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Viral vectors for neural circuit mapping and recent advances in trans-synaptic anterograde tracers

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Summary

Viral tracers are important tools for neuroanatomical mapping and genetic payload delivery. Genetically modified viruses allow for cell-type specific targeting, and overcome many limitations of non-viral tracers. Here we summarize the viruses that have been developed for neural circuit mapping, and we provide a primer on currently applied anterograde and retrograde viral tracers with practical guidance on experimental uses. We also discuss and highlight key technical and conceptual considerations for developing new safer and more effective anterograde trans-synaptic viral vectors for neural circuit analysis in multiple species.

In Brief

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Xu et al. reviews the viruses that have been developed for neural circuit mapping, and provide a primer on currently applied anterograde and retrograde viral tracers with practical guidance on experimental uses.

INTRODUCTION

A connectome is a comprehensive map of neural connections. Guided by earlier established macroscale wiring diagrams that provide a global overview of central nervous system (CNS) circuit architecture, a central goal of modern neuroscience research is to understand the cell-type specific connections between different regions and the detailed circuit organization within them. In neural circuits, classes of neurons can now be subdivided on the basis of their gene expression profiles, physiology and morphology (Luo et al., 2018). Information transfer between brain regions is largely mediated through synaptic transmission of populations of individual neurons. The typical organization of a neuron is a cell body with dendrites that receive and integrate incoming information, and a projecting axon that sends information to the dendrites of other neurons via chemical synaptic transmission. Variations on this organization can occur, such as axo-axonal synapses and electrical synapses between different parts of neurons. Synapses at the terminals of axons most often form connections to the dendrites of other neurons. Neural tracing experiments map these connections between neurons. Anterograde tracers allow the visualization of neurons and their targets, typically beginning in cell bodies and travelling down axons to their post-synaptic targets. Retrograde tracers progress in the opposite direction, from the terminals of axons back to their cell bodies. Anterograde and retrograde transport of neural tracers owes their directional specificity to differential intracellular transport pathways in neurons (Maday et al., 2014).

Naturally occurring viruses have been used for neural circuit tracing for decades by exploiting the natural properties of viral propagation and transmission. Genetic modifications of such viruses have led to many improvements for neuroscience applications. In addition to anatomical mapping, genetically modified viral tracers have greatly facilitated functional studies of cell-type specific and circuit-specific neural networks in the brain. Certain viruses, such as some serotypes of adeno-associated virus can be injected systemically into the blood stream and then can cross the blood–brain barrier to infect the whole brain (Chan et al., 2017; Zincarelli et al., 2008). Alternatively, viruses may be directly introduced into a specific brain region using injection, through pressure or Iontophoresis (Harris et al., 2012; Oh et al., 2014; Winnubst et al., 2019).

In this article, we first provide a comparative introduction of anterograde and retrograde viral and non-viral tracers for neural circuit analysis, while describing neural circuit tracing history and background. We describe the specific viruses used for neuroscience research, and provide essential information to guide readers on their choice of viral tracers, including caveats where appropriate. We then focus on anterograde-directed viral vectors and their applications for axonal tract tracing and trans-synaptic labelling. Last, we discuss recent progress, outstanding questions and future directions related to anterograde-specific monosynaptic viral tracing tools.

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CONVENTIONAL TRACERS FOR NEURAL CIRCUIT MAPPING

Neuroscientists have been mapping the structure of brain connections for ~170 years. Starting in the 1850s, the first method of neuroanatomical tract tracing was lesion-based, relying on Wallerian degeneration of axons that progressively degenerate in an anterograde fashion and reveal tracts that project distally from the site of the lesion. In the peripheral nervous system, Wallerian degeneration occurs as regenerative processes begin within a week of the lesion, whereas in spinal cord and other central nervous system tissues, very little regeneration occurs post lesion. Subsequently, "Wallerian-like" degeneration features were described for neurodegenerative diseases that include aspects of impaired axonal transport in Amyotrophic lateral sclerosis and Alzheimer's disease, for which retrograde degeneration is more pronounced. In the 1940s Walle Nauta developed a silver staining method for enhancing degeneration-based signals, which was the dominant approach until the 1960s when chemical tracers were developed. These tracers included radiolabeled amino acids that allowed auto-radiographic tracing of axonal connections in the CNS (Cowan et al., 1972; Lasek et al., 1968; Taylor and Weiss, 1965) and 2-deoxyglucose were used to spectacular effect to map functional divisions of visual cortex such as ocular dominance columns without the requirement of damaging the tissue of interest (Hubel et al., 1977; Tootell et al., 1988). Shortly thereafter, scientists developed and identified a suite of retrograde tracers including horseradish peroxidase (HRP), cholera toxin β-subunit (CTB), tetanus toxin and fluorogold, and anterograde tracers such as a biotinylated dextran amines (BDA) and phytohemagglutinin-L (PHA-L), as well as those that labeled both inputs and outputs, such as wheat germ agglutinin (WGA) [see reviews in (Lanciego and Wouterlood, 2011; Nassi et al., 2015)]. In the mid-1980s, stable fluorescent retrobeads with low-toxicity were also developed for retrograde tracing experiments (Katz et al., 1984). The use of these conventional tracers over the past few decades was essential for developing our current understanding of neuroanatomy and global neural connectivity in the brains of different species.

Non-viral conventional tracers are efficient tools for visualizing bulk neural connections, with the only specified parameter being the location of cell bodies or axon terminals. They typically come in a readily-injectable form and experiments can be performed in any animal of interest as they do not require specific transgenic lines or genetic methods. However, the utility of non-viral conventional tracers is mostly limited to mapping global connectivity. Most conventional tracers do not have sufficient resolution to reveal connectivity at the level of molecularly defined cell types; nor do they reveal whether axons that terminate in a particular location make synaptic contacts onto particular cell types or onto cells that in turn connect to other cells. Conventional tracers can remain extracellular at the injection site, and they generally label all of the cell bodies at the injection site or projecting there.

In contrast, viruses can be targeted to specific cell types using genetic strategies, for example, via specific expression of a viral receptor (Choi et al., 2010; Wickersham et al., 2007b) or by expressing recombinase proteins in specific cells to direct productive infection or payload delivery to those cells (DeFalco et al., 2001; Lo and Anderson, 2011). In some cases, viruses can be modified to express small molecule tracers such as WGA within target cell types of interest (Gradinaru et al., 2010). These technical enhancements have led to the

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replacement of chemical tracers by targeted viral-genetic methods in modern neuroscience research.

VIRUSES USED FOR NEURAL CIRCUIT TRACING

The use of viral tools in neuroscience is highly interdisciplinary, so in this section we provide essential background in virology to guide users in their choice of viral tracers and to help interpret experimental data. Viruses are small infectious agents that replicate only inside the living cells of an organism; they can infect all life forms. A complete and infectious virus particle, known as a virion, consists of nucleic acid surrounded by a protective coat of protein called a capsid. The capsid is made from proteins encoded by the viral genome and its shape serves as the basis for morphological distinction. Some virus families have an additional covering, called the envelope, which is usually derived in part from modified host cell membranes. Capsid proteins and viral envelope glycoproteins are involved in virus attachment and interactions with receptors present on the surface of the host cell, which determines viral tropism, that is, the specificity of a virus for a particular host tissue. Viral pseudotyping, the practice of replacing the envelope glycoproteins or capsid proteins of one virus with those of another, is a strategy that can be used to create viral vectors with new tropism and trafficking properties. Manipulation of these viral surface proteins can improve the transduction efficiency of viral vectors, and can expand or restrict their tropism.

Viruses are classified by a combination of molecular and structural features, including nucleic acid class, the presence or absence of an envelope, and their genome replication mode (Flint et al., 2015; Knipe and Howley, 2013). Figure 1 shows the major families of animal viruses, including the viruses used for neuroscience and other biomedical applications. Viruses are generally divided into DNA and RNA viruses that contain either a single-stranded or a double-stranded genome. A single-stranded genome consists of a string of unpaired nucleotides; a double-stranded genome includes a string of complementary paired nucleotides. For most viruses with RNA genomes and some with single-stranded DNA genomes, the single strands are said to be either positive-sense or negative-sense, depending on whether they are complementary to viral messenger RNA (mRNA). Positive-sense viral RNA can be directly translated by the host cell. Negative-sense viral RNA is complementary to mRNA and thus must be converted to positive-sense RNA by a viral RNA-dependent RNA polymerase before translation. For DNA viruses, positive-sense single-stranded DNA is identical in sequence to the viral mRNA and is thus a coding strand, while double-stranded DNA consists of both template and coding strands. The production of mRNA and viral genome replication differ accordingly among different types of DNA and RNA viruses (Figure 1, bottom portion).

The viruses used in neuroscience research are most often genetically modified or recombinant strains of wild type viruses (Frampton et al., 2005; Lee et al., 2017; Naso et al., 2017). These include adeno-associated virus, adenovirus, herpes simplex virus and pseudorabies virus, lentivirus and other retroviruses, Sindbis virus and Semliki Forest virus, rabies virus and vesicular stomatitis virus, vaccinia virus, and baculovirus. Tables 1 and 2

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show the comparative properties of current viruses used for gene delivery and applied for neural circuit tracing, respectively.

Viral vectors for gene delivery and cellular labeling

Here we describe the viral vectors summarized in Table 1. Adeno-associated virus (AAV), a member of the *Parvoviridae* family, has a single-stranded, positive-sense DNA genome of ~5 kilo base pairs (kb). AAV virions are small (~20 nm), naturally replication defective, nonenveloped viruses with a roughly spherical shape. Wild type AAV infects both dividing and non-dividing cells and can remain latent in host cell DNA by integrating into the host cell genome in a site-specific manner (the AAVS1 locus in the human chromosome 19) (Hamilton et al., 2004; Kotin et al., 1990). Recombinant AAV (rAAV) has been widely used in neuroscience due to its broad tropism, relatively long-term expression in non-dividing cells including neurons and lack of pathogenicity in animal models. Unlike wild type AAV, the genomes of recombinant AAV vectors typically do not undergo integration into the host DNA but are primarily maintained as a circular form known as an episome in the nuclei of transduced cells (Colella et al., 2018). One disadvantage of rAAV vectors is their small transgene capacity (approximately 4.8 kb), but they have many advantages, including the ease with which the capsid proteins can be modified to bias transduction to different cell types (Pillay et al., 2016; Pillay et al., 2017). rAAVs are often used in combination with Cre/loxP and Flp/FRT recombinase technologies, which generally apply to all DNA viruses.

Viral titer and capsid-receptor interactions are key determinants in how efficiently AAV enters cells and uncoats its single DNA strand. The subsequent synthesis of the complementary strand by host cell machinery is a slow process that affects the onset of transgenic expression and transduction efficiency of rAAVs. A dimeric, or self-complementary AAV (scAAV) has been designed to overcome this limitation. Compared with single-stranded rAAVs, scAAV vectors are 5 to 140-fold more efficient transducing agents than conventional rAAVs *in vitro*, and result in rapid and higher levels of gene payload expression *in vivo* (McCarty et al., 2001). However, the packaging capacity of scAAV (<2.5 kb) is half that of single-stranded AAVs, further restricting the number of genes and regulatory elements that can be packaged.

Adenoviruses (AdVs) are members of the *Adenoviridae* family. They are medium-sized (90–100 nm), spherical nonenveloped viruses with a double stranded DNA genome that is between 26 and 48 kb. Advantages of adenovirus-derived vectors include high transduction efficiency in most mammalian cells, and a lack of dependence on active host cell division (Lee et al., 2017). A disadvantage is that they are generally more toxic than AAV. The most commonly used adenoviral vectors are derived from human adenovirus type 5 (AdV5). The improvements of adenoviral vectors involved removal of viral replication genes and non-structural genes, and reduction of immunogenicity. The most recent generation of adenoviral vectors are “gutless”, as they are stripped of all viral coding sequences. These high capacity adenoviral vectors (HC-AdVs) allow transgenic insertions of up to 36 kb. While HC-AdVs lack viral elements that induce a host immune response, a complementary or helper virus is needed to provide the necessary proteins *in trans* for the packaging of HC-AdVs. Adenovirus vector-mediated gene expression is transient, ranging from two weeks to a few

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months. Therefore, they are not suitable for long-term studies but serve well for experiments that need high-level and transient expression.

Canine adenovirus type 2 (CAV2) is a useful tool for neuroscience, largely because it efficiently infects neurons at their axon terminals and travels retrogradely to their cell bodies (Del Rio et al., 2019; Kremer et al., 2000). CAV2 variants carrying the gene for Cre recombinase (CAV2-Cre) or genetic labels, permit retrograde expression, but can be more toxic than AAV or AAV2-retro at similar levels of transduction (Kremer et al., 2000; Tervo et al., 2016).

Retroviruses, including lentiviruses, belong to the *Retroviridae* family. Their virions are spherical, enveloped and are 80–100 nm in diameter, with two copies of a single-stranded positive sense RNA genome (about 7–10 kb). For most other viruses, viral DNA is transcribed into RNA that is then translated into protein. In contrast, retrovirus genomic RNA is first reverse-transcribed into DNA and is then integrated into the host cell genome. After genomic integration, the host cell RNA polymerase II transcribes the viral genes into mRNAs. Lentiviral vectors are characterized by their relatively late onset of transgene expression. Most retroviruses cannot infect non-dividing cells such as neurons, but lentiviruses can infect dividing and non-dividing cells regardless of their proliferation status. HIV-based vectors make up the majority of lentiviral vectors used in research today, including their use as the most common gene delivery vehicle to establish stable cell lines. Lentiviral vectors for CNS gene transfer are usually based on the HIV-1, the virus that causes Acquired Immuno Deficiency Syndrome (AIDS). Due to safety concerns, vectors derived from other lentiviruses, such as the non-primate equine infectious anemia virus and feline immunodeficiency virus, have been developed as gene transfer vectors for CNS targets (Poeschla, 2003). Another notable retroviral vector was derived from the mouse retrovirus, murine leukemia virus. This vector has proved to be an important tool to label newborn neurons for lineage tracing applications (Price et al., 1987; Turner and Cepko, 1987; Yu et al., 2009). It also has been used for circuit mapping and functional studies of new born neurons in the adult brain (“adult neurogenesis”) (Ge et al., 2006; van Praag et al., 2002; Vivar et al., 2012).

Lentiviral tropism is largely defined by the glycoproteins on their envelope that mediate viral binding to the host cell surface receptors. A wide range of envelope glycoproteins, including those from vesicular stomatitis virus and rabies virus have been used to pseudotype lentiviral vectors (Mazarakis et al., 2001).

Sindbis virus and Semliki Forest virus are two members of the *Togaviridae* family. Members of this family are spherical enveloped viruses (65 - 70nm in diameter) with a single-stranded positive-sense RNA genome of 10 - 13 kb, which has a 5' cap and a 3' poly(A) tract and can serve directly as mRNA in a host cell. Wild-type strains of both viruses infect neurons in the CNS, induce apoptosis and cause encephalitis in rodents, although Sindbis virus is non-pathogenic in humans (Fazakerley et al., 1993; Nargi-Aizenman and Griffin, 2001). Vectors based on Sindbis virus or Semliki Forest virus have been developed to rapidly express high levels of transgenes (in hours) in brain tissue *in vitro* and *in vivo* (Ehrengruber et al., 1999; Kebschull et al., 2016). Transgene expression is transient, however, due to the high

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cytotoxicity of these viruses (Nivitchanyong et al., 2009). Recombinant Sindbis virus and Chikungunya virus, a member of the Semliki Forest Virus subgroup have been used to image viral neuroinvasion and host–pathogen interactions in zebrafish (Palha et al., 2013; Passoni et al., 2017; Zhang et al., 2019).

Vaccinia virus (VACV) is a large enveloped virus belonging to the *Poxviridae* family. Poxviruses are unique among DNA viruses because they replicate only in the cytoplasm of the host cell, outside of the nucleus (Condit et al., 2006; Schramm and Locker, 2005). VACV has a linear, double-stranded DNA genome approximately 190 kb in length. Poxvirus virions lack the symmetry features common to other viruses, and they appear as "brick shaped" membrane-bound particles with a size of roughly 360 × 270 × 250 nm. VACV has been used to deliver transgenes to neuronal cells, resulting in efficient but transient transgene expression (Moss and Earl, 2002; Pettit et al., 1995; Wu et al., 1995). The large size of the vaccinia virus genome permits insertion of multiple genes under the control of one or multiple gene regulatory elements.

Baculoviruses belong to the *Baculoviridae* family, whose members naturally infect invertebrates. The viruses contain a double-stranded DNA genome ranging from 80 to 180 kb. The baculovirus–insect cell expression system has become one of the most widely used systems for routine production of recombinant proteins (Kost et al., 2005). The baculovirus protein expression is noteworthy in that it produces high levels of recombinant proteins that are properly folded. Natural baculoviruses cannot infect mammalian cells, but expanded tropism can be enabled via modification of the baculoviral envelope glycoprotein (Barsoum et al., 1997). Baculovirus gene transfer into mammalian cells, known as BacMam, is based on a genetically engineered, VSV-G pseudotyped baculovirus that contains a DNA cassette for transgene expression driven by a mammalian specific promoter (Boyce and Bucher, 1996). Transduction rates of up to 80% of targeted cells are reliably achieved with BacMam at high multiplicity of infection without apparent neuro-cytopathic effects. VSV-G pseudotyped baculoviruses also mediate efficient gene transduction in the cerebral cortex of mice by direct injection *in vivo* (Tani et al., 2003). In contrast to other commonly used viral vectors, engineered baculoviruses have the unique property of replicating in insect cells while being incapable of initiating a replication cycle and producing infectious virus in mammalian cells. The viruses can be readily manipulated, accommodate large insertions of foreign DNA, and have a good biosafety profile (Kost and Condreay, 2002). We foresee growth in the broad use of baculovirus-mediated transduction in neuroscience research as these favorable properties become more widely appreciated.

Transneuronal / trans-synaptic viral vectors

We next describe the viral vectors summarized in Table 2. Herpes simplex virus (HSV) (human origin) and pseudorabies virus (PRV) (porcine origin) belong to the *Herpesviridae* family. Members of this family are enveloped, spherical to pleomorphic viruses with a size of 150-200 nm in diameter. They have a double-stranded DNA genome that ranges from about 125 to 240 kb. Herpesvirus DNA is transcribed into mRNA within the infected cell nucleus. One very appealing feature of herpesviruses as vectors is that HSV type 1 (HSV1)

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is capable of delivering up to 50 kb of transgenic DNA when used as a vector; thus HSV1 is one of the largest viral delivery vectors.

The name of “pseudorabies” implies a functional relationship between PRV and rabies virus, but these two viruses are very different. PRV is a herpes virus, not a rabies virus (see below). The reason for this confusing nomenclature originated from the observation that PRV produced CNS infection and led to severe neurological disease (Aujeszky's disease) in farm species at a time when few viruses besides rabies virus were known to invade the brain (Geerling et al., 2006) and some of the PRV symptoms resembled those caused by rabies virus. HSV1 and rabies virus have been tested and used for circuit analysis in monkeys. In contrast to rabies virus and HSV1, PRV does not typically cause infections in primates (Card and Enquist, 2014; Geerling et al., 2006).

Rabies virus and vesicular stomatitis virus (VSV) belong to the *Rhabdoviridae* family. Members of this virus family are enveloped, negative-sense single-stranded RNA viruses with bullet- and rod- shaped geometries (about 75 nm wide and 180 nm long). Their genomes range from 11–15 kb in length. Rhabdovirus viral replication is cytoplasmic. Entry into the host cell is achieved by attachment of the viral glycoproteins to host receptors, which mediates clathrin-mediated endocytosis. Replication follows the negative-stranded RNA virus replication model (Ortin and Martin-Benito, 2015). Wild type rabies infections of the CNS, while often organismally lethal, do not appear to cause widespread neuronal cell death. Wild type and genetically modified rabies viruses have been widely used for retrograde trans-synaptic labeling in a variety of species including rodents and non-human primates (see below).

VSV is related to rabies virus; most VSV strains are highly cytotoxic (van den Pol et al., 2002). VSV can achieve infection and gene expression in a wide range of organisms, including vertebrates and invertebrates. In particular, the envelope glycoprotein from VSV confers to various vectors the ability to transfer genes to a broad range of different cell types, including mammalian and non-mammalian cells. Other viruses such as lentiviruses, can show enhanced uptake when pseudotyped with the rabies or VSV glycoprotein (VSV-G) (Desmaris et al., 2001; Mazarakis et al., 2001). After injection into the brain, lentiviral vectors pseudotyped with VSV envelope glycoprotein (VSV-G) transduce neurons with subsequent spread of the expressed protein throughout the cell's axons and dendrites, while rabies virus glycoprotein pseudotyping of lentiviral vectors enables retrograde axonal transport and access to the nervous system after peripheral delivery (Mazarakis et al., 2001). In the CNS, VSV enables trans-neuronal tracing of neural circuitry in different directions dictated by the viral envelope glycoprotein derived from either VSV or rabies virus (Beier et al., 2013; Beier et al., 2011).

In summary, the viral vectors modified from the viruses described above differ in their genome type, maximum genetic payload, their ability to integrate into the host genome, infectivity, and cellular tropism (Tables 1 and 2). We include Experimental Planning flowcharts in Figure 2 to provide practical guidance to interested readers to aid their experimental choice of viral reagents, when combined with the information described below. Genetic modifications of wild-type viruses typically involve the removal of genes required

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for viral replication, and/or the replacement with reporter genes under the control of ubiquitous or cell-type specific gene regulatory elements (Chan et al., 2017; Dimidschstein et al., 2016). Strategies for selective DNA recombination via Cre-LoxP and Flp-Frt to control cell-type specific expression cannot be applied for RNA viruses. These are important features for users to consider for their specific applications.

BACKGROUND AND HISTORY OF VIRUS-MEDIATED CIRCUIT TRACING

The use of viruses for neural circuit tracing has a long history. Here we provide a relevant summary of previous topical reviews (Enquist, 2002; Geerling et al., 2006; Kuypers and Ugolini, 1990; Loewy, 1998). In the 1920s, Goodpasture and Teague provided the earliest support for herpesvirus transmission to the brain along the optic nerves to produce encephalitis in rabbits. In the 1930s, Albert Sabin, who later gained fame for the development of the live attenuated polio vaccine, made the important observation that neurotropic viruses enter the brain via preferential neural pathways in mice. VSV and eastern equine encephalitis viruses enter the olfactory pathway, whereas PRV travels in the sympathetic and trigeminal pathways without apparent olfactory infection. These observations were followed in the 1970s and 1990s by demonstrations of axonal transport of herpesviruses to neuronal cell bodies and transneuronal spread in the CNS [see review in (Enquist, 2002; Geerling et al., 2006)].

These earlier studies highlighted one of the greatest advantages of using neurotropic viruses for neural circuit mapping: they replicate in each infected neuron, robustly labeling each level of hierarchical neural circuits through self-amplification (Kuypers and Ugolini, 1990; Martin and Dolivo, 1983). However, the nature of viral replication and spread introduced a new concern over whether viral infections could be contained within specific neural circuits or whether they propagate non-specifically. Ugolini and colleagues injected HSV1 into peripheral nerves and showed that it produced a transneuronal infection in the rat brain (Kuypers and Ugolini, 1990). But they also found that the virus spreads locally and nonspecifically among neighboring glial cells and neurons (Ugolini et al., 1987). This dampened enthusiasm for viral-mediated tracing methods, until an attenuated PRV strain (Bartha PRV) was shown to produce highly specific retrograde transneuronal infections (Enquist, 2002; Loewy, 1998). Further consideration suggests that the improved synaptic specificity of PRV Bartha may be related to its reduced toxicity. Non-attenuated viral reagents could rapidly kill cells, causing virus to spill into the extracellular space, which spreads to nearby cells without synaptic specificity. Subsequent studies revealed specific genetic alterations responsible for the retrograde specificity and reduced pathogenicity of Bartha PRV. Mutations in any "Us" genes eliminate anterograde spread of PRV in neural circuits (Brideau et al., 2000; Enquist, 2002). Bartha PRV has been further modified into powerful retrograde viral tracing tools for neural circuit analysis in non-primate species including mice and rats (Card and Enquist, 2014; Card et al., 2011; DeFalco et al., 2001; Kobiler et al., 2010).

Genetic modifications also have led to many improvements in rabies virus, VSV and HSV1, including reduced cytotoxicity, addition of marker genes, control of synaptic spread and pseudotyping for infection of selected cell types (Beier et al., 2013; Beier et al., 2011;

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Callaway and Luo, 2015; Chatterjee et al., 2018; Ciabatti et al., 2017; Ohara et al., 2009; Osakada et al., 2011; Sun et al., 2014; van den Pol et al., 2009; Wickersham et al., 2007a; Wickersham et al., 2007b). For example, the glycoprotein gene (G)-deleted rabies virus (RV G) has its envelope glycoprotein gene deleted from its genome but can be grown in complementing cell lines so that the rabies glycoprotein coats the deletion mutant. Such a modified virus can infect neurons as a wild type rabies virus would, but cannot spread beyond the initially infected cells (Etessami et al., 2000; Wickersham et al., 2007a). Because the glycoprotein is not involved in transcription and replication, RV G can still express its remaining genes and replicate its viral genome within infected cells. Genes for fluorescent proteins inserted in the RV G genome are expressed at high levels in infected cells, revealing fine structure of dendrites and axons. RV G is an excellent tool for studying detailed morphology of neurons projecting to injection sites within the mammalian brain on a short term basis up to 2 weeks, which is additionally useful when combined with electrophysiological analysis (Wickersham et al., 2007a; Yamawaki and Shepherd, 2015). Similarly, a recombinant VSV with its G-gene deleted (VSV G) replicates only in the initially infected cells without transneuronal spread, but expresses transgenes, generating neuronal label with Golgi-staining like morphological details (van den Pol et al., 2009).

EnvA pseudotyping of rabies virus allows for the directed delivery of genetically encoded reporters and effectors to presynaptic neurons designated by their inputs (Chatterjee et al., 2018; Marshel et al., 2010; Osakada et al., 2011; Sun et al., 2019; Sun et al., 2014; Suzuki et al., 2019; Wertz et al., 2015). Specifically, the deletion mutant rabies virus can be pseudotyped with the avian sarcoma leucosis virus glycoprotein EnvA. This EnvA-RV G can only infect neurons that express avian tumor virus receptor A (TVA), an avian receptor protein that is absent in mammalian cells unless it is provided through exogenous gene delivery, for example, by AAV transduction or using transgenic mice. The deletion-mutant rabies virus can then be trans-complemented with rabies glycoprotein in the same TVA-expressing cells, thereby enabling its retrograde spread to direct presynaptic neurons. The absence of rabies glycoprotein in these presynaptic cells prevents further viral proliferation.

Within packaging size limits, nearly any gene of interest can be expressed from the modified rabies virus genome, making it possible to link circuitry to function by virtue of the genetic payload. The time window for such functional studies is abbreviated by the deleterious effects of viral infection and strong, unregulated transgene expression. With recent improvements, rabies virus-based monosynaptic tracing systems have been used to identify and manipulate presynaptic neurons that synapse onto genetically targeted cell types in specific regions throughout the central and peripheral nervous systems (Callaway and Luo, 2015; Chatterjee et al., 2018; Ciabatti et al., 2017; Ginger et al., 2013; Kim et al., 2016; Osakada et al., 2011; Reardon et al., 2016; Sun et al., 2014).

PROPERTIES OF ANTEROGRADE VERSUS RETROGRADE VIRAL TRACERS

Viral tracers can be classified as anterograde or retrograde tracers. The retrograde viral tracers include retrograde-transporting viruses and retrograde transneuronal or trans-synaptic

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viruses. Viruses that are used to label projection cell types are termed retrograde-transporting viruses (Figure 3). These viruses enter through axon terminals and are effectively transported in vesicles by dynein retrograde transport back to the cell body, where they are then released. These viruses include CAV2 (Del Rio et al., 2019; Schwarz et al., 2015), a specific serotype of AAV, termed rAAV2-retro (Tervo et al., 2016), lentiviruses pseudotyped with rabies virus glycoprotein or chimeric variants thereof (Mazarakis et al., 2001), immediate early (IE) gene-deleted pseudorabies viruses (Oyibo et al., 2014), and spread-deficient, G-deleted rabies virus (Wickersham et al., 2007a). In addition, most serotypes of AAVs exhibit some capability for retrograde uptake (Rothermel et al., 2013). AAV6 in particular travels retrogradely in rat and monkey brain (Salegio et al., 2013; San Sebastian et al., 2013).

AAVs administered through injection are always maximally efficient at the injection site and can infect axons of passage. This can complicate data interpretation: a transduced neuron, distal to the injection site, may have an axon that terminates in the injection site or simply passes through it (Rothermel et al., 2013; Vite et al., 2003). While this may be a property of many vectors, this is not a major issue for G-deleted rabies or rabies-virus-glycoprotein pseudotyped lentivirus (Wickersham et al., 2007a). Synaptic specialization appears to be needed for their efficient uptake.

The viruses that propagate from neuron to neuron in the retrograde direction are termed retrograde transneuronal or trans-synaptic viruses (Figure 4A; Table 2). These viruses typically include rabies, VSV, HSV1 and PRV, which have the ability to traverse multi-synaptic pathways and the ability to replicate thereby amplifying signals at each step in the process. A specific monosynaptic circuit mapping application through trans-complemented RV G is illustrated in Figure 5 (A-D). The terms “transneuronal” and “trans-synaptic” are frequently used interchangeably, but they have different meanings. Trans-synaptic viruses are those that are believed to label neurons by virtue of their synaptic connectivity. Transneuronal transmission can happen in any number of ways without synaptic contacts, for example though volume transmission or non-synaptic cell-cell contacts. Please see a discussion about potential caveats and raised issues about synaptic specificity and utility of rabies virus and herpesvirus mapping in a critical review article (Beier, 2019).

In contrast to retrograde viral tracers, anterograde viral tracers include viruses for neuron axonal labeling and viruses for transmission from neuron to neuron in the anterograde direction (anterograde transneuronal or trans-synaptic viruses). rAAVs are perhaps the first choice of many neuroscientists for anterograde axonal labeling. The Allen Mouse Brain Connectivity Atlas, a project to map neural connections at several hundred sites across the entire mouse brain, was based on pathway tracing with AAV vectors. rAAV1 was used as an anterograde axonal tracer; transduced neurons expressed a fluorescent marker (EGFP or tdTomato) that filled their axons, revealing the structures that they innervate. This project mapped an unprecedented number of axonal connections in a uniform and standardized brain space (Oh et al., 2014; Wang et al., 2014). The MouseLight project at HHMI Janelia Campus used rAAVs to sparsely label sub-populations of neurons. Tens of cortical neurons could be routinely labeled with high intensity in each mouse brain, facilitating reconstruction of extensive, brain-wide axonal arborizations of diverse projection neurons

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(Economo et al., 2016; Winnubst et al., 2019) (also see Ref. (Lin et al., 2018) for a dual AAV expression system that enables strong and sparse labeling of individual neurons). The full reconstruction of 1,000 projection neurons in the motor cortex, thalamus, subiculum, and hypothalamus revealed new cell types and organization of long-range connectivity in the mouse brain (Winnubst et al., 2019). MAPseq (Multiplexed Analysis of Projections by Sequencing) is a RNA-Seq method that uses Sindbis viral vectors for high-throughput mapping of neuronal projections (Kebschull et al., 2016). Recombinant Sindbis virus can rapidly produce high expression levels of random RNA sequences ("barcodes") in infected neurons, so that axons are filled with barcode mRNA. Each putative projection area is dissected, and the barcode mRNA, if present, is extracted and sequenced. By reformulating projection mapping as a problem of sequencing, MAPseq harnesses advances in high-throughput sequencing technology to permit efficient axonal projection mapping. Similarly, BARseq uses barcoded Sindbis virus in combination with *in situ* sequencing for high throughput mapping of long-range projections of thousands of spatially resolved neurons (Chen et al., 2019). In addition to AAVs and Sindbis virus, other gene delivery vectors modified from adenovirus, lentivirus, Semliki Forest virus and vaccinia virus can be used for neuron axonal labeling *in vivo* and *in vitro*, each with its own advantages and disadvantages (Table 1).

While progress has been made using viruses to label axonal projections, this technique does not reveal the post-synaptic partners of labelled neurons. Anterograde transneuronal or trans-synaptic viruses can provide this information (Figure 4B; Table 2). Few viruses have been demonstrated to exhibit exclusively anterograde trans-synaptic labeling. Wild-type HSV and PRV vectors typically propagate both anterogradely and retrogradely. In contrast, the most frequently used anterograde trans-synaptic virus is the H129 strain of the herpesvirus, HSV1, which spreads preferentially in the anterograde direction (Dix et al., 1983; Zemanick et al., 1991). A Cre dependent H129 strain has been used to map output projections of specific cell types (Lo and Anderson, 2011; Sun et al., 2019), as illustrated in Figure 5 (E-L). However, like wild type H129, disadvantages of this genetically modified H129 strain include strong cytotoxicity and high virulence (see below). The H129 strain also exhibits delayed retrograde trans-synaptic transmission (Archin and Atherton, 2002; Archin et al., 2003; Wojaczynski et al., 2015). A second virus used for anterograde projection mapping is VSV. Recombinant VSV vectors expressing either the native VSV glycoprotein or the glycoprotein from lymphocytic choriomeningitis virus (LCMV-G) have been used to map circuits in mice and other species (Beier et al., 2011; Tong et al., 2014). However, previous reports had also noted putative retrograde or non-specific transmission of VSV vectors (Lundh, 1990; van den Pol et al., 2002). Thus the extent of anterograde specificity of this vector needs to be further explored.

AAV vectors are replication incompetent and therefore lack the signal amplification that is currently necessary for efficient trans-synaptic labelling. However, Zingg et al. (2017) provided evidence that highly concentrated AAV1 and AAV9 are capable of transneuronal spread (Zingg et al., 2017). The putative synaptic specificity of this vector is less well-defined than for herpesviruses or rhabdoviruses, given that the assay used to detect the synaptic preference can only show a two-fold enhancement over non-synaptic transmission (Zingg et al., 2020). The underlying mechanisms of trans-synaptic trafficking are not yet

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known. One possibility is a form of transcytosis where the AAV particle is taken up at one side of the cell and released at another side of the cell after intracellular transport without un-coating or actually establishing infection within the first cell. Consistent with this potential mechanism, AAV9 was shown to cross the blood brain barrier by endothelial transcytosis (Foust et al., 2009; Merkel et al., 2017).

Transneuronal spread of highly concentrated AAVs is expected to be inefficient (Zingg et al., 2017), but even trace amounts of AAV1/9-Cre crossing from one cell to another are sufficient to drive robust Cre-dependent transgene expression in selected postsynaptic neuronal targets in Cre reporter mouse lines (Centanni et al., 2019; Wang et al., 2018a; Yao et al., 2018). Nevertheless, we need to be aware of the caveat that, as noted for wild type AAVs (Zhao et al., 2017), AAV-Cre is also able to retrogradely spread to presynaptic neurons, resulting in Cre-dependent transgene expression in presynaptic neurons. This would complicate the interpretation of data particularly from brain regions that are reciprocally connected.

In genetically tractable models, synaptic activation-defined transgene expression can be used for anterograde tract labeling and trans-synaptic anterograde tracing (Talay et al., 2017). For example, trans-Tango is a synthetic signaling pathway that is introduced into all neurons in the animal. This pathway converts receptor activation at the cell-surface into reporter expression through site-specific proteolysis; specific labeling is achieved by presenting a tethered ligand at the synapses of genetically defined neurons (Talay et al., 2017).

RECENT ADVANCES IN ANTEROGRADE MONOSYNAPTIC TRACING

After broadly covering the viral vectors for neural circuit mapping, we now focus on introducing recent advances in HSV1 H129-based trans-synaptic anterograde tracers. H129 virus is arguably the most promising anterograde neuronal tracing tool (Lo and Anderson, 2011; Sun et al., 1996; Wojaczynski et al., 2015). Wild type H129 was an isolate from the brain of a human patient who suffered from viral encephalitis (Dix et al., 1983). Zemanick and colleagues showed that H129 was transported preferentially in the anterograde direction by following an injection into the motor cortex of cebus monkeys (Zemanick et al., 1991). Subsequent studies in rat CNS confirmed the preferential anterograde transport of H129 through neural circuits (see review in (Wojaczynski et al., 2015)). Peripheral HSV1 infection in humans typically produces relatively harmless fever blisters or cold sores in the oropharyngeal region. Similarly, primary peripheral infection of macaques results in very little morbidity or mortality (Simmons, 2010). However, intracerebral inoculation in monkey cortex induced motor symptoms of infection after 2-3 days (Zemanick et al., 1991). While uncommon in humans and macaques, HSV1 lethality is common among rodents, tree shrews, and owl monkeys (Berkowitz et al., 1994; Deisboeck et al., 2003; Marks and Carpenter, 1973). This lethality presents rapidly; the lytic HSV1 life cycle takes only about 18 hours (Dong et al., 2020; Salameh et al., 2012). When using H129 capable of polysynaptic spreading, infected mice generally die in 3-7 days, depending on the site of infection and dosage (Bolovan et al., 1994; Lo and Anderson, 2011; Sedarati and Stevens, 1987).

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Current genetically modified H129 viruses are significantly limited by high virulence and cellular toxicity. An important research direction is to develop anterograde tracers without these limitations that can be used in a variety of animal models. While almost all of currently available anterograde viral tracers label weakly and require immunostaining for robust detection, we recently generated a virus that expresses high levels of a fluorescent label without the requirement for immunostaining for robust detection (Zeng et al., 2017). This virus, H129-G4, was obtained by inserting binary, tandemly-connected EGFP cassettes into the H129 genome. The EGFP fluorescent label of H129-G4 is sufficiently strong that morphological details of labeled neurons, including dendrites, spines and axonal fibers can be clearly visualized (Figure 6) (Li et al., 2017; Wang et al., 2018b; Yu et al., 2017; Zeng et al., 2017).

Guided by earlier work that developed monosynaptic retrograde rabies virus vectors (Sun et al., 2014; Wall et al., 2010; Wickersham et al., 2007b), the H129 genome can be modified so that viral replication is restricted to targeted neurons, and anterograde spread of the virus is limited to a single synaptic step. This modification has the collateral benefit of reducing virulence and lethality of recombinant viral vectors. A strategic plan to devise conditionally replicating recombinant H129 for monosynaptic anterograde tracing can be broken down into steps (Figure 7 A-C). The first step is to generate replication defective H129 recombinants that can infect neurons but cannot replicate in them. Second, viral replication competency can be restricted by rescue in targeted neurons using exogenous co-expression of the required viral gene via helper AAV. Thus, we can enable monosynaptic anterograde spread of the virus from initially infected neurons to directly connected postsynaptic partners. This strategy was implemented in the work of Zeng et al. (2017), and an important proof of principle outcome is illustrated (Figure 7 D-G). Mutant H129 vectors (H129- TK) were generated by deleting the thymidine kinase (TK) gene from the H129 genome. TK synthesizes thymidine monophosphate by catalyzing the phosphorylation of thymidine, an essential step for the synthesis of thymidine triphosphate (TTP), which is necessary for DNA synthesis. In proliferating cells, this endogenous TK compensates for viral TK deficiency to enable viral replication. However, cellular TK activity in non-proliferating neurons and other cells is low or non-existent. This results in insufficient TTP and strongly impairs viral genome replication, thus preventing H129- TK from spreading among post-mitotic neurons. With trans-complementation via a TK-expressing helper AAV, H129- TK can undergo genome replication, viral protein synthesis and virion assembly. Newly generated virions can then travel down axons, cross a single synapse, and enter postsynaptic neurons. This new tool also has been applied for anterograde tracing from serotonin receptor 2c-expressing cells in ventral CA1 to their long-distance direct postsynaptic cells in the Edinger–Westphal nucleus (Yao et al., 2018).

The work of Zeng et al. (2017) was extended from the study by Lo & Anderson (2011), who developed the first conditional H129 tracer by replacing endogenous H129 TK gene with a loxP-STOP-loxP-tdTomato-2A-TK cassette via homologous recombination. Although it is termed H129 TK-TT, the TK gene was not deleted, but rather made conditional on Cre-recombinase activity. Consequently, in the presence of Cre-recombinase, H129 TK-TT acts as a polysynaptic anterograde tracer expressing tdTomato first in the starter neurons, then to their directly connected post-synaptic partners, and subsequently to their second- and higher-

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order partners. In comparison, Zeng et al. (2017) developed a TK deletion mutant, H129- TK-tdT by deleting the TK from the H129 genome. In the presence of TK complementary expression from a helper AAV that can be Cre- or non-Cre dependent, transcomplemented H129- TK-tdT propagates from starter neurons to monosynaptically connected neurons and labels them with tdTomato. A key difference between transcomplemented H129- TK-tdT and recombinant H129 TK-TT is genetically controlled monosynaptic versus unregulated multi-synaptic spread.

The above-described technology is sufficient for monosynaptic, anterograde delivery of a genetic material, but it still has limitations. Replication of trans-complemented H129- TK leads to significant cytotoxicity within 3-5 days after infection. In some brain regions after time, H129- TK invades axon terminals, leading to some retrograde labeling *in vivo* (Zeng et al., 2017). Furthermore, the postsynaptic viral fluorescent label of H129- TK still requires immunostaining enhancement for robust detection.

Towards overcoming these aforementioned limitations of H129- TK, we deleted from the H129 genome the UL53 gene that encodes glycoprotein K (gK), a virion envelope protein that is key to viral egress and spread (Allen et al., 2014; David et al., 2012; Foster and Kousoulas, 1999; Hutchinson and Johnson, 1995; Jayachandra et al., 1997). Preliminary data suggest that this new deletion H129 mutant (glycoprotein K deletion, H129- gK) has the two desirable features. First, the absence of gK allows unimpaired viral genome replication. Although gK removal prevents viral budding and spread in the absence of a helper virus, H129- gK can express high levels fluorescent proteins, eliminating the requirement of immunostaining for detection. Second, gK removal can improve anterograde-specific infectivity of the H129 virus as demonstrated by *in vitro* microfluidic tests. This is consistent with the earlier finding that a gK mutant HSV1 is unable to infect via axons (David et al., 2012). Once validated, the newly generated H129 gK-G4 may prove to be a much improved anterograde viral tracer. When they are ready for distribution, these new anterograde tracers will be made available through our service platform at the University of California, Irvine (https://cncm.som.uci.edu/).

LOW-TOXICITY ANTEROGRADE MONOSYNAPTIC TRACERS

Beyond applications of anatomical tracing, low-toxicity anterograde monosynaptic vectors could be utilized to deliver functional payloads such as genetically encoded effectors/ actuators in targeted neural circuit elements in an anterograde direction in the brain. Ideally, data acquisition with such tools should last weeks or months; so long-term post-infection cell health is crucial for the longitudinal collection of high-quality data. The use of conditionally-replicating or functionally-defective H129 mutants lowers *systems*-level toxicity by eliminating polysynaptic viral spread. An important next step is to reduce *cellular*-level toxicity by modifying viral virulence genes to extend the use of H129 recombinants for long-term, functional gene expression. This goal sharpens the need to reduce viral toxicity by engineering changes in previously identified H129 genes that are associated with cytotoxicity and pathogenicity (Burton et al., 2002; Lachmann, 2004; Manservigi et al., 2010).

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Reduction in toxicity, accomplished by slowing the kinetics of viral gene expression, could result in loss of signal strength for labeling. To prevent this, parallel genetic engineering approaches should be directed towards increasing viral labeling efficacy while maintaining anterograde monosynaptic spread and reducing toxicity. One approach to boosting signal strength is to insert multiple copies of transgenes into the viral genome. We have made H129-G4 based variants (H129- TK-G4, H129 gK-G4) containing four copies of EGFP genes to improve labeling signal strength. A second approach is to use the tTA/TRE-mediated transcriptional amplification system in which the viral vector encodes a transcription factor, the tetracycline-dependent transcription activator (tTA), driven by a tTA-responsive promoter (Baron et al., 1997; Cetin and Callaway, 2014; Daigle et al., 2018). When tTA and a functional effector (e.g., ChR2-EYFP) are placed downstream of a tTA-responsive promoter, a tunable positive feedback loop is generated, resulting in high levels of effector expression. Other promising alternative approaches may be developed in similar ways to the development of nontoxic, double-deletion-mutant rabies viral vectors (Chatterjee et al., 2018). A fully developed H129- TK / H129 gK system with greatly reduced toxicity would be broadly applicable to drive expression of fluorescent proteins, optogenetic and chemicogenetic actuators, and genetic calcium and voltage indicators (Daigle et al., 2018; Garg et al., 2019; Madisen et al., 2015; Madisen et al., 2012) for the functional dissection of specific neural circuits. This is critical because in the absence of functional analysis of neural circuits, the connectome is limited to an anatomical map.

CONCLUSIONS

Viruses have substantial value for neural circuit mapping. We have reviewed the properties of the viruses used for the study of neural circuits, and compared their strengths and limitations. While retrograde trans-synaptic viral tracers, typified by genetically-modified rabies virus, have achieved much success, genetically modified anterograde trans-synaptic viral tracers are less developed and currently exhibit relatively poor performance and greater toxicity. We expect that advances in H129-based anterograde viral tracing tools will catalyze new scientific discoveries in neuroscience research. We foresee that interdisciplinary collaborations between virologists and systems neuroscientists will produce an array of anterograde monosynaptic H129 tools with high labeling efficacy and low toxicity. These new anterograde monosynaptic viral tracers will facilitate neural circuit analysis across a wide range of model species.

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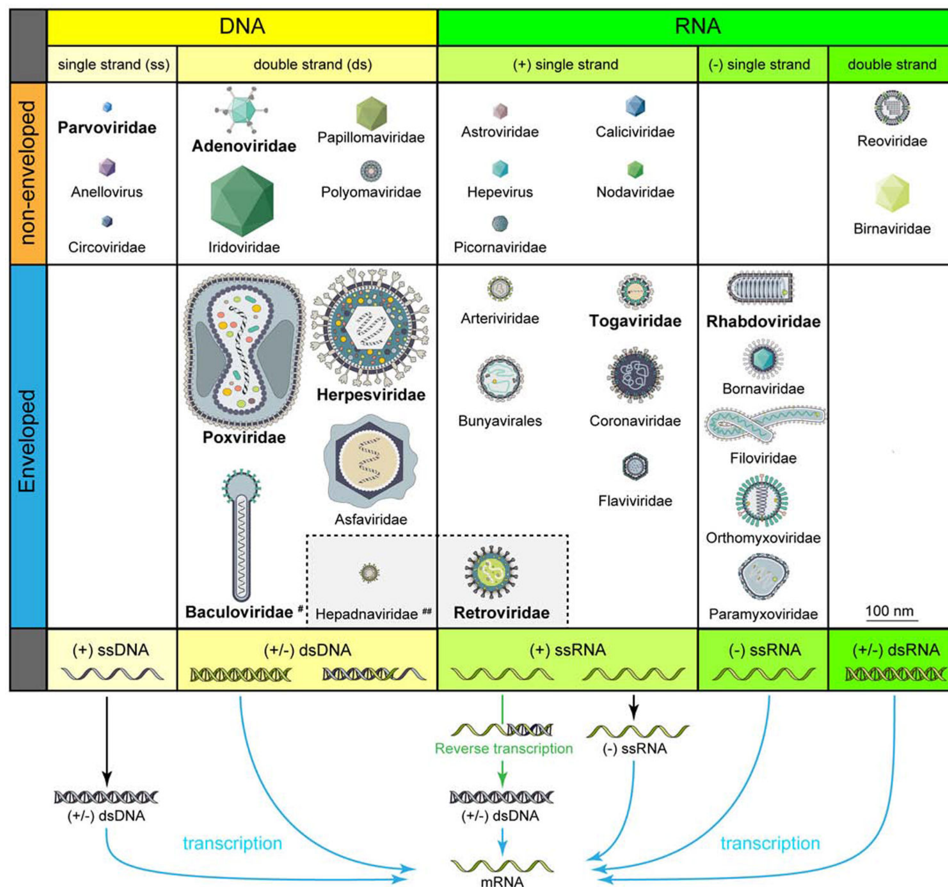

Figure 1. Illustration of major families of animal viruses, distinguished by their genome type and viral structure, and gene expression and replication mode.

This figure is adapted and rendered based on relevant information from the book chapters in S.J. Flint et al. (2015) Principles of Virology (4th edition, ASM Press, Washington, DC), and Knipe & Howley (2013) Fields Virology (6th edition, Wolters Kluwer / Lippincott Williams & Wilkins). The virion images are newly reconstructed based on S.J. Flint et al. (2015). The virus family (**bolded**) belongs to the viruses often used in neuroscience research. # denotes that the *Baculoviridae* family does not naturally infect vertebrates. ## denotes that the *Hepadnaviridae* family uses a unique reverse-transcription process which is not shown in the illustration for simplicity.

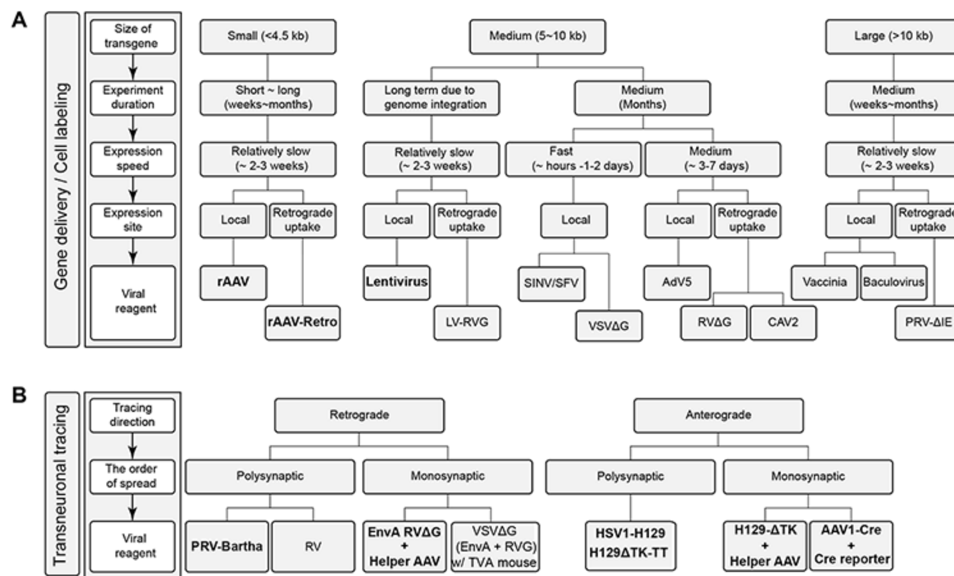

Figure 2. Experimental planning flowcharts are provided to aid experimental choices of viral reagents.

The bold font indicates the viral reagent often used in neural circuit studies. Please see the main text for related references. rAAV, recombinant AAV; rAAV-Retro, retrograde transporting AAV; LV-RVG: lentivirus pseudotyped with rabies virus glycoprotein protein (RVG); SINV, Sindbis virus; SFV, Semliki forest virus; AdV5, adenovirus type 5; RV G, rabies virus with glycoprotein gene deletion; CAV2, canine adenovirus type 2; PRV-Bartha: pseudorabies virus strain Bartha; PRV- IE: PRV-Bartha with IE gene deletion; RV: rabies virus; VSV G: vesicular stomatitis virus with the glycoprotein coding gene deletion; VSV G (EnvA + RVG): G-deleted VSV pseudotyped with EnvA and RVG; HSV1-H129: herpes simplex virus 1 strain H129; H129 TK-TT: recombinant H129 with Cre dependent expression of TK and tdTomato; H129- TK: recombinant H129 with TK gene deletion.

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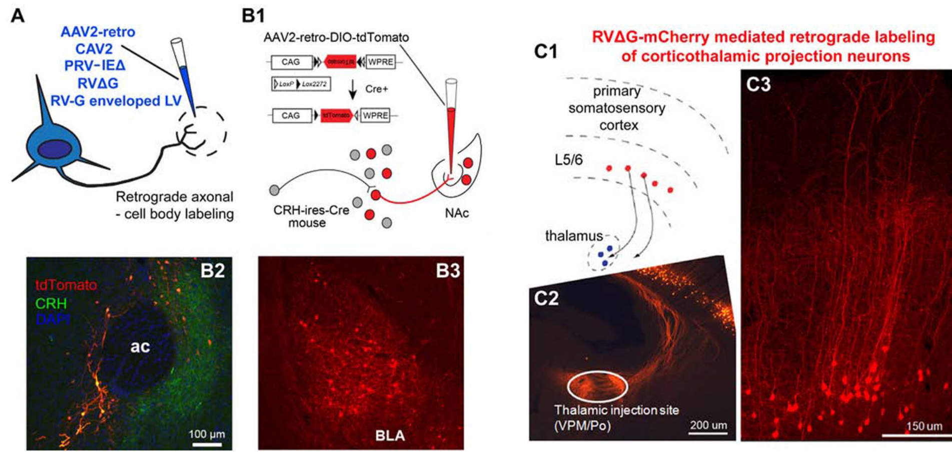

Figure 3. Retrograde-transporting viruses and specific illustrations of labeling projection cell types through axonal retrograde transport.

(A) Schematic illustration of retrograde labeling of neuronal cell bodies via axonal uptake of a retrograde transporting virus. The listed viruses are replication deficient, and they cannot spread out of the initially infected neuron. (B1-B3) rAAV2-retro-mediated labeling demonstrates a new CRH+ input pathway from the amygdalar complex to the nucleus of accumbens (NAc). (B1) Schematic of the retrograde-labeling virus and the location of the NAc injection site in CRH-IRES-Cre mice. To reduce clutter, only one of the retrogradely labeled neurons (red) in the input-mapped region is illustrated with its axonal terminal in the NAc (the AAV2-retro injection site). There is local AAV2-retro labeling of neurons (red) at the NAc injection site. (B2) Endogenous CRH+ cells in the NAc are infected by the rAAV2-retro. (B3) A significant portion of the brain-wide CRH+ projections originate from the amygdala nuclei including the basolateral amygdala (BLA). Images are modified with permission from our published work (Itoga et al., 2019). (C1-3) Corticothalamic neurons in mouse primary somatosensory cortex labeled via the injection of glycoprotein gene-deleted rabies virus expressing mCherry (RV G-mCherry) in somatosensory thalamus. In C1, only two of the retrogradely labeled corticothalamic neurons are illustrated with their axonal projections toward the thalamus (the RV G injection site). Images are based on unpublished data from the Xu lab.

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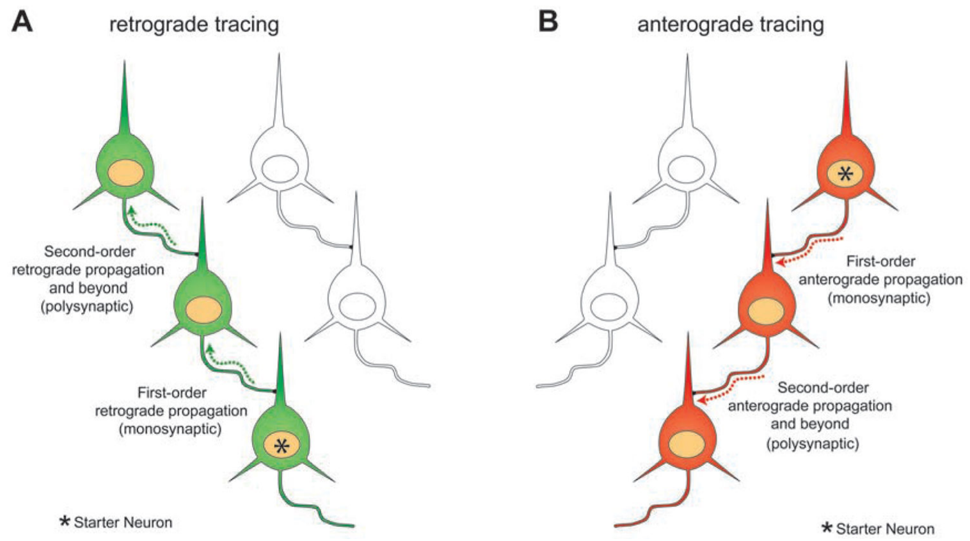

Figure 4. Schematic illustrations of transneuronal or trans-synaptic viral tracing.

(A) Retrograde tracing follows the synaptic connection from the postsynaptic starter neuron to its presynaptic partners. (B) Anterograde tracing follows the synaptic connection from the presynaptic starter neuron to its postsynaptic partners.

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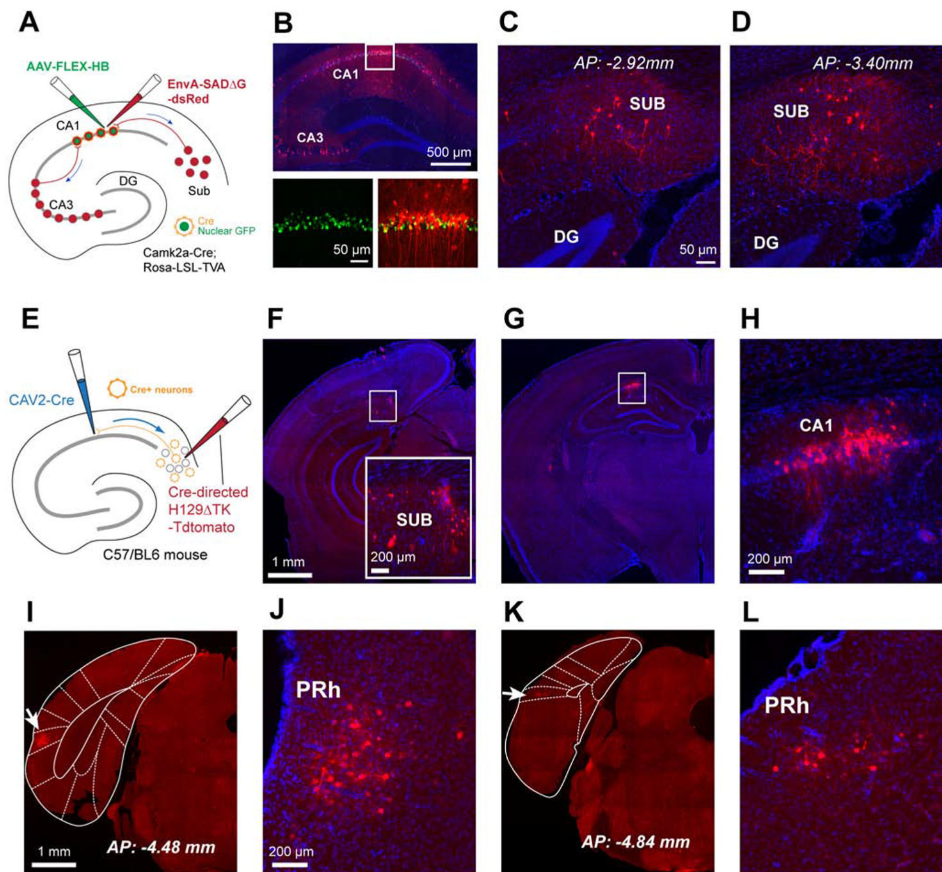

Figure 5. Specific circuit mapping applications by retrograde monosynaptic rabies virus tracing and anterograde herpes simplex virus (H129) tracing.

(A-D) Direct subiculum (SUB)-CA1 back-projections are shown by monosynaptic retrograde rabies tracing. This experiment was independently repeated in 12 mice, each with similar results. (A) The scheme for our Cre-dependent, monosynaptic rabies tracing approach. Using Camk2a-Cre; TVA mice, we mapped direct presynaptic input connections onto Camk2a-Cre expressing excitatory neurons in hippocampal CA1 in the intact brain. Starter neurons in dorsal hippocampal CA1 are shown (B, top panel), labeled by both EGFP and dsRed expression from both AAV and rabies infection (B, bottom panels). Their presynaptic partners (e.g., local interneurons and CA3 neurons) are labeled with the red fluorescent protein dsRed from the rabies virus infection. (C-D) Retrogradely labeled Subiculum (SUB) neurons presynaptic to CA1 excitatory neurons are seen in sections of dorsal SUB at different anterior-posterior positions (C, AP: –2.92 mm; D, AP: –3.40 mm). (E-H) Time-limited anterograde-directed HSV tracing supports SUB-CA1 projections. We used the conservative time control of 48 h post-injection to limit labeling to directly connected postsynaptic neurons. This experiment was independently repeated in 5 mice, each with similar results. (E) The scheme for anterograde tracing by combined use of CAV2-Cre injection in CA1 and the injection of Cre-dependent H129 (H129 TK-tdTomato) in SUB to map projections of CA1-projecting SUB excitatory neurons. Note that the combined use of different viruses is becoming more appreciated in the field. (F) H129 infected neurons at the injection site in the SUB are shown in red; DAPI staining in blue. (G-H) Postsynaptic

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neuronal labeling is robustly seen in hippocampal CA1 ipsilaterally at 48 hours post H129 viral injection. (I-L) Besides CA1, postsynaptic neuronal labeling by H129 is seen in the perirhinal cortex (PRh) ipsilaterally. This experiment was independently repeated in 5 mice, each with similar results. (I) An example of PRh labeling, with a white arrow pointing to the atlas aligned brain structure. (J) An enlarged view of perirhinal neuronal labeling in (I). (K-L) Perirhinal labeling from a different animal. Abbreviation: DG, dentate gyrus. Images are modified from our published work (Sun et al., 2019) with Springer Nature permission.

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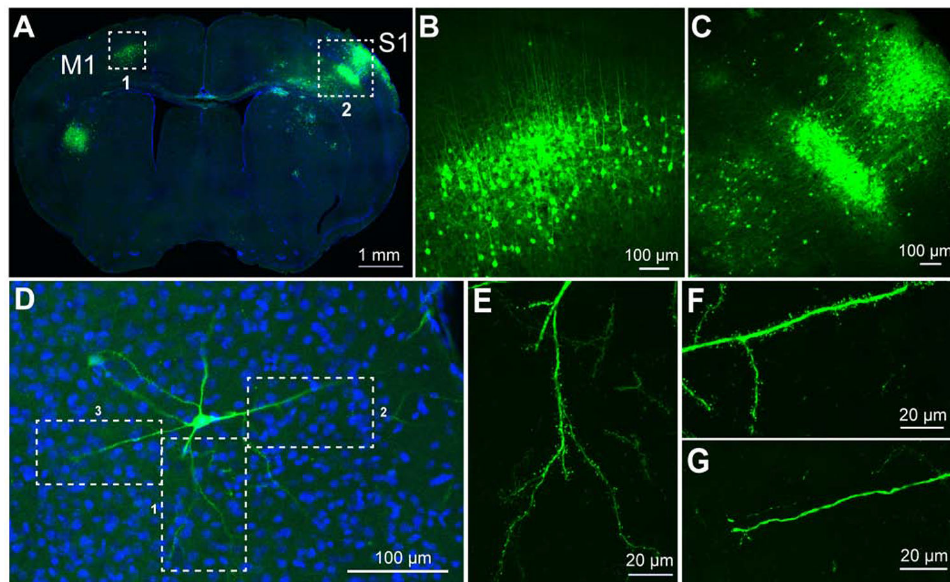

Figure 6. H129-G4 is an anterograde, polysynaptic tracer that drives strong EGFP expression, which allows for visualization of detailed neuronal morphological features without immunostaining enhancement.

H129-G4 was injected into the primary motor cortex of a C57BL/6 mouse. A coronal brain section image (A) was obtained at 4 days post-infection. The boxed areas in (A) are presented in the right panels (B-C) at higher magnification. A representative H129-G4 labeled single neuron is shown in (D). High magnification images of the dendritic segments with individual spines (E and F) and the axon (G) are presented in the right panels. Images are modified from our published work (Zeng et al., 2017) under the Creative Commons license with Springer Nature.

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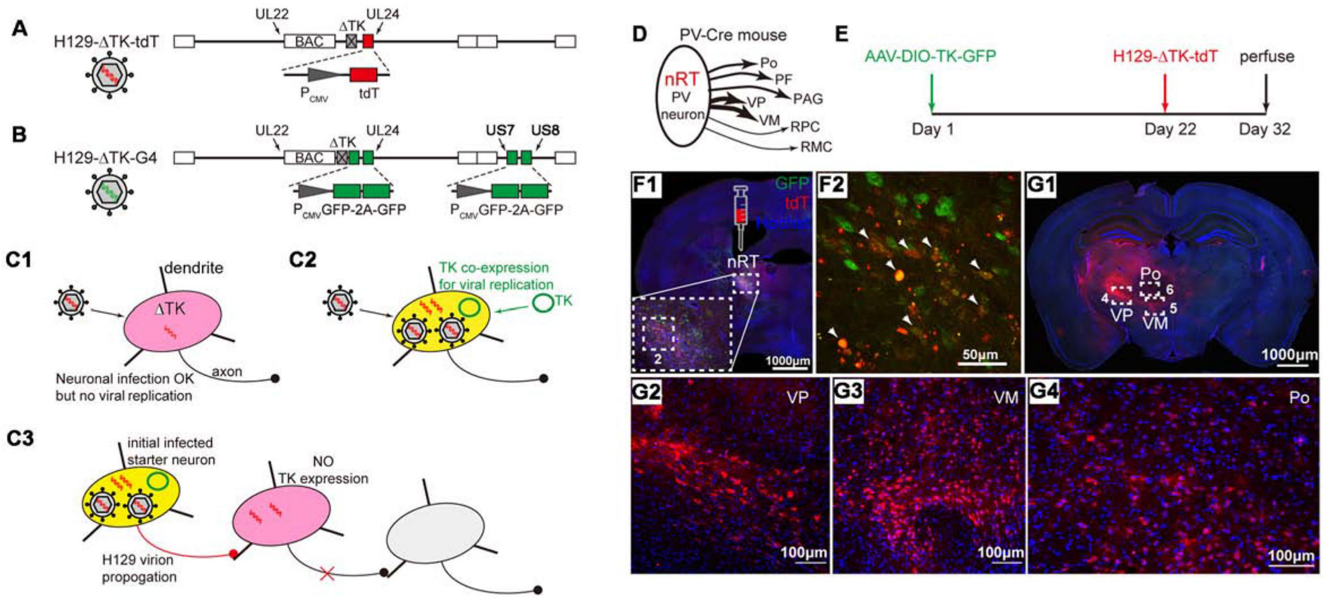

Figure 7. Illustration of the anterograde monosynaptic viral tracing system. (A-C) A conceptual framework for our development of deletion mutant H129-based anterograde monosynaptic viral tracers.

(A-B) Genetic engineering of the thymidine kinase (TK) deletion mutant H129 (H129- TK) that is replication defective in non-proliferating cells such as neurons. The mutants are constructed by deleting the TK gene and adding the tdTomato (tdT) or 4xEGFP gene to the H129 genome. (C1) H129- TK can initially infect and enter neurons, but the deletion mutant virus is replication incompetent. (C2) The mutant virus can replicate in neurons co-expressing exogenous TK by way of a TK-expressing helper AAV. As indicated, this conditional replication results in tdTomato expression from the recombinant H129 genome, along with EGFP expression from the helper AAV genome in initially infected neurons. (C3) H129- TK/AAV-TK targeted co-expression limits viral replication to initial infected starter neurons from which viral progeny propagate to label direct postsynaptic neurons. As labeled postsynaptic neurons lack TK expression, the viral label cannot spread beyond anterograde monosynaptically connected cells. (D-G) H129- TK with Cre-dependent helper AAV enables mapping of output connections of specific neuron types, as illustrated by tracing the monosynaptic projection targets of parvalbumin-expressing neurons in the reticular nucleus of thalamus (nRT) of PV-Cre mice. The schematic (E) shows the timeline of injection of AAV-DIO-TK-GFP, and H129- TK-tdT (injected to the same site 21 days apart). The animals were perfused at 10 days post-injection of H129- TK-tdT, brains were extracted and tdTomato label was enhanced with immunostaining. Example brain section images are shown in (F-G). The image of the injection site in nRT is shown in (F1), and a small region (label "2") is shown in the inset of (F1) and at higher magnification in (F2). The initially infected starter neurons express both tdTomato and GFP, indicated with the white arrows. Note that this section image was acquired at day 3 after H129- TK-tdT injection. (G1) Monosynaptic anterograde label of nRT-PV neurons in a brain section. (G2-G4) Representative regions mapped by H129- TK-tdT, including ventral posterolateral (VP), ventral posteromedial (VM), posterior nucleus (Po) of the thalamus. See Zeng et al. (2017) for postsynaptic neuronal labels in long-range projection targets. Images are modified from

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Table 1.

Properties of commonly used viral vectors for gene delivery in neuroscience applications

Virus	Adeno-associated virus (AAV)	Adenovirus (AdV)	Canine adenovirus 2 (CAV2)	Lentivirus	Sindbis virus / Semliki Forest virus (SFV)	Vaccinia virus	Baculovirus
Family	Parvoviridae	Adenoviridae	Adenoviridae	Retroviridae	Togaviridae	Poxviridae	Baculoviridae
Enveloped	No	No	No	Yes	Yes	Yes	Yes
Genome type	(+) ssDNA	dsDNA	dsDNA	(+) ssRNA	(+) ssRNA	dsDNA	dsDNA
Genome size	~5 kb	~36 kb	~31 kb	~ 10 kb	~12 - 13 kb	~190 kb	~135 kb
Vector capacity	~4.8 kb	~8-36 kb	~30 kb	~8 kb	~6 kb	~30kb	>38kb
Biosafety level	BSL1	BSL2	BSL1	BSL2	BSL1/2	BSL2	BSL1
Infectivity	Infects dividing and non-dividing cells; different serotypes have different tropisms and different invading paths, low inflammatory potential	Infects dividing and non-dividing cells, high inflammatory potential (but low for high-capacity AdVs)	Preferentially invades from axonal terminals of neurons in the CNS, relatively low inflammatory potential	Infects dividing and non-dividing cells, low inflammatory potential	Infects diverse host cell types, preferentially infect neurons in CNS, fast expression	Infects many types of mammalian cells and some invertebrate cells.	Naturally infects insect cells. Tropism could be expanded via modification of envelope glycoproteins.
Model system	*in vitro* (low efficiency), *in vivo* (preferred)	*in vitro, in vivo*	*in vitro, in vivo*	*in vitro* (preferred)*in vivo* (low efficiency)	*in vitro, in vivo*	*in vitro, in vivo*	*in vitro, in vivo*
Advantages	Nonpathogenic, safe transgene delivery, many serotypes with different tropism, low immunogenicity	Efficient transduction of most cell types and tissues	Not a human pathogen, efficient retrograde transport.	Stable and persistent transgene expression via host cell genome integration	Sindbis virus is non-pathogenic in humans; SFV generally produces mild symptoms in humans. Both produce fast and high level of transgene expression.	Expression occurs in the cytoplasm, eliminating splicing of transcripts; synthesis of the desired proteins in mammalian cells.	Can be readily manipulated to accommodate large insertions of foreign DNA, and have a good biosafety profile.
Disadvantage(s)	Low payload capacity	Transient transgene expression	Native tropism targets specific projection cell types	Low payload capacity, potential for oncogenesis; slow onset of transgene expression	Generally high cytotoxicity, mostly used for transient expression	Laboratory workers are recommended to be vaccinated. The yield of the protein of interest is generally lower when compared with the baculovirus expression system.	Not yet fully characterized for neuroscience applications
Species applications	Human, non-human primates, cats, mice, rats, ferrets, song birds; inefficient in zebra fish or xenopus	Human, non-human primates, mice, rats,	Mice, rats, non-human primates	Human, non-human primates, mice, rats, song birds; inefficient in zebra fish or xenopus	Mice, rats, zebra fish, xenopus	Humans, non-human primates, mice, rats, and xenopus	Mice, rats and zebra fish

Note: please see the main text for related references

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Table 2.

Properties of commonly used transneuronal / trans-synaptic viral tracers

Virus	Herpes simplex virus type 1 (HSV1)	Pseudorabies virus (PRV)	Rabies virus (RV)	Vesicular stomatitis virus (VSV)
Family	Herpesviridae	Herpesviridae	Rhabdoviridae	Rhabdoviridae
Enveloped	Yes	Yes	Yes	Yes
Genome type	dsDNA	dsDNA	(−) ssRNA	(−) ssRNA
Genome size	~150 kb	~142 kb	~12 kb	~11 kb
Vector capacity	~50 kb	~50 kb	~ 4 kb (largest insert so far)	Likely similar to rabies virus
Biosafety level	BSL2	BSL2	BSL2	BSL2
Infectivity	HSV1 infects a broad range of host cells, and shows fast transneuronal spread in the CNS.	Neurotropic PRV infects many host species, and show transneuronal spread in the peripheral and central nervous systems.	Rabies virus is a neurotropic virus, and infects a broad host range including almost all mammals.	VSV has a very broad cell tropism and replicates rapidly in various cell lines derived from different species.
Model system	*in vitro, in vivo*	*in vitro, in vivo*	*in vitro, in vivo*	*in vitro, in vivo*
Advantages	Large packaging capacity, strong neural tropism, multiple replication defective and replication competent strains. Anterograde neuronal circuit transmission for H129.	PRV Bartha strain only shows retrograde spread; genetically modified strains are excellent anatomical tools.	Rabies virus is a specific retrograde trans-synaptic tracer. The genetically modified RV-based monosynaptic tracing system is well developed and widely used.	Broad cell tropism, relative independence on cell cycle, rapid replication, high virus yields. Instances of VSV infection of humans are rare. Pseudotyped VSV with RV-glycoproteins shows retrograde specific transmission.
Disadvantage(s)	High cytotoxicity with most strains (reduced toxicity for replication defective strains); may spread both retrograde and anterograde ways.	Generally high cytotoxicity; no infection in primates.	Laboratory workers are recommended to be vaccinated, due to the potential infection risk by rabies. Relatively high toxicity with short term transgene expression.	VSV is highly cytopathic for host cells. Further modifications are required for wider neuroscience applications *in vivo*. Potential bi-directional transmission.
Species applications	Human, non-human primate, mice, rats, tree threw, song birds, zebra fish and xenopus	Mice, rats, zebra fish	Non-human primates, mice and rats, zebra fish	Non-human primates, mice and rats

Note 1: please see the main text for related references. Note 2: all rabies virus tracers currently used in neuroscience studies are derived from laboratory passaged attenuated strains. In the US and other countries, these viral reagents can be studied under BSL-2 guidelines, with and without genetic modifications. However, higher BSL conditions may be required for wild type strains (rabies virus isolated from dogs or wild animals).