

Original Article

Motor cortex-lateral hypothalamus circuit regulating the sympathetic outflow to kidney by melanocortineric signaling

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Abstract: Previous investigations have suggested the existence of a neural circuit that coordinates metabolic states between the lateral hypothalamus (LH) and cerebral cortex. Evidence suggests that MC4R-GFP neurons in the LH coexpress the leptin receptor, and some findings indicated an important physiologic role for the melanocortin-4 receptors (MC4R) in the regulation of renal sympathetic traffic by leptin. Our studies supported the melanocortineric nature of the motor cortex by using a mouse line in which green fluorescent protein (GFP) is expressed under control of the MC4R gene promoter, and combining double immunohistochemistry and retrograde tracing technique. PRV-614/MC4R double-labeled neurons were detected in the LH and motor cortex, suggesting that tight linkage between LH and cerebral cortex may be enabled by the melanocortineric pathway. Motor cortex-lateral hypothalamus circuit may also produce autonomic disturbances characteristic in major renal disease by renal sympathetic responses.

Keywords: Lateral hypothalamus, motor cortex, melanocortineric pathway, melanocortin-4 receptors, virally mediated trans-synaptic tracing

Introduction

Understanding the neural basis of renal metabolic response remains a fundamental goal of neuroscience, of as much importance to studies of renal processing as it is to clinical studies of the renal disorders. Sympathetic system is a neural mediator of the kidney to internal stimuli, e.g., blood volume, special circulating hormone and blood glucose [1-4]. Previous report showed that mouse spinal, locus coeruleus, locus subcoeruleus, subthalamic nucleus, and pedunculopontine neurons retrogradely traced with PRV-614 from kidney expressed immunohistochemically detectable melanocortin-4 receptors (MC4R) [5-13]. In addition, some investigations indicated an important physiologic role of the MC4R in the regulation of renal sympathetic traffic to leptin [14, 15], the adipose-derived hormonal signal of body energy stores [16-18], and the renal sympathoexcitatory responses to leptin were dependent on the

MC4R [14, 19]. These findings thus revealed a major role for MC4R action in the modulation of renal sympathetic response, suggesting that there exist the importance of MC4R-expressing neurons in the regulation of renal sympathetic signaling that is crucial to leptin action and metabolic control.

Recent data suggests that the lateral hypothalamus (LH) is not merely regulating feeding, but also has many roles in regulation of the autonomic and sympathetic nervous systems as well as in modulating motivated behavior [20]. Louis GW et al had reported that the leptin receptor-expressing neurons in the LH play important physiological and behavioral roles [21], and is a crucial integrator of energy balance and motivated behavior [20]. Previous investigations have suggested the existence of a neural circuit that coordinates metabolic states between LH and cerebral cortex, and when metabolites are inefficiently utilized dur-

ing metabolic dysfunction with leptin resistance, LH neurons can be activated by triggering sympathetic innervation to elevate compromised catabolism [22]. Otherwise, evidence suggests that MC4R-GFP neurons in LH coexpress the leptin receptor [23]. These studies indicate that the melanocortinergic activity of neurons between LH and cerebral cortex may influence renal metabolic states. However, the exact neurosubstrate between LH and cerebral cortex underlying the regulation of renal metabolic states by the central melanocortin system has not been well defined.

The goal of this study was to use transneuronal retrograde tracing with PRV-614 [24-32], which is characterized by a reduced cytopathogenicity, to trace the neural pathways that regulate renal activity and to identify the separate populations of neurons in motor cortex and lateral hypothalamus that are anatomically linked to the sympathetic innervations of the kidney.

Material and methods

Animal care

The transgenic MC4R-GFP mice were produced as described in detail previously [27, 28, 33, 34]. All animal housing conditions, surgical procedures and postoperative care techniques were conducted according to the NIH guidelines for the Care and Use of Laboratory Animals, and approved by the Institutional Animal Care and Use Committee.

PRV-614 injection

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 injected into kidney in all animals using a previously described approach [9, 25, 35, 36]. Briefly, a laparotomy was performed to expose the upper pole of the kidney. Six 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 30-gauge needle connected to a Hamilton syringe (10 μ l) under microscopic guidance. All surgical procedures were performed using aseptic techniques. After PRV-614 injection, they were housed individually.

Immunohistochemistry

In preparation for immunohistochemistry the MC4R-GFP mice (8-10 weeks of age) were treated according to NIH guidelines in research. After a survival time of 5-6 d, the mice were deeply anesthetized with ketamine and xylazine. Then, they were transcardially perfused with 0.9% saline and 4% paraformaldehyde-borate fixative. The brains were removed, blocked and cut coronally in 30- μ m sections, and consecutively mounted onto glass slides. As a note, our study focused on the LH and motor cortex.

The sections were washed with immunobuffer (0.4% Triton-X in Tris-PBS) for 3 \times 10 min and blocked with 2% normal donkey serum in immunobuffer for 1 h at room temperature. A band pass filter for Cy3 was used to identify cells infected by PRV-614. The sections were then incubated with a chicken polyclonal antibody against GFP (ab13970, 1:1,000; Abcam) for 24 h at 4°C. Sections were then rinsed with 0.01 M PBS (3 \times 10 min), and followed by incubation with Alexafluor 488-conjugated donkey anti-rabbit IgG (1:800; Molecular Probes, Eugene, OR) in 0.01 M PBS containing 0.4% Triton X-100 for 1 h. All sections were mounted onto gelatin-coated slides, air dried overnight, and cover-slipped with mounting media.

Immunofluorescence was imaged using an Olympus IX81 photomicroscope connected to epifluorescence with a filter set for visualization. PRV-614-expressing cells are identified with red fluorescence; MC4R-GFP-positive cells are recognized by green fluorescence; double-labeled neurons are presented as yellow. The regions in which positive cells were located were defined with reference to the atlas [37].

Results

Specific expression of PRV-614 in the motor cortex and lateral hypothalamus

At 5 d survival time (n=5), PRV-614-labeled cells was located in the LH (5 d survival time, **Figure 1**). Additionally, three of the animals surviving 5 d began to show PRV-614 labeling in the motor cortex. At 6 d survival time, PRV-614 positive neurons were observed in the motor cortex (6 d survival times, **Figure 2**). From 5 d to 6 d, the most substantial increase seen in the number of PRV-614-infected cells was in the LH and motor cortex.

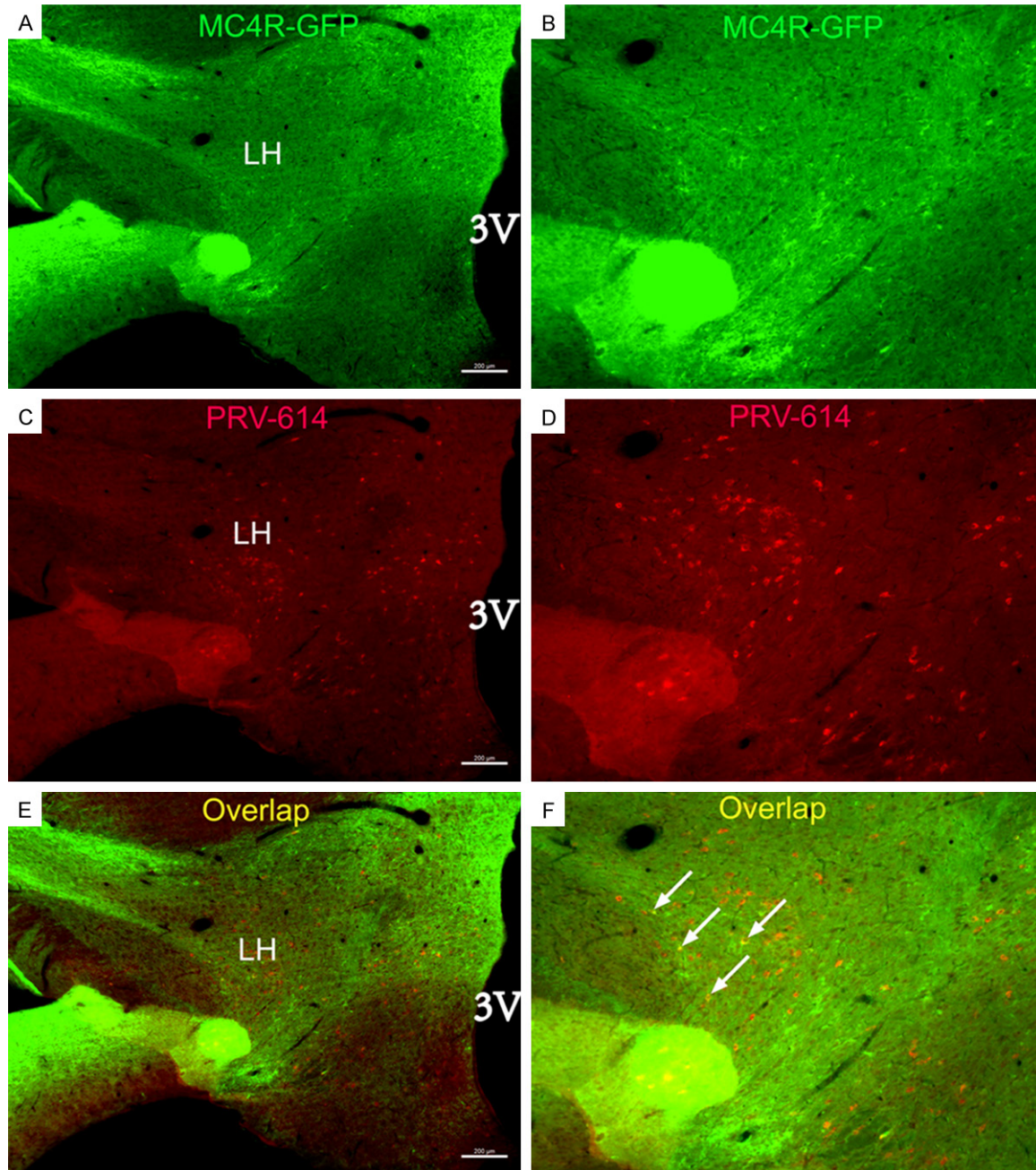


Figure 1. PRV-614/MC4R-GFP dual labeled neurons in the lateral hypothalamus 5 d after PRV-614 injection into kidney. (A) MC4R-GFP expressing neurons (green); (C) PRV-614 expressing neurons (red) in same section as (A); (E) overlap of (A and C), depicting distribution of MC4R-GFP-IR and PRV-614-bearing neurons. (B, D and F) Amplified views of (A, C and E), respectively. Arrows indicate double-labeled neurons (yellow). LH, lateral hypothalamus. Scale bar: 200 μm for (A, C) and 100 μm for (B, D-F).

PRV-614 and MC4R-GFP co-expression in the motor cortex and lateral hypothalamus

We assayed MC4R-GFP expression in the fore-brain, and observed a large number of GFP-labeled cells in the motor cortex and LH. We found that PRV-614/MC4R-GFP dual labeled

neurons were present in the LH and motor cortex (6 d survival times, **Figure 2**).

Discussion

Our experimental approach using PRV recombinants and MC4R-GFP transgenic mice provided

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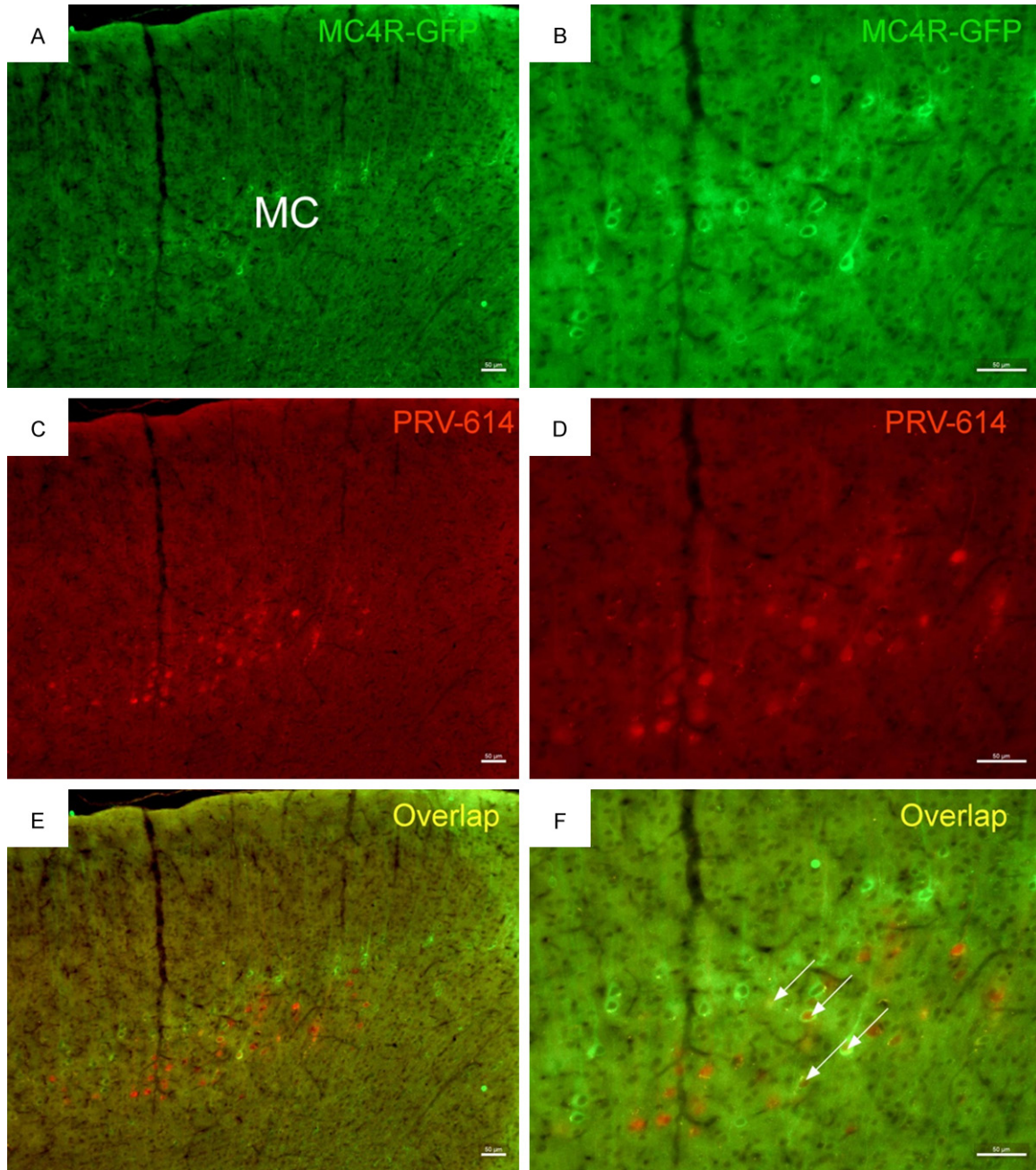


Figure 2. PRV-614/MC4R-GFP dual labeled neurons in the motor cortex 6 d after PRV-614 injection into kidney. (A) MC4R-GFP expressing neurons (green); (C) PRV-614 expressing neurons (red) in same section as (A); (E) Overlap of (A and C), depicting distribution of MC4R-GFP-IR and PRV-614-bearing neurons. (B, D and F) Amplified views of (A, C and E), respectively. Arrows indicate double-labeled neurons (yellow). MC, motor cortex. Scale bar: 50 µm.

a powerful method to define the identity and organization of central circuits, and phenotypically identified populations of neurons in the LH and motor cortex synaptically linked to kidney, which were in agreement with a previous immunohistochemical study [38]. Our data suggested that PRV-614/MC4R-GFP neurons in the LH-cerebral cortex circuit may primarily involve

in the sympathetic regulation of renal metabolic state.

Previous studies in rat and mouse documented that neurons in the LH involved in the control of the sympathetic outflow to the kidneys [39, 40]. It's well-known that LH has many neurochemical sources, including catecholaminergic-, mel-

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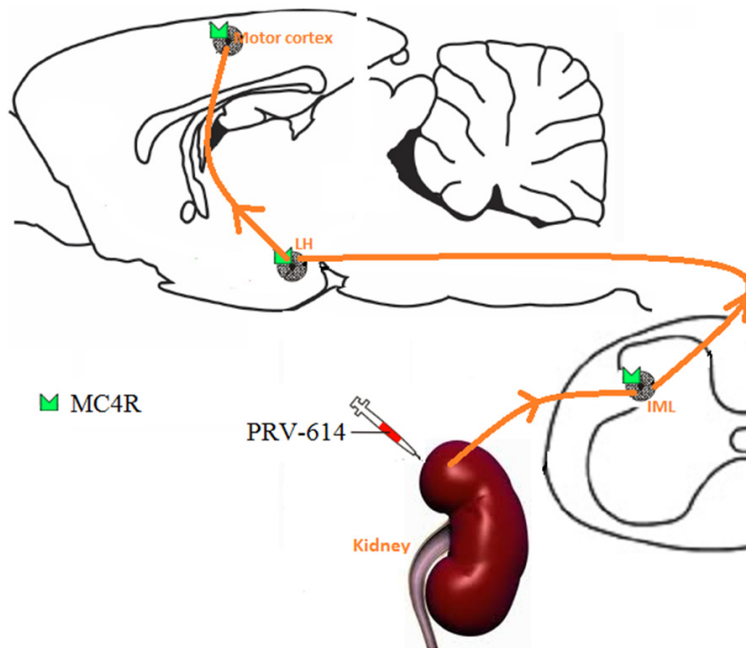


Figure 3. Summary diagram showed the motor cortex-lateral hypothalamus circuit regulating the sympathetic outflow to kidney by retrograde transsynaptic transport. The kidney has become a model system in which to study sympathetic function. There is no evidence that between motor nerve and the parasympathetic nervous system provides any innervations to kidneys, so the neurotropic pseudorabies virus (PRV)-614 was injected into the left kidney. Injection of PRV-614 into the kidney resulted in retrograde infection of neurons in the motor cortex and LH, and PRV-614/MC4R-GFP dual-labeled neurons were detected in the motor cortex and LH. LH, lateral hypothalamus, MC4R, melanocortin-4 receptor.

anocortinergetic-, orexinergic-, neurotensin-, leptin receptor-expressing neurons [20, 23, 41]. Though the melanocortinergetic property of neurons in cerebral cortex has been previously described [34], it is not clear that mouse cerebral cortex neurons retrogradely traced with PRV-614 from kidney also express immunohistochemically detectable MC4R. The retrograde transsynaptic tracer pseudorabies virus (PRV) has been widely used as a marker for synaptic connectivity in the brain. With the development of PRV as an alternative to tract tracing tools, our understanding of complex neuronal circuitry, particularly the innervations of kidney, has greatly advanced [42, 43]. By using a mouse line in which GFP is expressed under control of the MC4R gene promoter, we systematically investigated MC4R signaling in the LH and motor cortex by combining double immunohistochemistry and retrograde tracing techniques of PRV-614 for direct visualization under fluorescence microscope [6, 35, 36, 44]. We found that injections of PRV-614 into the kidney

resulted in retrograde infection of neurons in the LH and motor cortex, respectively, at different postinoculation times, which was consistent with Cano G et al's study showing that LH areas labeled with PRV were infected at the intermediate survival interval while cerebral cortex areas were infected at the late survival interval [40].

At the same time, PRV-614/MC4R double-labeled neurons were detected in the LH (Figure 1), which was in line with Cui H et al's report showing that LH contained MC4R-positive neurons in which coexpressed neurotensin as well as the leptin receptor [23], suggesting that MC4R-expressing neurons in the LH mediate renal sympathetic response and may contribute importantly to the control of energy balance in the kidney. In addition, PRV-614/MC4R dual-labeled neurons were observed in the motor cortex (Figure 2), which

was in agreement with a previous immunohistochemical detection showing that the cerebral cortex exhibited moderate to high levels of GFP immunoreactivity using adult male MC4R-GFP transgenic mice [34], suggesting that MC4R-expressing neurons in the motor cortex may implicate in homeostatic regulation of renal energy balance. Our data showed that there existed a neural circuit that coordinates metabolic states between LH and cerebral cortex (Figure 3), suggesting that tight linkage between renal metabolic states and cerebral cortex may be enabled by MC4R-expressing neurons in the lateral hypothalamus.

Conclusion

Based on our present findings, we hypothesize that the PRV-614/MC4R-GFP dual neurons in the LH and MC mediate renal sympathetic responses, suggesting that tight linkage between LH and cerebral cortex may be enabled by the melanocortinergetic pathway. Motor cor-

tex-lateral hypothalamus circuit may also produce autonomic disturbances characteristic in major renal disease by renal sympathetic responses.

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Disclosure of conflict of interest

None.

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