

Review Article

The connectome from the cerebral cortex to the viscera using viral transneuronal tracers

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Abstract: As an emerging framework in neuroscience, brain connectomics is well suited for investigating key questions on brain complexity by combining viral transneuronal tracing and whole brain graphic methodologies using analytical tools of network science. Transsynaptic viral tract-tracing in the toolbox of neural labeling methods has been a significant development in the connectomics field to decipher the circuit-level architecture of the cerebral cortex. In the present work, we reviewed the current methods enabling structural connectivity from the viscera to the cerebral cortex mapping with viral transneuronal tracers and showed how such neuroanatomic connectomic data could be used to infer new structural and functional information in viscera-cerebral cortex circuits.

Keywords: Brain, connectomics, viscera, cerebral cortex, viral transneuronal tracer

Introduction

The brain can control visceral functions using complex neural circuits. Viral tracers are known to represent a potent tool for anatomical and physiological analyses of specific brain-viscera neural pathways. The neurotropic viral tracers include both retrograde and anterograde transneuronal tracers with poly or mono synaptic methods. A variety of retrograde trans-polysynaptic viral tracers (e.g., the pseudorabies virus (PRV) [1-3]), monosynaptic retrograde labeling (e.g., the glycoprotein-deleted rabies (Δ G-rabies) virus vector [4-6]), and anterograde trans-polysynaptic viral tracers (e.g., herpes simplex virus 1, strain H129 (HSV-1-H129) [7-9]), have widely been used for identifying neural pathways underlying distinct behaviors or brain functions.

Viral-based transneuronal tracing technologies are broadly used as neuroscience research tools for the anatomy of both the central and

peripheral nervous systems [2, 3, 10, 11]. Highly sensitive viral transneuronal tracers are available for connectomic studies [12]. Therefore, understanding in depth neurotropic viral properties and genetically modified viral tools is essential for constructing connectomic studies from internal organs to the brain. For the direction viral tracers take, describing connections 'from the viscera to the cerebral cortex' is crucial. However, it should be noted that this is a retrograde direction, and, in our view, it would be much more logical to state that the connections run from the cerebral cortex to the viscera (Reviewer's suggestion). Indeed, connectivity mapping may be done from the viscera to the cerebral cortex as well as from the cerebral cortex to the viscera. In this review, we describe connections from the cortex to the viscera using retrograde transneuronal transport.

Over the past few decades, the genetically modified technique has brought revolutionary breakthroughs to fluorescence labelling imag-

ing [13-16]. Using this powerful molecular labelling technique, the viruses are genetically engineered to serve as vectors for fluorescent protein expression in host cells, which enables the analysis of neuroanatomic localization and evaluation of the biological function. For example, the recombinant strains of the pseudorabies virus (PRV) Bartha are conjugated to either green (PRV152-EGFP) or red (PRV614-RFP) fluorescent protein [17-24], which provides a good foundation for intracellular targeting of the labeled neurons in future connectomic studies [1].

Using viral-genetic methods, neuroscientists can label polysynaptic tracing of restricted neuronal networks across multiple synapses for monitoring neuronal activity [10, 25-28], as well as dual or three genetically engineered viral tracers for transneuronal tracing. These developments of transneuronal tracers give rise to a major step forward in our understanding of brain connectomics. The duration of the labeling period upon application of the virus in different viscera is summarized in **Table 1**.

Based on the use of neurophilic viruses as neuronal markers, connectomic studies have shown that viruses can specifically transfer between connected neurons by replicating in recipient neurons and entire functional networks including first-, second-, third-order neurons, etc. For example, Stanley [29] mapped the hierarchy of synaptically linked multineuronal efferent pathways following sites of PRV infection over time after injection of PRV152 into the liver. He found five infection phases based on the postinfection pattern at various times, suggesting that there exist five chains (also named five order synapses) of synaptically connected neurons from the CNS to the liver.

The properties and application of the commonly used neurotropic viral tracers

The use of viral transneuronal tracers will help significantly in unraveling connections not only in the somatic part of the nervous system but also in its visceral part and in elucidating to what extent these connections are linked. Based on the inherent property of propagating among synaptically linked neurons, the commonly used neurotropic viral tracers are divided into the retrogradely transported virus [30, 31], including PRV, rabies virus (RV) and many

adeno-associated virus (AAV) serotypes (such as the rAAV2-retro vector) [32], and the anterogradely transported virus, including HSV-1-H129 [7-9], vesicular stomatitis virus (VSV) [33], and AAV1 [11, 34, 35].

Some studies from Strick [36-38] demonstrate retrograde trans-polysynaptic transport of the N2c strain of RV (CVS-N2c), whereas a report of Choi and Callaway [39] suggests that a novel type of retrograde gene transfer RV vector is only an anterograde monosynaptic tracer. Callaway and Luo [40] advocate that the glycoprotein-deleted rabies (Δ G-rabies) virus vector is commonly used for monosynaptic retrograde labeling, but the high cytotoxicity of this vector is limited in its application to anatomical and physiological analyses of specific neural pathways.

For the use of such tracing tools, further research is necessary to examine the advantages and disadvantages of using PRV and RV. For example, the types of viruses suitable for retrograde infection of specific organs, i.e., cell and tissue specificity of viral tracers; the specificity of transneuronal tracing of synaptically linked neural circuits using recombinant viral strains, i.e., risk of runaway second-order or higher order virus infection. Development of better recombinant strains with higher cell/tissue specificity and labeling efficiency is thus essential for pushing forward the field of connectomic studies using viral tracing.

Current methods for viscera-brain connectome studies

Elucidating connectomics at the whole-brain level is attracting increasing attention [41-44], as a variety of technological solutions for different biological applications have been optimized. We have defined the viscera-brain connectome as the specific region in the CNS that projects to the viscera via synaptic connections. Based on this definition, we found that the viscera-brain connectome is composed of multiple, spatially separate subareas.

Nonetheless, it is well known that the viscera-brain connectome is important in defining specific sources of the central command for the neural regulation of these viscera. A variety of new methods are being developed to perform computer-assisted high-throughput image ac-

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Table 1. Duration of the labeling period upon virus application in different viscera

Species of the animal model	Species of the virus	Labeling period	Application site	Labeling destination	Reference
Mice	HSV-1	2-3 days	stomach	Nodose ganglia, enteric ganglia	[116]
Mice	RV	3-5 day	diaphragm/intercostal muscles	spinal cord, pons and medulla, cerebellum, mesencephalon, diencephalon, and telencephalon	[117]
Rat	RV	3-3.5 days 4-5 days	Orbicularis Oculi muscle	trigeminal nuclei, oculomotor nucleus lateral hypothalamus, cerebral cortex	[118]
Rat	RV	3-4 days 3-4 days ~89.5 h	stomach	dorsal motor nucleus of the vagus The nucleus of the solitary tract the insula, medial prefrontal cortex	[69]
Rat	RV	3-4 days ~114.5 h ~82 h ~92 h ~99 h	stomach (vagus nerves cut) kidney	Rostral ventrolateral medulla primary motor cortex, primary somatosensory cortex, secondary motor cortex RVLM NTS primary motor cortex, primary somatosensory cortex, secondary motor cortex	[37]
Rat	RV	3 days	adrenal medulla	layer V of cerebral cortex	[38]
Rat	PRV	3 days	heart	stellate ganglia, sympathetic chain ganglia	[119]
Rat	PRV-263	3 days	kidney	sympathetic chain ganglia	[120]
Rat	H129	3 days	stomach	Nodose ganglia	[121]
Rat	PRV-Bartha	3 days	stomach	brainstem	[122]
Rat	PRV	2 days	bladder and colon	major pelvic ganglion	[123]
Rat	PRV	3 days	bladder wall	DRG	[124]
Rat	PRV-mRFP1, PRV-EGFP	4 days	kidney	sympathetic postganglionic neurons in celiac ganglia	[87]
Cat	RV	61.5-120 h	diaphragm	spinal cord, medulla, and pons, midbrain, diencephalon, and telencephalon	[125]
Cebus monkeys	RV	4-5 days 5-6 days	adrenal medulla	corticospinal tract primary motor cortex, dorsal and ventral premotor areas, supplementary motor area, Medial prefrontal areas	[99]
monkeys (Cebus apella)	RV		adrenal medulla	layer V of cerebral cortex	[38]
rhesus monkeys	RV	~3 days 4-5 days >5 days	hand muscle	Int and DRG of lower cervical and upper thoracic segments of the spinal cord the red nucleus (RNm), and CM cells in layer V of M1 CM cells in in layers III and VI of M1	[126]
rhesus monkeys	RV	4-4.5 days	orbicularis oculi	ventrolateral premotor, motor cortices (M1-M4), pre-supplementary motor cortex, somatosensory cortices, prefrontal cortex	[127]
rhesus monkeys	RV	2.5 days 3 days 3.5 days	lateral rectus eye muscle	brainstem cell groups Cerebellar nuclei Cerebellar cortex	[128]
Macaca fascicularis	RV	3-4 days	lateral rectus muscle	superior colliculus neurons	[129]
guinea pigs	RV	50 h-86 h	medial rectus	oculomotor nucleus, medial vestibular nucleus, Scarpa's ganglion, Purkinje cells	[130]

quisition and analysis to obtain viscera-brain connectome. There are various technologies of viscera-brain connectome studies as shown below.

The first is the two dimensional connectomic analysis mapping based on a standard atlas of the rat brain [45]. Rabies virus (RV) is a retrograde transneuronal tracer which transports in a time-dependent fashion and can identify a chain of synaptically connected neurons [46-49]. For example, after injecting the rabies virus (RV) into the rat kidney, Levinthal and Strick [37] used a computer-based charting system (MD2, Accustage) [48] and their self-designed laboratory software to create kidney-cortex connectomic maps that displayed the distribution of RV-labeled neurons on a two dimensional surface [50].

The second are the high-throughput light sheet tomography [51, 52] and the micro-optical sectioning tomography (MOST) [53-57]. Chen [1] reconstructed and compared the distinct distribution patterns and quantitative statistics of the brain-wide PRV-infected neurons projecting to the skeletal muscle by using the high-throughput light sheet tomography platform. Sun [58] utilized monosynaptic rabies viral tracers in combination with fluorescence MOST to generate a whole-brain atlas of direct long-range inputs to GABAergic interneurons in the medial prefrontal cortex (mPFC) of male mice, thus providing the anatomical foundation for understanding the functional organization of the mPFC.

Connectivity of the stomach and the cerebral cortex

Neural pathways from the stomach are essential for normal gastrointestinal function [59]. Previous reports have suggested the importance of the parasympathetic and sympathetic control over the stomach function [60, 61]. The parasympathetic output to the stomach often promotes efficient digestion [62-64] whereas the sympathetic output to the stomach inhibits digestive activity [65-67]. The vagus nerve is known to be a crucial organ-brain connection that monitors the gastrointestinal physiological functions. Studies by Williams [68] clarified the key roles of vagal afferents in mediating particular gut hormone responses, suggesting the

importance of vagal sensory neurons in sensing gastrointestinal inputs.

Emerging evidence indicates that the cerebral cortex directly influences the parasympathetic and sympathetic control of the rat stomach [69]. Levinthal and Strick [69] used RV as a transneuronal tracer to study stomach-cerebral cortex connectivity. They injected RV into the rat stomach to identify the areas of the cerebral cortex that are most directly connected to the stomach (**Figure 1**). When the rabies virus was injected into the ventral stomach wall, it was transported in the retrograde direction to infect the CNS via sympathetic and parasympathetic pathways. In the sympathetic network, RV firstly infected the celiac ganglion (CG) (i.e., first-order neurons) that innervates the stomach. Then the virus was transported transneuronally in the retrograde direction to label the spinal cord intermediolateral cell column (IML) (i.e., second-order neurons) that synapse onto the infected CG neurons and rostral ventrolateral medulla (RVLM) (i.e., third-order neurons) of the brainstem that synapse onto the infected IML second-order neurons. At longer survival times, the RV may go to the next step of retrograde transneuronal transportation and label layer V of the motor cortex (i.e., fourth-order neurons) that synapse onto the infected third-order RVLM neurons.

In the parasympathetic network, RV infected transneuronally in the retrograde direction to the dorsal motor nucleus of the vagus (DMV) (i.e., first-order neurons) via the vagal ganglion (VG) that innervates the stomach. Then the virus was transported to label the nucleus of the solitary tract (NTS) (i.e., second-order neurons) that synapse onto the infected DMV neurons. At longer survival times, the virus moved from the second-order neurons in the NTS to the third-order neurons in Layer V of the insular cortex (**Figure 1**).

Connectivity of the heart and the cerebral cortex

Normal cardiac function is contingent upon a complex hierarchy of CNS regulation [70]. Armour indicated that cardiac peripheral and central neurons are in constant communication with one another such that, for the most part, they behave as cardiac neuronal hierarchy [71, 72]. Rajendran identified peripheral neural cir-

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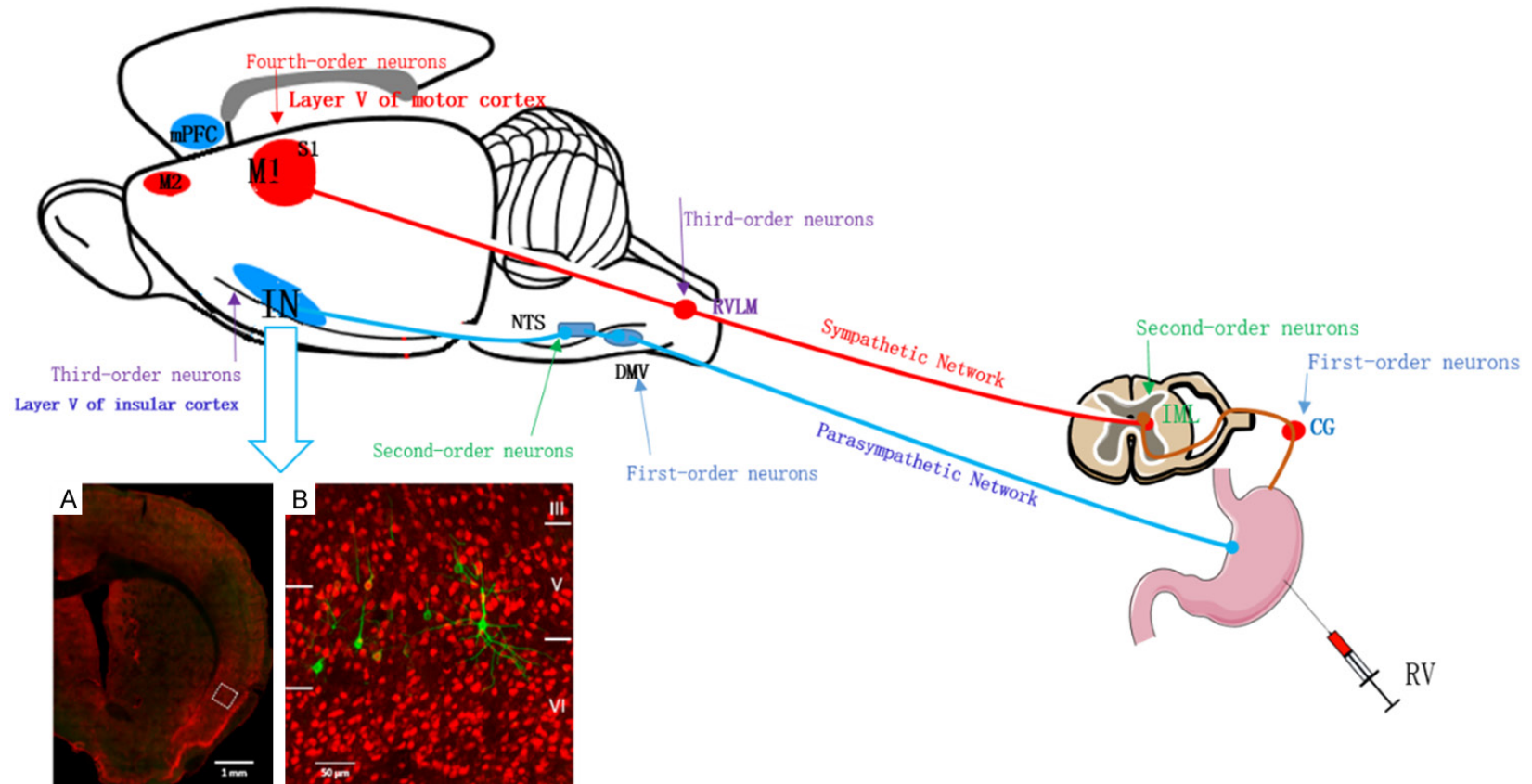


Figure 1. Schematic drawing of the connectivity from the stomach to the cerebral cortex. When the rabies virus is injected into the ventral stomach wall, it is transported in the retrograde direction to infect the CG (i.e., first-order neurons) that innervates the stomach via the sympathetic network. Then RV is transported transneuronally in the retrograde direction to label spinal cord IML neurons (i.e., second-order neurons) that synapse onto the infected CG neurons and the RVLM neurons (i.e., third-order neurons) that synapse onto the infected IML second-order neurons. At longer survival times, RV can undergo another stage of retrograde transneuronal transportation and label layer V of the motor cortex (i.e., fourth-order neurons) that synapse onto the infected third-order neurons. In the parasympathetic network, transport of RV in parasympathetic circuits labeled first-order neurons in the DMV, second-order neurons in the NTS, and third-order neurons in Layer V of the insular cortex. CG, celiac ganglion; DMV, dorsal motor nucleus of the vagus; NTS, nucleus of the solitary tract; RVLM, rostral ventrolateral medulla.

cuits that regulate the heart rate using optogenetic and viral vector strategies [73]. Stellate ganglion (SG), a sympathetic ganglion formed by the fusion of the inferior cervical ganglion and the first thoracic ganglion, relays vital information from the heart to the brain about the physiological state [74-80]. It is well-established that the sympathetic nervous system is a major information highway for the heart signaling health and disease whereas parasympathetic responsive neural circuits regulate cardiac physiology.

The cerebral cortex is involved in processing sensory information and motor function. Electrical stimulation of the prefrontal cortex or insular cortex is known to elicit changes in heart rate [81-84], however, its mechanisms of neural circuits remain elusive. Transsynaptic viral labeling of the neural tract-tracing method has been a significant development in the connectomics field to decipher circuit-level architecture of the cerebral cortex. Tracing with PRV has proven to be especially useful for connectivity of the heart and cerebral cortex.

Ter Horst and Postema [85] reported forebrain parasympathetic control of the heart activity by retrograde transneuronal viral labeling in spinal cord-transected rats. Before injecting PRV into the left ventricular myocardium of the heart, spinal cord transection C8-T1 was performed. This enabled PRV to transneuronally transport via parasympathetic networks and PRV labeled first-order neurons in the DMV, second-order neurons in the NTS, and third-order neurons in Layer V of the dysgranular insular cortex. Thereby, a network of 3 interconnected neurons may allow the output of the cerebral cortex to influence parasympathetic control of the heart. Transport of PRV in the sympathetic pathway labeled first-order neurons in the sympathetic ganglia (SG), second-order neurons in the intermediolateral column (IML) in the spinal cord, third-order neurons in the rostral ventrolateral medulla (RVLM) and fourth-order neurons in Layer V of the motor cortex (**Figure 2**). These results demonstrate the power of transneuronal tracing with PRV to unravel multi-synaptic circuits from the heart to the cerebral cortex.

Connectivity of the kidney and the cerebral cortex

Retrograde transneuronal transport of neurotropic viruses including the pseudorabies virus

(PRV) and rabies virus (RV) has been used previously to interrogate the distribution of neurons that are disynaptically linked to the kidney [17-22, 86-92]. The adaptation of PRV for transneuronal tracing in rats and mice enabled us to reveal kidney-brain connections in chains of three or more synaptically linked neurons [87, 93, 94]. Many studies examined kidney-cerebral cortex neural circuits following the PRV injection into the kidney. Liu reported that PRV-614 immunopositive neurons were located in the cerebral cortex 6-7 days after injecting PRV-614 into the kidney [3, 22].

Transneuronal tracing of RV provides a renaissance in our understanding of kidney-cerebral cortex neural circuits. Levinthal and Strick [37] used retrograde transneuronal transport of the rabies virus from the rat kidney parenchyma to identify the areas of the cerebral cortex for the neural regulation of the kidney and found that an important network of five interconnected neurons was sufficient to allow the output of the cerebral cortex to influence sympathetic control of the kidney (**Figure 3**). When RV was injected into the kidney, it was transported in the retrograde direction to infect the sympathetic ganglia (SG) (i.e., first-order neurons) that innervate the kidney. Then RV was transported transneuronally in the retrograde direction to label spinal cord IML neurons (i.e., second-order neurons), where sympathetic preganglionic neurons (SPNs) that project to the kidney are located and synapse onto the infected SG neurons.

In the sympathetic network, RV was transported transneuronally in the retrograde direction to label RVLM neurons (i.e., third-order neurons) that synapse onto the infected IML second-order neurons and the NTS neurons (i.e., fourth-order neurons) that synapse onto the infected third-order RVLM neurons. When the survival time was extended, RV underwent another stage of retrograde transneuronal transport and labeled layer V of the motor cortex (i.e., fifth-order neurons) that synapse onto the infected fourth-order neurons. Moreover, in the corticospinal tract pathway, RV was transported to spinal cord interneurons (Ins) (i.e., third-order neurons) that synapse onto the infected SPNs second-order neurons. Next, the virus moved from third-order neurons in the Ins to fourth-order neurons in layer III of the motor cortex (**Figure 3**).

Connectomics from cortex to viscera

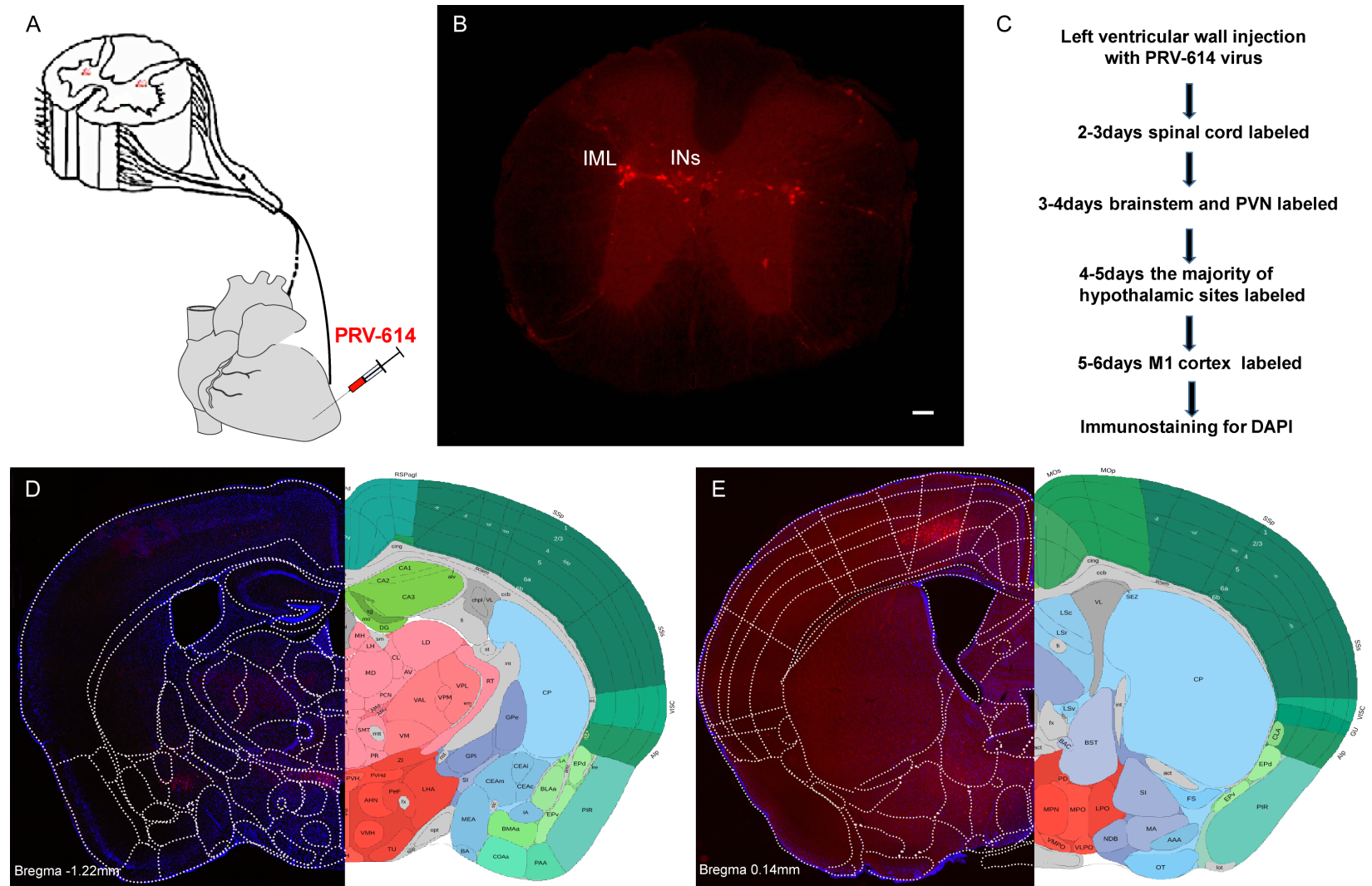


Figure 2. Schematic drawing of the connectivity from the heart to the cerebral cortex. **A.** Schematic representation of a spinal cord cross-section and the heart of the mouse. The left ventricle of the heart was injected with recombinant strains of the PRV Bartha virus conjugated to red (PRV-614-RFP) fluorescent protein. **B.** Low magnification confocal image of the thoracic spinal cord illustrating SPNs and INs retrogradely labelled with the neurotropic PRV-614-RFP 3 days after the injection. Following different post-injection times, viral replication leads to a strong amplification of the reporter protein in the infected neurons revealing their detailed morphology. Scale bar 100 μ m. **C.** Overview of PRV-614 labelling and processing protocol. **D, E.** Sagittal brain atlas images (20 μ m) from the Allen Institute that encompass the CEA (Bregma 1.22 mm) and layer V of M1/S1 (Bregma 0.14 mm). IML, intermediolateral nucleus of the spinal cord. INs, interneurons. M1, primary motor cortex. S1, primary somatic sensory cortex. SPNs, sympathetic preganglionic neurons. The fluorescent plots were from our unpublished results.

Connectomics from cortex to viscera

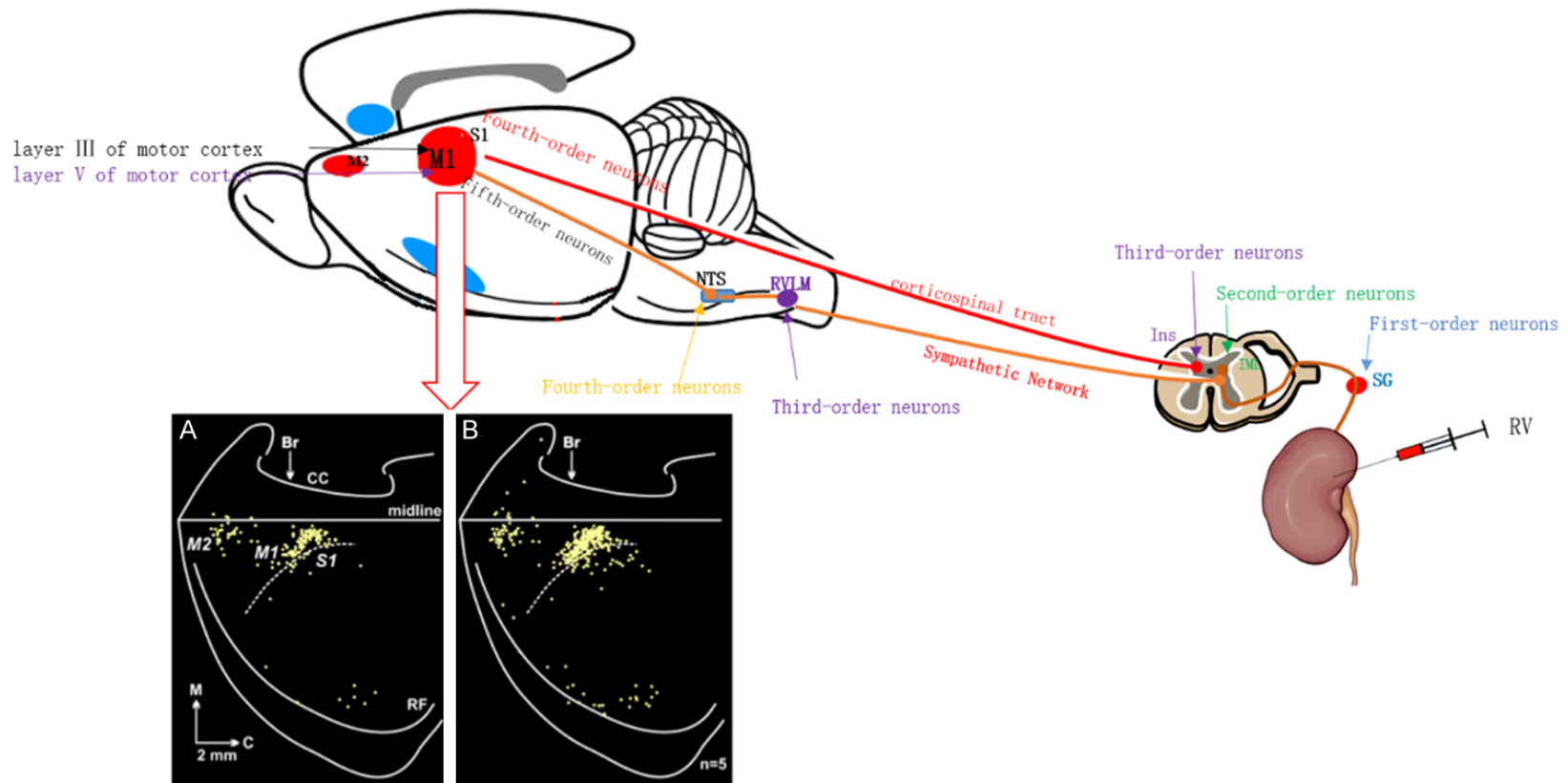


Figure 3. Schematic drawing of the connectivity from the kidney to the cerebral cortex. When the rabies virus is injected into the kidney, it is transported in the retrograde direction to infect the SG (i.e., first-order neurons) that innervate the kidney. Then the virus is transported transneuronally in the retrograde direction to label spinal cord IML neurons (i.e., second-order neurons) that synapse onto the infected SG neurons. At longer survival times, the RV can undergo another stage of retrograde transneuronal transportation and label all those third-order neurons that synapse onto the infected second-order neurons. For example, the RV can move from fourth-order neurons in layer V to fifth-order neurons in layer III. Similarly, the RV can move from third-order interneurons in the RVLM to fourth-order cortical neurons in layer V. In the corticospinal tract pathway, the RV was transported to spinal cord interneurons (Ins) (i.e., third-order neurons) that synapse onto the infected SPNs second-order neurons. Next, the virus moved from third-order neurons in the Ins to fourth-order neurons in layer V of the motor cortex. Ins, interneurons; NTS, nucleus of the solitary tract; RVLM, rostral ventrolateral medulla 1; SG, sympathetic ganglia. The fluorescent plots were from Levinthal and Strick (*J Neurosci.* 2012).

Connectivity of the adrenal medulla and the cerebral cortex

A major challenge facing connectomics neuroscience is to unravel the complex matrix of neural connections that characterize anatomical circuits within the CNS. The cerebral cortex is known to receive and process information related to the sensory and motor pathways [95-98]. Retrograde transneuronal transport of RV has proven to be useful at identifying the microarchitecture of complex neural circuits in the cerebral cortex [46-48].

The adrenal medulla has been recognized as a major sympathetic effector [99-101]. More recently, many neuropsychological processes have been attributed to the adrenal medulla-cerebral cortex axis and these include anxiety, depression, cognition, and affection [102-110]. Previous studies have indicated that there are neurons in the CNS that contribute to the transneuronal innervation of the adrenal medulla [111]. A study by Dum provided a comprehensive and critical resource on the connectivity between the adrenal medulla and cerebral cortex [99] and the connectivity from the cortical motor areas to the adrenal medulla is likely mediated by corticospinal and corticobulbar-spinal pathways [112-114]. In the corticospinal pathway, RV was transported to the spinal cord SPNs (1st-order neurons), interneurons (Ins) (2nd-order neurons), layer V of motor cortex (3rd-order neurons) and layer III of motor cortex (4th-order neurons). In the corticobulbar-spinal pathway, RV was transported to SPNs (1st-order), brainstem (2nd-order), layer V (3rd-order) and layer III of motor cortex (4th-order) (**Figure 4**).

Dum showed that motor, cognitive and affective areas of the cerebral cortex influence the adrenal medulla [38]. After RV injections into the adrenal medulla of monkeys (*Cebus apella*), three distinct connectomic networks including cortical areas involved in movement, cognition and affection were observed [38]. In the motor network, the cortical motor areas consist of M1, dorsal (PMd) and ventral (PMv) premotor areas, S1 on the lateral surface and the supplementary motor area (SMA), and dorsal cingulate motor (CMA_d) on the medial wall (**Figure 4**). In the cognitive network, the cortical cognitive areas include the rostral cingulate motor (CMA_r) and ventral cingulate motor

(CMA_v) areas on the medial wall (**Figure 4**). In the affective network, the cortical affective areas consist of areas 24c, 32 and 25 on the rostral medial wall in the monkey (**Figure 4**).

Connectivity of the other viscera and the cerebral cortex

Cortical innervation of the mouse liver has been experimentally investigated by Stanley [29] using PRV strains expressing different reporters together with BAC transgenesis and immunohistochemistry. Seven to eight days after hepatic PRV152 infection, M1 cortical infection occurred, indicating that the central multisynaptic outflow pathway to the liver comprises cerebral cortex regions that involve the M1. Further research is needed to understand the neural substrate that links the cerebral cortex to the function of the liver. Yao [115] reported retrograde and trans-synaptic identification of a cluster of cortical L5 pyramidal neurons after PRV injection into the bladder wall, suggesting that urination (also called micturition) may be regulated by a neural network that is distributed in both subcortical and cortical regions.

Conclusion

Over the past several decades, viral transneuronal labeling in the toolbox of neural tract-tracing technologies has provided new sights for understanding the neural connectomic substrate that links the cerebral cortex to the function of internal organs. New technologies are beginning to emerge providing connectivity mapping from the viscera to the cerebral cortex combined with viral transneuronal tracers. These technologies demonstrate how such neuroanatomic connectomic data could be used to infer new structural and functional information in the viscera-cerebral cortex circuits.

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Connectomics from cortex to viscera

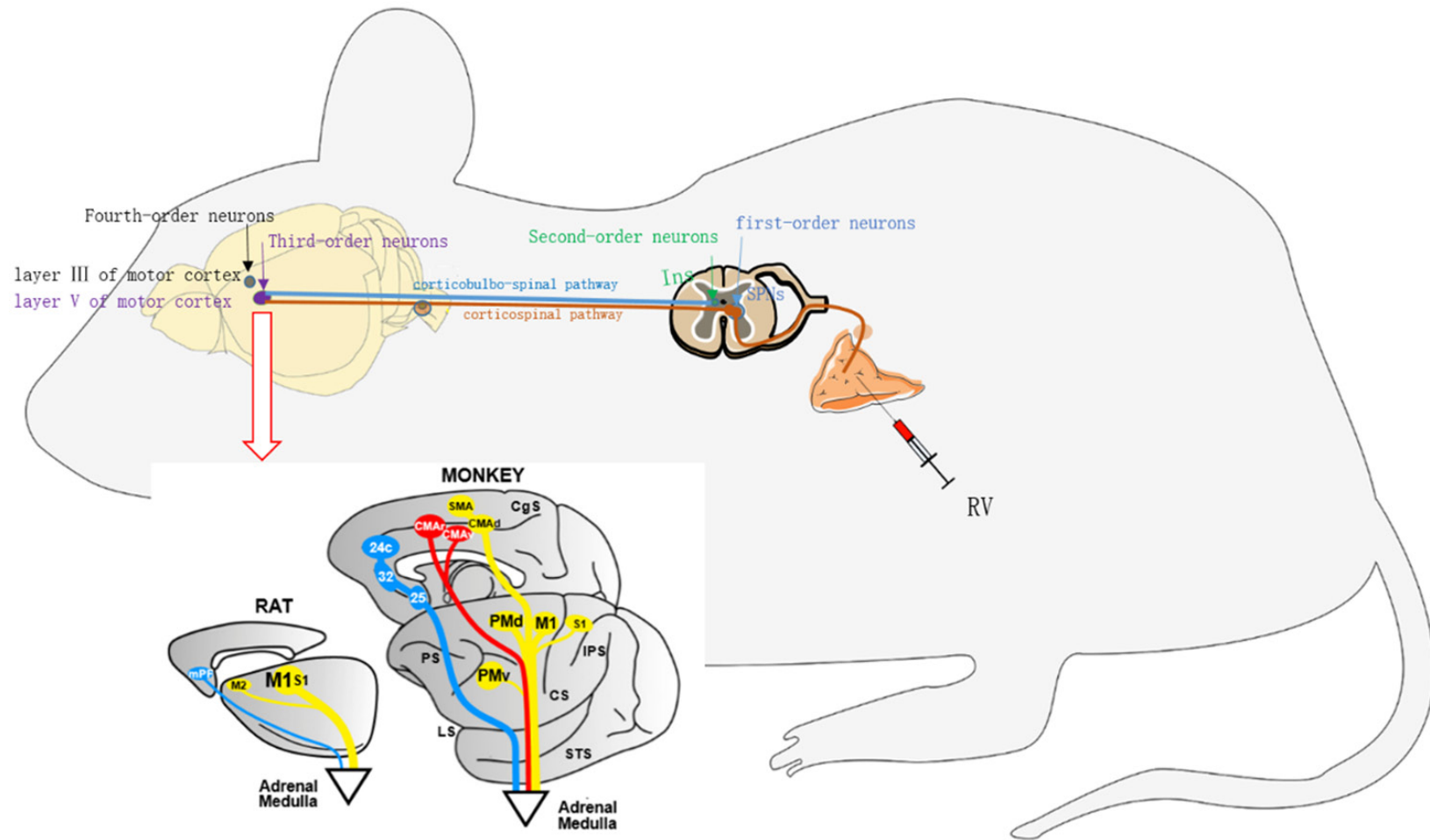


Figure 4. Schematic drawing of the connectivity from the adrenal medulla to the cerebral cortex. When the RV is injected into the adrenal medulla, it is transported in the retrograde direction to infect the SPNs (first-order neurons) that innervate the adrenal medulla. Then the virus is transported transneuronally in the retrograde direction to label all those second-order neurons including Ins of the spinal cord, brainstem, and hypothalamus. Yellow line (the motor network): the cortical motor areas consist of M1, dorsal (PMd) and ventral (PMv) premotor areas, S1 on the lateral surface and the supplementary motor area (SMA), and dorsal cingulate motor (CMAv) on the medial wall. Red line (the cognitive network): the cortical cognitive areas include the rostral cingulate motor (CMAr) and ventral cingulate motor (CMAv) areas on the medial wall. Blue line (the affective network): the cortical affective areas consist of areas 24c, 32, and 25 on the rostral medial wall in the monkey. Ins, interneurons; SPNs, sympathetic preganglionic neurons.

Disclosure of conflict of interest

None.

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