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REVIEW

Trans-synaptic Neural Circuit-Tracing with Neurotropic Viruses

Jiamin Li[1,2] · Taian Liu[1] · Yun Dong[1] · Kunio Kondoh[3,4] · Zhonghua Lu[1]

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Abstract A central objective in deciphering the nervous system in health and disease is to define the connections of neurons. The propensity of neurotropic viruses to spread among synaptically-linked neurons makes them ideal for mapping neural circuits. So far, several classes of viral neuronal tracers have become available and provide a powerful toolbox for delineating neural networks. In this paper, we review the recent developments of neurotropic viral tracers and highlight their unique properties in revealing patterns of neuronal connections.

Keywords Neurotropic virus · Central nervous system · Neural circuit · Trans-synaptic tracer · Retrograde tracing · Anterograde tracing

✉ Kunio Kondoh
kkondoh@nips.ac.jp

✉ Zhonghua Lu
zh.lu@siat.ac.cn

[1] Guangdong Provincial Key Laboratory of Brain Connectome and Behavior, The Brain Cognition and Brain Disease Institute, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen 518055, China

[2] University of Chinese Academy of Sciences, Beijing 100049, China

[3] Division of Endocrinology and Metabolism, Department of Homeostatic Regulation, National Institute for Physiological Sciences, National Institute of Natural Sciences, Myodaiji, Okazaki, Aichi 444-8585, Japan

[4] Japan Science and Technology Agency, PRESTO, Myodaiji, Okazaki, Aichi 444-8585, Japan

Introduction

The human brain, comprising close to 100 billion neurons, is the most mysterious and complex organ in the body. These neurons connect synaptically with each other to form a large number of functional modules that encode perception and process information, and generate behavioral, physiological, and emotional responses. Elucidating how these modules, namely neural circuits, are anatomically defined is a prerequisite for understanding their functions. Over the past decades, many methods and techniques have been developed to explore this question. One of the most powerful tools is the neurotropic viruses that invade neurons and spread among synaptically-linked neurons.

Many viruses have a long history of artificial modification as gene vectors. This has greatly expanded the capacities of these viruses for the visualization and manipulation of neural circuits *via* combination with various fluorescent, optogenetic, chemogenetic, and other tools. Since the first strain of herpes simplex virus (HSV) was isolated and demonstrated to have a neurotropic character and to spread between neurons through synaptic connections rather than *via* perineural spaces [1, 2], the past decades have witnessed the generation and application of many powerful viral tracers in neuroscience research. Compared with traditional tracers (e.g. tritiated amino-acids, wheat-germ agglutinin, fragment C of tetanus toxin, rhodamine isothiocyanate, neurobiotin, and fluorescent lipophilic cationic indocarbocyanine dyes), neurotropic viruses have several advantages: (1) they can be transmitted across synapses; (2) the transmission direction is definitive – they naturally spread in retrograde, anterograde, or both directions; (3) their proliferation after spreading avoids the decline of signal intensity with spread; and (4) they are compatible with diverse genetic

markers. Currently, the common viral tools for neuronal tracing include HSV [3], pseudorabies virus (PRV) [4], rabies virus (RV) [5], vesicular stomatitis virus (VSV) [6], and adeno-associated virus (AAV) [7] (Table 1) [8]. During evolution, viruses developed various survival strategies that endow them with unique attributes that have been used to develop versatile viral tracers (Fig. 1). The aim of this review is to briefly survey the development of these neurotropic viruses as neural circuit-tracing tools and to discuss their advantages and limitations in neuroscience research.

Herpesviridae

Herpesviridae are double-stranded DNA viruses with a large genome ($\sim$ 150 kb) that encodes $>$ 70 genes, so they have a large packaging capacity. The viral particle is composed of capsid, tegument, and envelope, varying in size from 200 nm to 250 nm. After infection of neural cells, herpesviridae remain mostly latent. The viral DNA is not integrated into the host genome and has little effect on the transcription of host genes. Thus, they can be exploited as safe vectors to deliver genes to the nervous system. Moreover, they have evolved the ability to spread bidirectionally along neural pathways, which makes herpesviridae an outstanding tool for tracing neuronal connections [9].

Herpesviridae comprise three subfamilies: alpha, beta, and gamma herpesviridae. PRV and HSV type 1 (HSV-1) that belong to the alpha herpesvirus family have been commonly used as neuronal tracers because they have a broader host range than other members of this family [10]. HSV-1 is the pathogen of herpes stromal keratitis and causes herpes simplex encephalitis when infecting the central nervous system [11].

The first strain of HSV-1 was obtained from a patient with lobar pneumonia in 1923 [1]. It was shown experimentally that HSV-1 could propagate from a rabbit's eye to its brain and eventually cause herpes simplex encephalitis. Two later studies by Goodpasture *et al.* [2] and Kristensson *et al.* [12] provided evidence that the virus is exclusively transported from nerve endings to neuronal cell bodies along axons rather than *via* perineural spaces. Cook and Stevens *et al.* [13] further demonstrated the trans-neuronal transfer of HSV-1. Based on these studies, Bak and Stevens *et al.* [14] first used HSV-1 to identify striatonigral projection neurons. Afterwards, an increasing number of researchers began to use HSV-1 to study the neuronal circuitry in a variety of neuroanatomical systems [15–18].

The virulence and direction of spread of HSV-1 is strain-dependent. For example, HSV-1 strains F, HF, and HFEM are less virulent in mouse brain than strain 17 syn^+ [19]. In the case of direction of spread, the McIntyre-B strain spreads exclusively in the retrograde direction, while strain H129 is preferentially transported anterogradely [20]. Although each strain of alpha herpesvirus has a different transmission pattern, their structure and genomes are largely similar [9].

Multiple modifications have been made to the genome of HSV-1 to enhance its performance in neuronal tracing. By inserting the *Escherichia coli lacZ* gene into the *thymidine kinase* (*TK*) gene locus of HSV, Ho and Mocarski [21] constructed a *TK*-deficient recombinant virus that shows milder cytotoxicity without damage to its infectivity and viral gene expression. Moreover, *in-situ* detection of the virus is facilitated by adding the chromogenic substrate X-Gal, rather than the often difficult immunohistochemical localization of viral antigens. Furthermore, the labeling of the infected cells has been made increasingly easy by the use of various fluorescent proteins [22, 23]. Simultaneously, the capacity of HSV-1 to bear

Table 1 Characteristics of viruses commonly used for trans-neuronal tracing.

Virus	Genome type	Genome size	Gene number	Transgene capacity	Envelope	Trans-synaptic direction	Onset of target gene expression
HSV H129	dsDNA	$\sim$ 152 kb	$\sim$ 80	$\sim$ 130 kb	Yes	Anterograde	$<$ 1d
PRV Bartha	dsDNA	$\sim$ 150 kb	$>$ 70	$\sim$ 130 kb	Yes	Retrograde	$<$ 1d
RV	-ssRNA	11.9 kb	5	$<$ 6 kb	Yes	Retrograde	$<$ 1d
VSV	-ssRNA	11–12 kb	5	$<$ 6 kb	Yes	Anterograde	$<$ 1d
AAV1	ssDNA	4.7 kb	5	$<$ 4.5 kb	No	Anterograde	3–5d

Genome size and gene number refers to the wild-type viral genome. The malleable viral genome can incorporate various foreign genes with the size limitations listed above. The direction of viral spread can be retrograde and/or anterograde. Retrograde tracing is the spread from postsynaptic neurons to presynaptic neurons by trans-synaptic transmission. Anterograde tracing is spread from presynaptic neurons to postsynaptic neurons by trans-synaptic transmission. HSV, herpes simplex virus; PRV, pseudorabies virus; RV, rabies virus; VSV, vesicular stomatitis virus; AAV, adeno-associated virus; ds, double-stranded; ss, single-stranded; kb, kilobase.

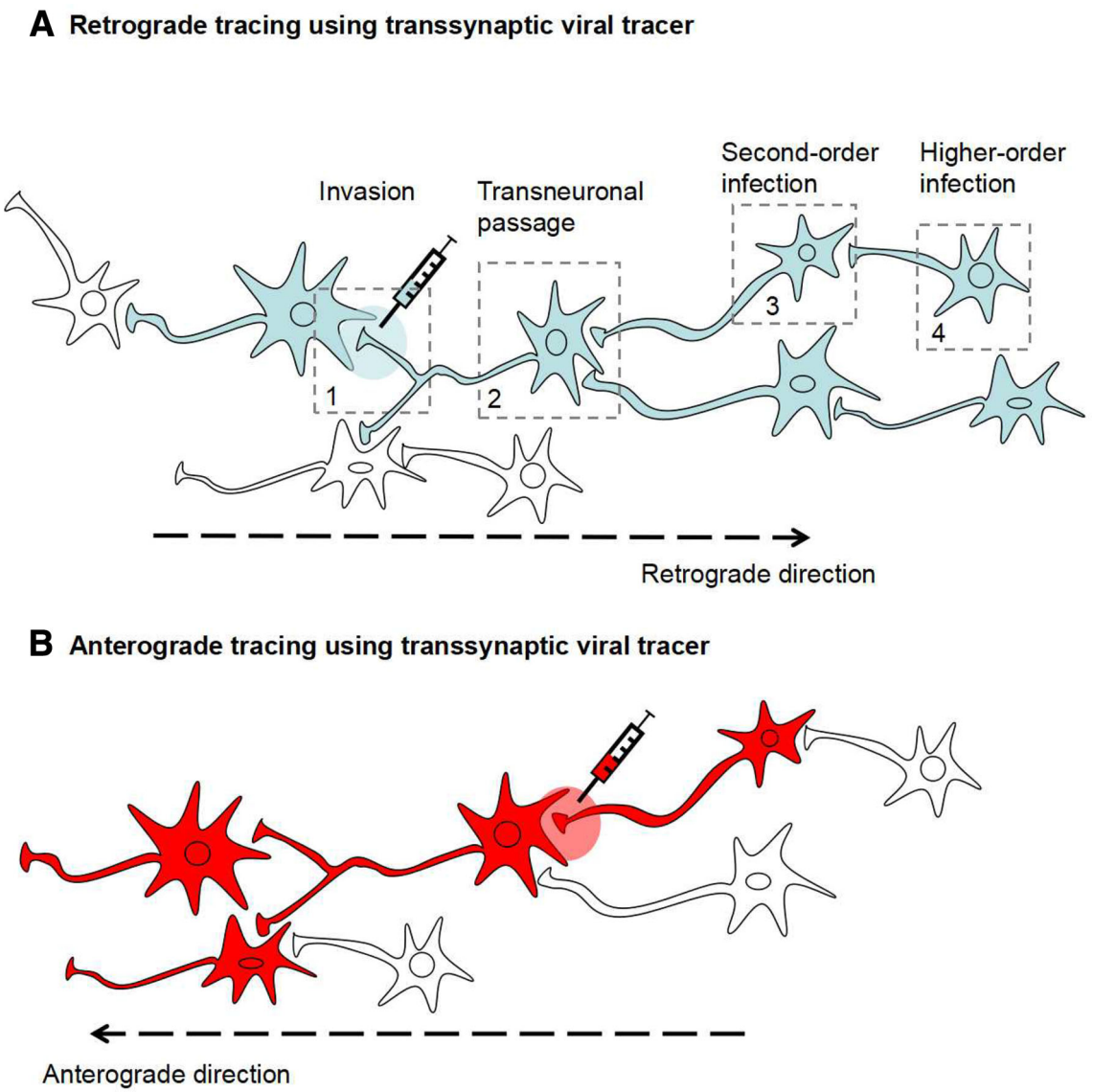

Fig. 1 Schematic of tracing using trans-synaptic viral tracers. **A** By injecting a retrograde viral tracer into region 1, the virus first infects the axons/dendrites/somata of first-order neurons in the injection site. Then, by trans-synaptic transmission in region 2, it spreads further to the synaptically-connected presynaptic second-order neurons in region 3. In this way, viral tracer can infect the presynaptic neurons connected with specific neurons and enable the tracing of chains of connected neurons. **B** Trans-neuronal anterograde tracing is the spread from presynaptic neurons to postsynaptic neurons by trans-synaptic transmission.

exogenous genes has been greatly expanded. Balan *et al.* [24] constructed different mutants of HSV-1 lacking the glycoprotein *gG*, *gE*, *gI*, or *gJ* to analyze their functions. They found that the gI-gE complex contributes little to attachment and penetration in the process of virus infection of cells. However, these glycoproteins are involved in the transmission between neuronal cells. Lilley and Coffin *et al.* [25] further reduced the toxicity of HSV-1 by deleting the immediate-early genes and inactivating other relevant genes. To date, improvements in the cytotoxicity and transmission efficiency of this virus are still in progress by functional analysis of the virus genome and, accordingly, genetic modifications [26].

Herpes Simplex Virus-1 Strain H129

As noted earlier, the H129 strain of HSV-1 preferentially spreads in the anterograde direction. Since Dix and Baringer *et al.* [27] reported this strain, a number of studies have applied this property to the anterograde tracing of neural circuits including the motor system, visual system, central viscerosensory pathways, and respiratory system [28–33]. To restrict the starter

neurons, Lo and Anderson *et al.* [34] developed an anterograde trans-synaptic tracer derived from H129 containing a Cre recombinase-dependent expression cassette of the *TK* gene (H129ΔTK-TT) (Fig. 2). In this modified strain, the endogenous *TK* gene that is essential for the replication and spread of HSV in non-mitotic cells such as neurons, was replaced by a loxP-STOP-loxP-tdTomato-2A-TK cassette through homologous recombination. When this recombinant virus infects neurons with cell-type-specific expression of Cre recombinase, Cre deletes the STOP sequence between the two *loxP* sites and therefore allows the expression of

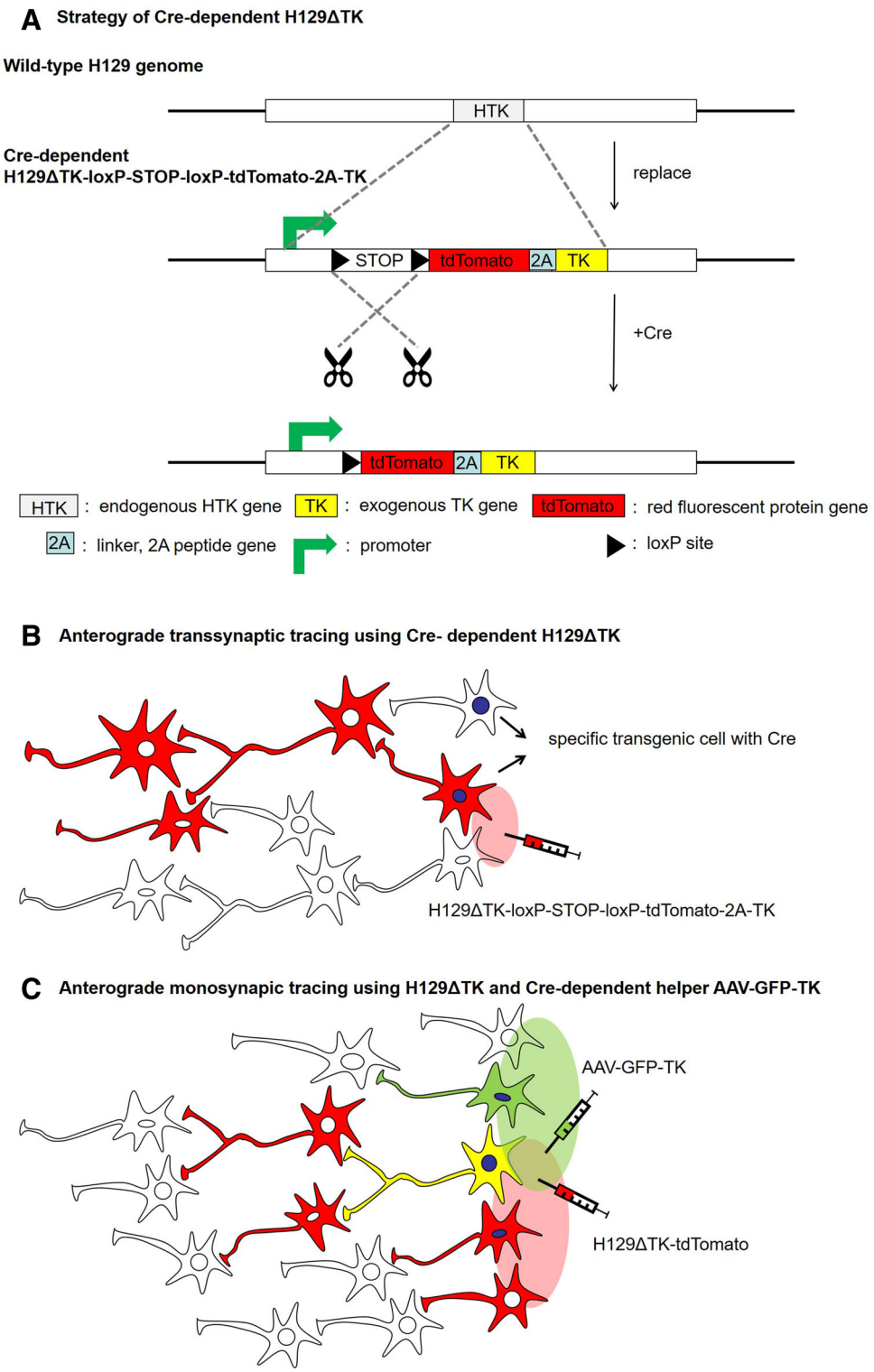

Fig. 2 Strategy and schematic of Cre- dependent H129ΔTK tracing. **A** H129 targeting strategy. Upper, schematic of the wild-type H129 genome; middle, schematic of the Cre-dependent H129ΔTK-loxP-STOP-loxP-tdTomato-2A-TK genome; lower, the targeting construct. The endogenous HTK gene was replaced with the expression cassette of the red fluorescent protein, tdTomato. Only in the specific subpopulation of cells with Cre recombinase (cell with a blue nucleus in B), the STOP sequence between the two loxP sites can be deleted and so allow the expression of tdTomato and the TK gene. Thus, the cell is labeled red and the virus can spread to the next cell. **B** By injecting Cre-dependent H129ΔTK-loxP-STOP-loxP-tdTomato-2A-TK, the virus infects neurons in the injection site. But only cells with Cre recombinase (blue nucleus) can be labeled with tdTomato fluorescent protein (red). The virus spreads from presynaptic neurons to postsynaptic neurons and so traces the specific neural circuit in an anterograde fashion. **C** Injection of H129ΔTK–tdTomato and Cre-dependent AAV-GFP-TK into the desired brain region. The starter neurons, which express both tdTomato and GFP, are indicated in yellow. The AAV helper virus that expresses TK in Cre-expressing cells is used to restrict the viral replication and transmission to only directly postsynaptic neurons.

tdTomato and TK [35]. Thus, the starter neurons can be easily distinguished.

The strategy using cell-type-specific expression of Cre also has limitations. One of the issues occurs when the injected virus infects axon terminals and is transported back to cell bodies that lie outside the injection site, which would almost certainly confound the interpretation of results. This problem is not restricted to H129, but applies to all neurotropic viruses that can infect axons [20, 34]. To address this issue, Dimitrov *et al.* [36] used an AAV that cannot infect axons to carry the *Cre* gene and label neurons at the injection site. This technique loses the cell-type specificity of Cre expression but ensures that only local neurons in the injection site express Cre recombinase and are therefore labeled by viral tracers. Furthermore, based on H129, Zeng *et al.* [37] developed an anterograde monosynaptic trans-neuronal tracer (Fig. 2). They deleted the *TK* gene and added the *tdTomato* gene into the H129 genome. The AAV helper viruses that express TK in the presence of Cre are used to enable the transmission of recombinant H129 to postsynaptic neurons. Since there is no TK expression in postsynaptic neurons, the spread of recombinant H129 is confined to directly postsynaptic neurons. Thus, tdTomato is expressed only in neurons directly postsynaptic to Cre-expressing neurons.

Pseudorabies Virus

PRV is an alpha-herpesvirus that causes Aujeszky's Disease in swine. Unlike HSV-1, PRV cannot infect higher primates, which renders it a safer tool in experimental practice [38]. Wild-type PRV (e.g., the Becker strain) is highly toxic and is transmitted bidirectionally [39]. Platt *et al.* [40] analyzed the toxicity of different strains of PRV and identified an attenuated Bartha strain that is less toxic to the host. Moreover, the Bartha strain only spreads retrogradely along neuronal pathways [41]. Further analysis of the PRV genome revealed that anterograde spread relies on *US7* (*gI*), *US8* (*gE*), and *US9* [10]. Deletion of any of these genes abolishes anterograde, but not retrograde, transmission of the virus. In addition, the absence of *gE* and *gI* reduces the virulence. The PRV-Bartha strain has a 3-kb deletion encompassing *US8* (*gE*), *US9*, and a large portion of *US7* (*gI*) and *US2* in its genome [42]. This mutation empowers PRV-Bartha as an efficient retrograde tracer with lower toxicity [10]. Numerous modifications are being made to its genome to make it a more versatile tracing agent [43].

On the basis of PRV-Bartha, Smith *et al.* [44] constructed PRV152 that expresses an enhanced green fluorescent protein (EGFP) and verified it in a study of the visual system. The recent development of genetically-encoded activity sensors also enables PRVs to report the activity of infected neurons. Boldogkoi *et al.* [45] developed a PRV that can report the activity of neurons by inserting a gene encoding the ratiometric Ca^{2+} indicator *TN-L15*. Since viral replication can potentially distort the electrophysiological properties of infected neurons, they further developed a time-shifted expression system to restrict the recording of neuronal activity to an early stage of infection [45].

The above strategy of Cre-dependent expression of TK was also introduced into PRV-Bartha so that researchers can perform retrograde trans-synaptic tracing from specific Cre-expressing neurons. This has shown great power in dissecting the neural inputs to the feeding center in the hypothalamus [46], the stress and olfactory systems [47], and the reward system [48]. A PRV-based retrograde monosynaptic trans-neuronal tracer has also been generated [47]. Similar to the HSV monosynaptic tracer system, the *TK* gene in the PRV Bartha genome is replaced by the *EGFP* gene. Then a lentiviral helper virus expressing TK in the presence of Cre is used to enable the transmission of recombinant PRV to presynaptic neurons. This monosynaptic tracer system can be combined with polysynaptic PRVs to distinguish direct and indirect inputs to Cre-expressing neurons. Since Livet *et al.* [49] reported the Brainbow strategy to randomly express multiple fluorescent proteins with distinct spectral properties in individual neurons based on Cre-dependent recombination, it has been used to label neurons and make them distinguishable from their neighbors. The Brainbow cascade was soon incorporated into the PRV genome by Kobiler *et al.* [50], and Card *et al.* [51, 52] used PRV263 bearing the Brainbow cassette to label the nephritic circuit in combination with a PRV (PRV267) or AAV that drives the expression of Cre. The strategy with the Brainbow PRV is useful for analyzing the integration and segregation of two separate neural pathways.

Neural tracing methods using PRVs have been applied not only to define existing circuits (e.g. the sympathetic [41, 53–58], visual [44, 59], respiratory [60], feeding [46], olfactory [47, 61], and reward-motivated systems [62]) but also to transplant-host circuitry [63, 64] and neuroplasticity after injury [65]. For example, Seiler *et al.* [63, 64] used PRVs to trace the synaptic connections between transplanted and degenerated retina, and Lane *et al.* [65] used PRVs to study neuronal connectivity in the lesioned spinal cord.

Some researchers have used dual viral trans-neuronal tracing to label multiple orders of neuronal connections. Kim *et al.* [66] used two antigenically-distinct recombinants of PRV in single and double infections of the rat central nervous system. They found that while the two viruses infect the same population of neurons, double-labelled neurons are quite sparse. Their observation could

be explained by "superinfection inhibition", that is, the first-infected virus inhibits subsequent infection by other viruses. When the viral strains used in experiments are non-isogenic, the more invasive strain within the neural circuits replicates faster and could prevent subsequent infection by the other viruses. Banfield *et al.* [67] developed two recombinant isogenic strains of PRV carrying a red or green fluorescent protein gene in order to avoid this uneven competition. The same strategy has also been used in RV tracers. Ohara *et al.* [68] achieved dual trans-neuronal tracing in the entorhinal-hippocampal pathway using RV. They found a strong negative correlation between the efficiency of double-labeling and the time interval between the first and second infections. Shortening the interval increases the ratio of double-labeling. McGovern *et al.* [29] also used dual H129 to map the lung sensory pathway. Although dual viral trans-synaptic tracing has the potential to define more complex connections of neural circuits such as divergent projections from single neurons [69], the mechanism of dual virus infection is not very clear yet. Therefore, false-negative results caused by superinfection inhibition may be obtained. Nevertheless, dual-virus trans-neuronal tracing will likely provide a powerful and sensitive method to study complex neural circuits.

Although HSVs are powerful neuronal tracers, they also have specific limitations. The main drawback is their cytotoxicity. HSVs can cause cell death and are commonly lethal to animals. Although their toxicity can be reduced by engineering viral genomes, such as *TK*-null H129 and PRV [70], this problem has not been solved completely. Oyibo *et al.* [71] developed a non-toxic, but replication-incompetent PRV by deleting the immediate-early gene *IE180* which is indispensable for viral replication. Further analysis by Wu *et al.* [72] revealed that superior cervical ganglia cells infected with the *IE180*-deleted PRV survive for 1 month without any cytopathic effects. Therefore, despite the lack of trans-synaptic transport capacity, *IE180*-deleted PRV has the potential to be developed into a less-toxic neurotropic tracer. The other problem is, as discussed above, that injected HSVs or PRVs can infect nerve terminals and be retrogradely transported to the cell body. This confounds the interpretation of results when dendrites and axons of Cre-expressing neurons coexist in the target region. Using Cre-dependent viruses with helper viruses that restrict the expression of Cre may overcome this problem.

Rhabdoviridae

Both RVs and VSVs used for neural circuit tracing belong to the family of Rhabdoviridae – negative-sense, single-stranded RNA viruses. They are small (180×75 nm) and the genome is ~ 12 kb and encodes only 5 proteins: nucleoprotein (N), phosphoprotein (NS or P), matrix protein (M), glycoprotein (G), and polymerase (L). It uses cytoplasmic machinery to synthesize its own protein to complete virus packaging and does not invade the nucleus of host cells [73].

Rabies Virus

RV is a neurotropic virus that causes lethal zoonotic disease. Its retrograde trans-synaptic transmission allows long-range spread from the infected peripheral site to the central nervous system [74]. *RVG* encodes the sole RV envelope protein G and RV can only infect neurons *via* receptors for RVG [5]. Unlike HSV, RV is transported exclusively in the retrograde direction and its infection is largely restricted to neurons and not glia. In addition, RV induces less apoptosis and infected animals show fewer pathological traits in contrast to HSV. All these features make RV an ideal tool for tracing neural connections [73].

Goodpasture [75] first demonstrated that RV travels along axons just like herpes viruses. Mebatsion *et al.* [76] reported that infection by RV relies on RVG, and Etessami *et al.* [77] demonstrated that RVG is necessary for the trans-synaptic spread of RV. Based on these findings that RVG plays a key role in the infectivity and trans-neuronal spread of RV, Wickersham *et al.* [78] and Wall and Callaway *et al.* [79] developed a monosynaptic trans-synaptic system derived from SAD-B19, an attenuated vaccine strain of RV (Fig. 3). This system exploits a recombinant RV in which the *RVG* gene is replaced by EGFP and pseudotyped with envelope glycoprotein (EnvA) from avian sarcoma-leukosis virus (ASLV-A), the cognate receptor of which is TVA. Since TVA is an avian receptor and does not exist in mammals, the recombinant RV [SADΔG-EGFP(EnvA)] cannot infect mammalian neurons. When two viruses, SADΔG-EGFP(EnvA) RV and a helper virus (AAV) that express TVA and RVG in a Cre-dependent manner are injected into the desired region, the RV can only invade cells with Cre expression and then spread to their upstream neurons. Choi *et al.* [80–82] further developed a selective monosynaptic tracing system that can infect neurons expressing a specific cell-surface ligand by using a viral receptor–ligand bridge protein (TVB-NRG). ErbB4 is the receptor for neuregulin (NRG) that is found exclusively on inhibitory neurons of the cerebral cortex. EnvB is the envelope glycoprotein from ASLV-B, and its receptor is TVB. Unlike the TVA system that requires the binding of TVA with its receptor EnvA to meduate viral infection, TVB can support virus entry into cells directly. Thus, the receptor–ligand bridge protein TVB-NRG can mediate the infection of cells with ErbB4 by a G-deleted RV bearing TVB-NRG. In addition, the

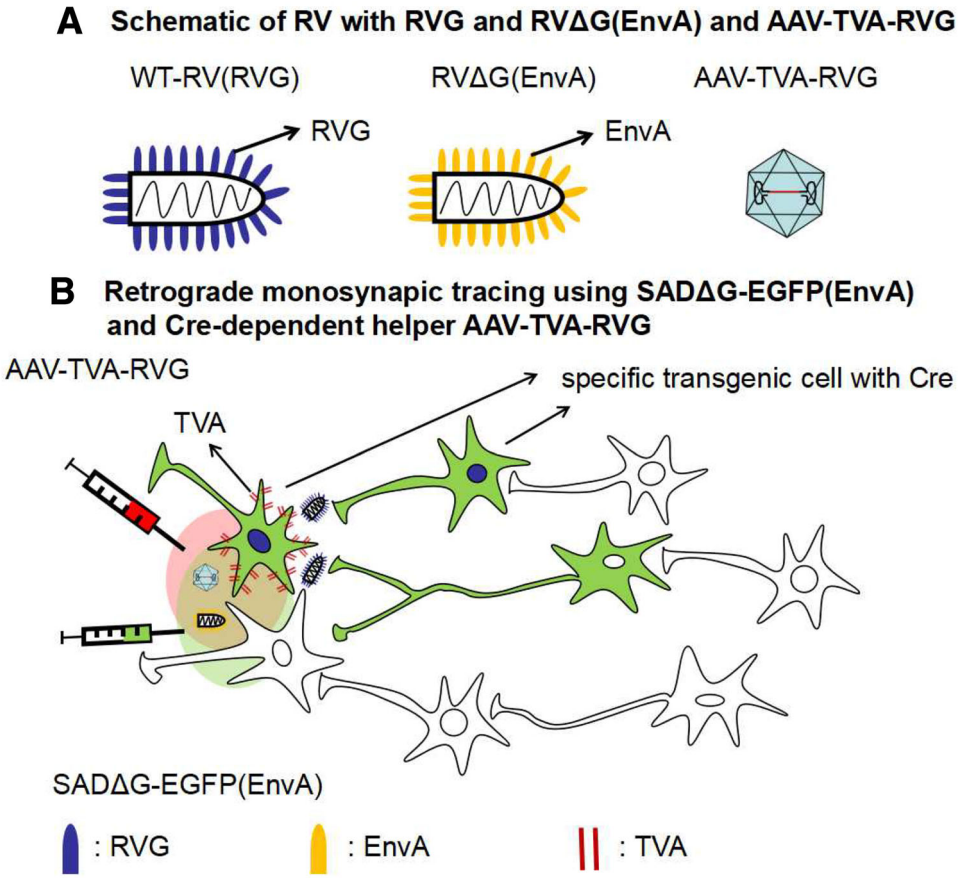

Fig. 3 Schematic of retrograde monosynaptic tracing using RVΔG(EnvA) and Cre-dependent helper AAV. **A** Rabies G protein (RVG) is necessary for the trans-synaptic spread of RV. By replacing RVG with EnvA protein, the recombinant RVΔG(EnvA) cannot infect neurons that do not express TVA, and cannot spread across synapses. **B** By injecting SADΔG-EGFP(EnvA) and Cre-dependent helper AAV-TVA-RVG into the target area, only the Cre-expressing cells can express TVA receptors and RVG and can be infected by SADΔG-EGFP(EnvA). In these cells, RVΔG can assemble into RV(RVG) which regains the capacity for trans-synaptic spread and realize retrograde monosynaptic tracing.

helper vectors to provide EnvB (the receptor of TVB) and RVG could also be a lentivirus or other means of delivery. Marshel *et al*. [83] used single-cell electroporation of DNA to deliver a fluorescent marker (mCherry), TVA receptors, and RVG into targeted cells. Then monosynaptic retrograde tracing was achieved by injecting the recombinant RV SADΔG-EGFP(EnvA) into the transfected cells [84, 85].

Kim *et al.* [86] developed an optimized RVG protein that consists of SAD-B19G and rabies Pasteur virus strain glycoprotein. They claimed that this chimeric G increases the efficiency of the long-distance transport of viruses compared to SAD-B19G. Miyamichi *et al.* [87] introduced a point mutation into TVA to reduce TVA activity in order to abolish SADΔG-EGFP(EnvA) infection of cells not expressing Cre. Although the numbers of starter cells are reduced, this strategy is suitable for analyzing local circuits.

To date, monosynaptic neuronal tracing with glycoprotein-deleted RV has been applied to various nervous systems/pathways [88] (e.g. the motor [84], olfactory [87, 89, 90], and visual systems [91, 92], and the dentate gyrus [93], hunger-driven [94], dopamine neuron [95], and sleep-wake regulation circuits [96]).

The application of RV-based tracing has been expanded to various research fields. For instance, Garcia *et al.* [97] used the RVΔG-EGFP(EnvA) system to trace the connectivity of embryonic stem cell (ESC)-derived neurons. They introduced TVA, RVG, and a fluorescent marker (tdTomato) into ESCs by electroporation, and after culturing the ESC-derived neurons, transplanted them into mouse brain. Then they injected SADΔG-EGFP (EnvA) into the transplantation region to trace the synaptic connectivity. Therefore, RV can contribute to understanding the development and differentiation of ESCs in the brain.

New RV-based strategies have also been developed to potentially trace both the input and output connections of specific brain regions and cell types. Schwarz *et al.* [98] developed two retrograde monosynaptic methods called TRIO and cTRIO. In these methods, they used canine adenovirus type 2 (CAV2) to provide recombinase (Cre or Flp). CAV2 is a non-trans-synaptic tracer that can transduce neurons through their axonal terminals and efficiently traffic back to the soma *via* retrograde axonal transport. In the TRIO system, by injecting CAV-Cre to one of the output regions, Cre is expressed in neurons that send axonal projections to the CAV2-injected area. By subsequently injecting RVΔG(EnvA) and Cre-dependent AAV that express TVA and RVG into the targeted area, RV can

infect only neurons in the targeted area that send axonal projections to the CAV2-injected regions, and are then transported to monosynaptically upstream neurons. cTRIO is a cell-type-specific system using CAV2 expressing Flp and AAV expressing TVA and RVG only in the presence of both Cre and Flp. With the help of the two viruses, RV can infect Cre-expressing neurons in the targeted area that send axonal projections to the CAV-injected region. Therefore, one can monosynaptically trace from a specific type of cell projecting to a specific brain area.

Although RV is less toxic than HSV, it is still lethal to experimental animals in most cases. It markedly shortens the time window for subsequent anatomical and physiological analysis, especially in studies where a prolonged period of observation is required, such as the study of aging and neurodegenerative diseases. To address this issue, a series of studies have been performed. Wirblich and Schnell [99] demonstrated that the expression level of RVG is not closely related to the pathogenicity of RV. Mori *et al.* [100] further showed that RVG itself is not cytotoxic by over-expression of RVG in the embryos of animal since no abnormality was found in the nervous system until adulthood. Haberl *et al.* [101] constructed a chimeric G protein composed of a VSV-G surface domain and an RV transmembrane and cytoplasmic domain to replace the regular RVG. This allows a longer time window for physiological experiments.

Reardon *et al.* [102] reported a CVS-N2c strain which is rather virulent and highly neurotropic. Unexpectedly, use of this strain in RVG-dependent monosynaptic tracing markedly reduces the neurotoxicity and increases the efficiency of retrograde trans-synaptic transmission. Compared with the traditional SAD-B19 strain, CVS-N2c extends the time window for physiological experiments from 5–7 days to nearly 28 days, during which cells remain viable. Ciabatti *et al.* [103] developed a self-inactivating RV-ΔG (SiR) which makes life-long access to neural circuit tracing possible. They fused the N protein of RV-ΔG with the PEST proteasome-targeting domain which directs the proteasomal degradation of protein. Therefore, viral protein expression or SiR disappear following the primary infection and trans-synaptic transport, resulting in the prevention of cytotoxicity. By expressing Cre or Flp by SiR, virus-infected cells permanently express genetic markers/tools without cytotoxicity. SiR infection does not show abnormal neuronal physiology for up to 5 months. A Tobacco Etch Virus cleavage site (TEVs) is interposed between the RV protein and the PEST proteasome-targeting domain. The TEVs linker can be specifically cleaved by the Tobacco Etch Virus protease (TEVp), resulting in separation of the RV proteins and PEST proteasome-targeting domain, thus preventing their degradation. Through the above machinery, TEVp can act as a switch to control the viral transcription-replication cycle. Chatterjee *et al.* [104] developed a non-toxic but non-trans-synaptic mutant RV (RVΔGL) by deleting the viral polymerase gene (L) in addition to glycoprotein (G). This strategy helps to maintain the viability of infected neurons for even 1 year. The size of L is very large, beyond the packaging capacity of AAV. However, by using other viral vectors, it may be possible to develop monosynaptic tracing systems using RVΔGL.

RV is an ideal retrograde neuronal tracer due to its hypotoxicity, wide host spectrum, and simple genomic structure. However, the unidirectional transmission of RV restricts its use in anterograde tracing. Another limitation is that it can only accommodate an insert up to 6 kb in size, far less than HSV (130 kb) [105], which greatly limits its application. Another drawback of RV is that its genomic RNA cannot be processed by Cre recombinase. Therefore, the polysynaptic tracing systems used for PRV or HSV cannot be applied with RV, and only monosynaptic tracing is available unless G protein is supplied in the downstream neuron.

Vesicular Stomatitis Virus

VSV is an arthropod-borne virus that can infect a broad range of mammals (e.g., rodents, cattle, swine, horses, and humans). VSV infection of livestock leads to significant disease characterized by vesiculation and ulceration around the mouth, hoofs, and teats. But this usually resolves in a few weeks without fatality [106, 107]. Naturally-occurring human infection with VSV is rare, and most such infections are asymptomatic, while some may have mild flu-like symptoms. So VSV is relatively less virulent to humans than HSV and RV [108].

The glycoprotein of VSV (VSV-G) is necessary for viral binding to target cells [109]. And VSV-G binds to phosphatidylserine that is a universal component of cell-surface membranes [108]. This enables VSV to infect almost all cell types. Immediately after entry into a cell, VSV replicates rapidly. The first progeny of viruses can be generated and released within 1.5 h [6], so it is very useful for the rapid expression of exogenous genes. The expression level of the exogenous genes carried by VSV depends strongly on their genomic position. Generally, the first gene locus yields the highest transcript level that is optimal for the insertion of marker genes [110].

Lundh [111] first reported the anterograde and retrograde trans-neuronal transmission of VSV after retinal injection. Beier *et al.* [112] constructed VSV vectors with different combinations of glycoprotein genes from other viruses and demonstrated that the direction of viral transmission depends on the glycoprotein. VSV with intrinsic glycoprotein is primarily transmitted in the

anterograde direction. Furthermore, pseudotyping of VSV with the G protein from lymphocytic choriomeningitis virus provides VSV with the ability of anterograde trans-synaptic transmission, while VSV with the G protein from RV [VSV(RV-G)] can be retrogradely transmitted. Similar to RV, VSV-G deleted- and EnvA pseudotyped-VSV has been developed and used with AAV expressing EnvA and RVG for monosynaptic tracing. Using this system, Beier *et al.* [110] analyzed the retrograde trans-synaptic spread of VSV with RVG. Although the recombinant virus can be specifically transmitted in the retrograde direction, the transmission efficiency appears to be lower than RV.

Mundell *et al.* [113] have successfully applied VSV (VSV-G) and VSV (RV-G) to a wide range of vertebrates, including mouse, monkey, seahorse, zebrafish, axolotl, and *Xenopus*. However, the virus did not infect so well in two invertebrates, *Drosophila melanogaster* and the box jellyfish, suggesting the likely absence of viral receptors in these invertebrate species.

Although VSV has been demonstrated to have the appealing compatibility to be pseudotyped with glycoproteins from other viruses, its efficiency is not always high. VSV is relatively innocuous for laboratory workers because it does not cause severe disease in humans. However, unlike RV, VSV infection triggers a dramatic immune response, while RV can often escape from immune surveillance [108]. Moreover, VSV is much more toxic to infected cells than RV because of its rapid and intensive transcription [108], and it displays distinct virulence at the cellular level and is lethal to experimental animals when directly injected into the brain. Besides its shortcomings, the early onset of gene expression in VSV ($\sim$ 1 h) still makes it beneficial in some experimental paradigms which need to be done within a narrow time window. Altogether, VSV is a useful tracer with good compatibility but still needs further improvement in virulence and efficiency of spread.

Adeno-associated Virus

AAV is a small (25 nm), non-enveloped, single-stranded DNA virus with a genome of $\sim$ 4.7 kb [114]. Humans are commonly infected by AAV, but it is largely non-pathogenic and causes few health problems [6]. Recombinant adeno-associated virus (rAAV) has been commonly used for gene transfer in various tissues [115]. For trans-synaptic neuronal tracing, Zingg *et al.* [7] reported that AAV serotype 1 and serotype 9 exhibit anterograde trans-synaptic spread under the condition of a high titer [116]. Coupled with the Cre-dependent reporter system, AAV1 can efficiently and specifically tag postsynaptic neurons. However, without viral replication, the mechanism by which AAV is transported anterogradely through synaptic connections is still not clear.

Conclusions

Neurotropic viruses have been widely used to map neural networks. Since they have different characteristics, such as different genome sizes, DNA or RNA viruses, and diverse virulence and biosafety levels, these features should be taken into account when choosing viral tracers for experiments. While remarkable progress has been made, there is still an unmet need for the optimization of these tracing tools. Many questions remain to be addressed. For example, we know little about the mechanisms of viral infection, trans-synaptic transmission, and the effects of the viruses on cells, especially on neurons *in vivo*. By revealing the underlying mechanisms, we will be able to generate optimized viruses with high efficiency and low cytotoxicity. Moreover, we may be able to modulate the directions of viral transport and gene expression in any desired viral strain in the future. Thus, more powerful viral tracers will continue to emerge to help delineate complex neural networks.

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