry visualization.

One of every four series from each animal was subjected to the double immunofluorescent staining procedure that was performed using standard procedures for visualization of putative enzymes in virally infected neurons. [23-25] PRV-614 infected neurons express the red fluorescent protein for direct visualization under fluorescence microscope. Briefly, free floating tissue sections were incubated at 4°C overnight in 0.02 M potassium PBS (KPBS; pH 7.4) containing 2% normal donkey serum, and 0.4% Triton X-100 (LKPBS). The tissue sections were then rinsed several times in 0.01 M PBS over a 1 h period followed by incubation with Alexafluor 488-conjugated anti-chicken IgG (1:1000; Abcam) in KPBS containing 0.4% Triton-X-100 for 1 h at room temperature. For TPH IHC, first antibody was sheep anti-TPH (1:2000; Chemicon International, Temecula, CA) and second antibody included biotinylated donkey anti-sheep IgG (H+L) (Lot no.68003, Jackson Imm-

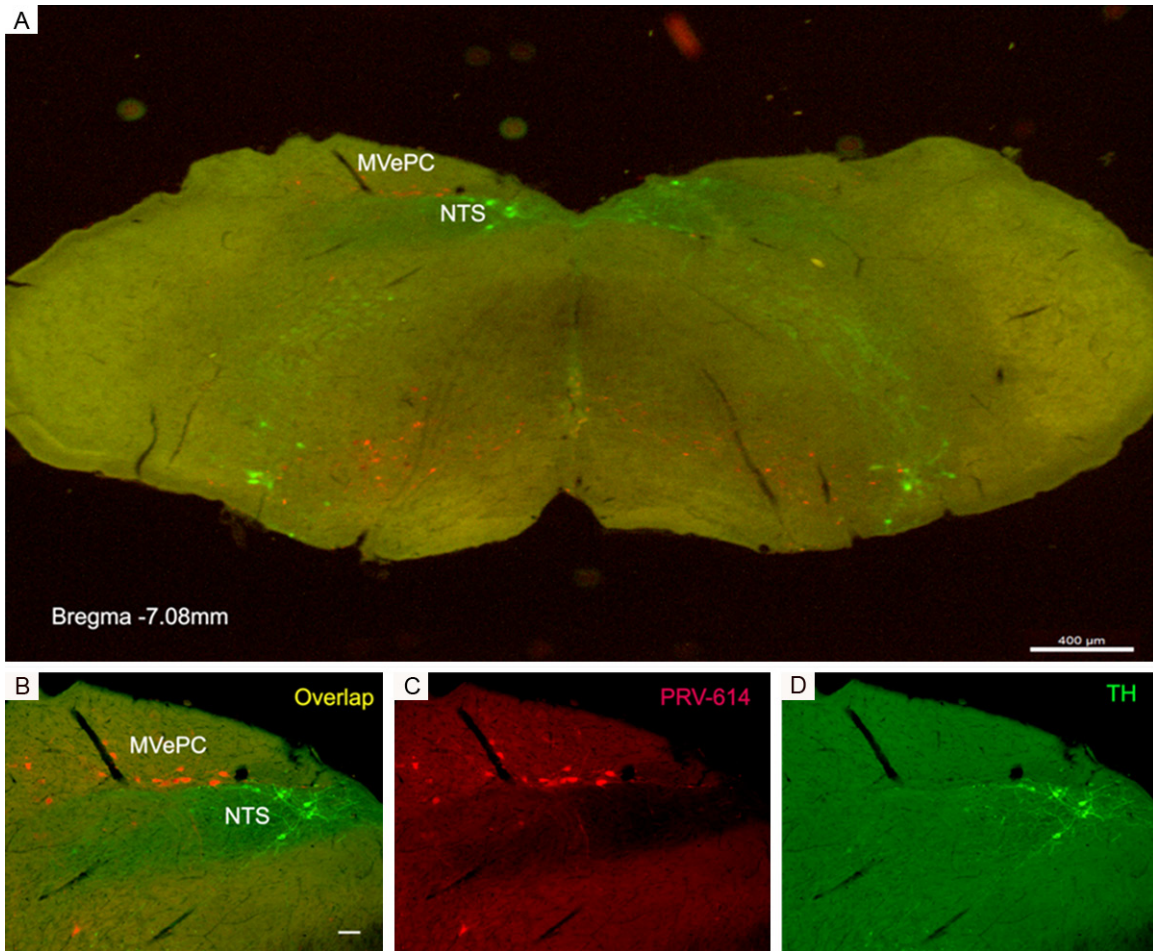


Figure 1. Fluorophor expression in caudal brainstem level at 5 d post-injection of the left kidney. (A) The distribution of PRV-614 infected and TH-immunoreactive neurons throughout the caudal brainstem is illustrated. (B) PRV-614-immunofluorescent cells does not co-localize within the TH-positive neurons. (C) PRV-614-immunofluorescent cells are present in the MVePC region. (D) TH-immunoreactive neurons and fibers are not present in MVePC region. Labeled neurons in MVePC were mapped to transverse sections from the mouse brain atlas of Franklin KB and Paxinos G. NTS: solitary nucleus, MVePC: the parvicellular medial vestibular nuclei. Bregma -7.08 mm. Scale bar 400 μ m for (A), 50 μ m for (B-D).

unoResearch Lab., West Grove, PA) and streptavidin Alexa Fluor 350 conjugate (Lot no.49248A, S-11249, Invitrogen, Molecular Probes, Eugene, OR). For TH IHC, first antibody was rabbit anti-TH (1:2000; Chemicon International, Temecula, CA) and second antibody was Cy3-biotinylated donkey anti-rabbit IgG (1:1000, Invitrogen, Molecular Probes, Eugene, OR). Sections were then rinsed thoroughly in 0.01 M PBS (3 \times 10 min). All sections were mounted onto gelatin-coated slides, air dried overnight, and coverslipped with mounting media. In negative control incubations, the primary antisera were omitted from the immunohistochemical reaction. This procedure completely eliminated neuronal staining.

Tissue analysis

Immunostained tissue sections were examined and photographed using an Olympus X81 photomicroscope equipped with epifluorescence and filters that selectively excited BODIPY-FL, CY3 or Fluor 350 and with a filter that allowed for the excitation of both fluorophors. The red 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 TH, 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.

High-magnification analysis was used to determine whether overlapping yellow or pink images were due to co-localization in the same neuron or an overlap of independently labeled neurons. The magnocellular and parvicellular sub-area of medial vestibular nuclei, in which infected or positive neurons, were located and defined by referencing the atlases of Franklin KB and Paxinos G [26].

Results

Mice that received PRV-614 injections in the left kidney had apparent outward signs of neurological deficiency throughout the survival period. All of the vestibular region and RVLM were examined to identify single- and double-labeled cells. Initial analysis focused on qualitative characterization of areas that contained double-labeled neurons, which were coexpressed with PRV-614 and TPH or TH.

Retrograde transneuronal infection of neurons in the vestibular nuclei mainly occurred in the magnocellular and parvicellular subdivisions of the MVe. Virus didn't find in the MVe on 3-4 d after PRV-614 injection into the kidney. Only scattered infected neurons were found in the MVe in animals analyzed four days following injection of virus into the kidneys. Five days following kidney injection we observed numerous infected neurons in the MVePC and MVeMC (**Figures 1A and 2A**). PRV-614/tyrosine hydroxylase (TH) double-labeled neurons located predominantly in MVeMC (**Figure 2B1-B3**) and not in MVePC (**Figure 1B-D**), whereas PRV-614/tryptophan hydroxylase (TPH) double-labeled neurons were not localized in MVeMC and MVePC (data not shown). Otherwise, PRV-614/tyrosine hydroxylase (TH) double-labeled neurons also located in RVLM (**Figure 2C1-C3**)

Discussion

Pseudorabies virus (PRV)-614 can propagate exclusively between connected neurons by strictly unidirectional (retrograde) transneuronal transfer without altering neuronal metabolism, due to its ability to function as a self-replicating marker [27, 28]. The principal findings of this study were as follow: (1) PRV-614 infected neurons were retrogradely labeled in the MVeMC and MVePC after tracer injection in the kidney; (2) PRV-614/TH double-labeled neu-

rons co-localized in the MVeMC not MVePC; (3) PRV-614/TPH double-labeled neurons did not found in the MVeMC and MVePC. These results presented here extended the role of the MVe in the regulation of renal sympathetic function by combining retrograde tracing with fluorescence immunohistochemistry for TH or TPH.

Recent studies using transgenic mice expressing green fluorescent protein under the control of the melanocortin-4 receptor promoter [29] and transneuronal labeling after destruction of the somatomotor innervations of the gastrocnemius muscles [30] supported the sympathetic nature of the vestibular labeling. Most physiological studies have implicated the vestibular signals as playing the predominant role in the regulation of peripheral blood flow through effects on the sympathetic nervous system [9, 10, 31-33]. Lee et al. reported that some lateral vestibular nucleus neurons were infected following the injection of PRV-614 into the gastrocnemius, and indicated these infected neurons likely were components of the neural pathway that mediated the response of hindlimb blood flow [30]. The present study observed an appreciable number of infected neurons in the MVe five day following the injection of PRV-614 into kidney, and PRV-614/TH double-labeled neurons co-localized in the MVeMC not MVePC, and PRV-614/TPH neurons did not found in the MVeMC and MVePC, suggesting that it was possible that the MVeMC was mainly involved in adjusting sympathetic outflow to kidney via catecholaminergic pathway. In addition, we found that PRV-614-immunofluorescent cells co-localized within the TH-positive neurons of the RVLM region, suggesting that MVeMC- RVLM-renal pathway may be catecholaminergic, which were in agreement with a previous fluorescence study in the catecholaminergic neurons of the RVLM, which was the principal vasomotor source of descending C1 projections to sympathetic preganglionic neurons (SPGs) in the spinal intermediolateral cell column (IML) [26], had a primarily modulatory function in blood pressure regulation [4].

We believe that our data represents an important contribution to the understanding of the normal physiological balance between the MVe and renal sympathetic activity. Specifically, it provides direct neuroanatomic evidence for identifying autonomic projections from the MVe

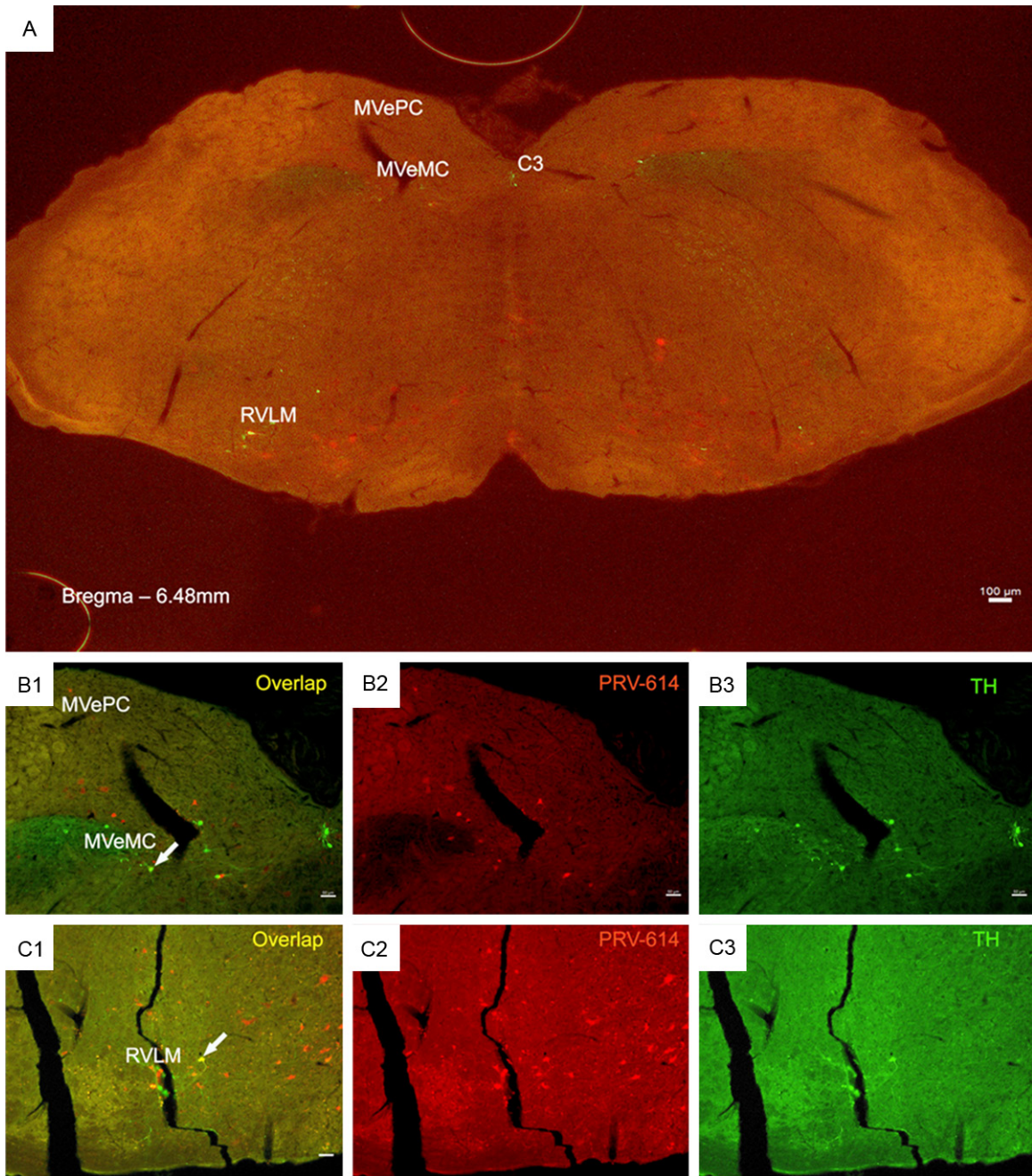


Figure 2. Fluorophor expression in caudal brainstem level at 5 d post-injection of the left kidney. (A) The distribution of PRV-614 infected and TH-immunoreactive neurons throughout the caudal brainstem is illustrated. (B1) PRV-614-immunofluorescent cells co-localize within the TH-positive neurons of the MVeMC region. (B2) PRV-614-immunofluorescent cells are present in the MVeMC and MVePC region. (B3) TH-immunoreactive neurons and fibers are present in MVeMC region. (C1) PRV-614-immunofluorescent cells co-localize within the TH-positive neurons of the RVLM region. (C2) PRV-614-immunofluorescent cells are present in the RVLM region. (C3) TH-immunoreactive neurons and fibers are present in RVLM region. Labeled neurons in the MVeMC and MVePC were mapped to transverse sections from the mouse brain atlas of Franklin KB and Paxinos G [26]. MVePC: the parvicellular medial vestibular nuclei, MVeMC: the magnocellular medial vestibular nuclei. RVLM: the rostral ventrolateral medulla. Bregma -6.48 mm. Scale bar 400 μ m for (A) 50 μ m for (B1-B3) and (C1-C3).

to the kidney, and a subset of neurons in the MVeMC, that participate in regulating sympa-

thetic outflow to kidney, is catecholaminergic not serotonergic.

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Disclosure of conflict of interest

None.

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