HHS Public Access

Author manuscript

Curr Protoc Neurosci. Author manuscript; available in PMC 2020 April 01.

Published in final edited form as:

Curr Protoc Neurosci. 2019 April ; 87(1): e67. doi:10.1002/cpns.67.

Overview: Recombinant Viral Vectors as Neuroscience Tools

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Abstract

Recombinant viruses are highly efficient vehicles for *in vivo* gene delivery. Viral vectors expand the neurobiology toolbox to include direct and rapid anterograde, retrograde, and trans-synaptic delivery of tracers, sensors, and actuators to the mammalian brain. Each viral type offers unique advantages and limitations. To establish strategies for selecting a suitable viral type, this article aims to provide readers with an overview of viral recombinant technology, viral structure, tropism, and differences between serotypes and pseudotypes for three of the most commonly used vectors

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CONFLICT OF INTEREST

Authors declare that they have no conflict of interest.

INTERNET RESOURCES

https://www.addgene.org/ Addgene is a non-profit plasmid repository and a copious source for obtaining viral transfer vector plasmids and packaged AAV particles – visit “Addgene Special Collections” section for categorized plasmid indexes.

https://www.janelia.org/ The Janelia research campus website provides researchers with the latest news about the developed molecular and imaging tools for neurobiological research.

http://www.gensat.org/cre.jsp GENSAT is a gene expression nervous system atlas that contains a list of several BAC-Cre recombinase driver lines. Transfer vectors for AAVs, retro, and lentiviruses can be designed to include recombinase sensitive sequences and to target specific neuronal populations in these strains.

in neurobiology research: adeno-associated viruses, retro/lentiviruses, and glycoprotein deleted rabies viruses.

Keywords

Virus; Gene Delivery; AAV; Retrovirus; Lentivirus; Rabies delta-G

INTRODUCTION

Viruses are designed by nature for *in vivo* gene delivery. Diversity and superior performance have made viruses a powerful tool in research. Recombinant viruses are used routinely to deliver sensors, reporters, tracers, recombinases, or gene regulatory elements to neuronal cells (Callaway, 2008; Crittenden, Heidersbach, & McManus, 2007; Rost, Schneider-Warme, Schmitz, & Hegemann, 2017). Optimized production methods and commercial availability of popular vectors have facilitated gene delivery and manipulation of the nervous system to support a more robust platform for experimentation (Lock et al., 2010; Osakada & Callaway, 2013; Salmon & Trono, 2007; Zolotukhin et al., 1999). Key aspects to understanding brain function are to study its architecture through neuroanatomy, to measure its activity through calcium imaging, and to affect neuronal output via optogenetics. Viruses are effective vectors for *in vivo* delivery of these valuable research tools.

The mammalian brain is organized into neural circuits that dictate cognitive processing and formulate action. To map the brain architecture, and evaluate neural activity at the single cell or population level, researchers rely on fluorescent markers, sensors, and neural circuit tracers (Gerfen, Economo, & Chandrashekar, 2018). Creation of Mouse Cre-recombinase driver lines and gene delivery via bacterial artificial chromosome constructs have enabled researchers to target specific neural sub-populations for functional and anatomical dissection (Gerfen, Paletzki, & Heintz, 2013; Gong et al., 2007). Viral vectors expand the neurobiology toolbox to include direct and rapid anterograde, retrograde, and trans-synaptic delivery of tracers, sensors, and actuators to mammalian brains (Callaway & Luo, 2015; Castle, Gershenson, Giles, Holzbaur, & Wolfe, 2014; Tervo et al., 2016). Three of the most common types of viruses used for gene delivery in neurobiology are adeno-associated viruses (AAVs), retro and lentiviruses, and glycoprotein deleted rabies virus (Rabies dG).

Each viral type offers unique advantages and limitations. AAV serotypes offer specificity in transduction or tropism, and elicits low immune response (Daya & Berns, 2008; Sonntag, Schmidt, & Kleinschmidt, 2010). Genes delivered by AAVs do not incorporate into their host chromosomes to cause insertional mutations and are maintained episomally as concatemeric circles. AAVs mediate lasting gene expression in neurons and glia (Penaud-Budloo et al., 2008). As a result, AAVs are among the favorites for gene delivery in research and are the most popular viral gene delivery system explored in clinical trials (Kaplitt et al., 2007; Naso, Tomkowicz, Perry, & Strohl, 2017).

Nevertheless, the major AAV shortcoming is its small packaging capacity - maximum of 5.2 kb of genetic material (Wu, Yang, & Colosi, 2010). Lentiviruses have the advantage of extended capacity for delivering genetic sequences of up to 9 kb and are capable of

transducing dividing and non-dividing cells. The lentiviral envelope is easily pseudotyped with membrane proteins and receptors for targeted transduction of cells. Upon virus entry, lentiviral RNA genome is reverse transcribed to DNA and randomly integrated into the host chromosomes. This is a major disadvantage of retro/lentiviral vectors since insertional mutations could lead to aberrant cell function or cell death. Although, a small number of random integrations are tolerated by cells and lentiviruses remain a robust vector for gene delivery in neurobiology research (Sakuma, Barry, & Ikeda, 2012; Sakurai et al., 2016).

To understand the underlying architecture of the brain, researchers often rely on fluorescent neural tracers delivered by glycoprotein deleted Rabies (Rabies dG) viruses (Callaway & Luo, 2015). Currently, rabies strains SADB19 and CVS-N2c genomes have been reconstituted by recombinant technologies and their transfer vectors can deliver 8.5 kb of genetic material. Rabies dG infects neurons and spreads trans-synaptically in a retrograde direction to identify connecting neurons and map signaling pathways in neural circuits. Recombinant technology has allowed researchers to modify rabies dG to create safer alternatives. Variations in rabies dG strains and genomic sequences are discussed in the Biology of Recombinant Viruses section.

This article aims to provide readers with an overview of viral recombinant technology, viral structure and tropism, and differences between serotypes and pseudotypes for AAVs, retro/lentiviruses, and glycoprotein deleted rabies virus (Rabies dG). Each section also contains examples of viral applications and strategies for selection of appropriate viral type for effective gene delivery.

Structure of Viruses

Viruses are compact structures that deliver genetic material to a host cell via transduction. Their structure is composed of a core and an outer shell. Viral genome is part of the core and carried as a single-stranded or a double-stranded RNA or DNA. Viral cores often include proteins that are arranged around the viral genome to provide structural support, enzymatic function, or to complement the host machinery for reproduction of the virus (Y. H. Chen, Keiser, & Davidson, 2018; Prasad & Schmid, 2012). Genes that encode core and capsid proteins are in the viral RNA/DNA genome and are expressed in host cells during viral propagation.

The outer shell of a virus protects its genome and facilitates contact with host surface proteins. Viruses are often categorized as enveloped or non-enveloped. All viruses contain a capsid or a nucleocapsid assembled from identical subunits called capsomeres. Often multiple capsid proteins interact to create a capsomere and form the outer shell of non-enveloped viruses. Enveloped viruses contain an additional plasma membrane layer. Viral proteins that punctate the outer shell of a virion in capsids or on the plasma membrane of enveloped viruses promote interactions between the viruses and their hosts and facilitate entry (Prasad & Schmid, 2012).

For AAVs, their single-stranded DNA is the only moiety that is deposited inside the capsid. The DNA genome can accommodate a maximum of 5.2 kb. Genetic sequences greater than

4.8 kb package poorly and have low yields (Wu et al., 2010). AAVs are small (25nm) non-enveloped viruses and their outer shells have a single capsid with an icosahedral symmetry. The major AAV capsid proteins are VP1, VP2, and VP3. Variations on the surfaces of the capsid proteins dictate AAV serotypes and tropism (Srivastava, 2016).

Retro/lentiviral cores are complex structures that consist of two single-stranded RNAs (viral genome), reverse transcriptases (converts viral RNA genome to DNA following transduction), nucleocapsids (protects RNA genome), integrases (inserts retro/lentiviral genome randomly into their host chromosomes), several matrix proteins, and proteases (needed for viral structural remodeling and protein maturation). The lentiviral RNA can carry up to 9 kb of genomic information. Longer RNA sequences do not form an infectious particle or a virion. The viral capsid protects the retro/lentiviral core and is surrounded by a layer of plasma membrane – the envelope coating. When a virus is packaged and buds from an infected cell, it is coated in the host plasma membrane and carries all the surface proteins of the host cell (Salmon & Trono, 2007).

The rabies virus is also an enveloped virus. Its viral core is assembled from a single-stranded RNA genome that can be up to 8.5 kb. The RNA genome is associated with nucleoprotein N (necessary for the polymerase to recognize the viral genome), P (phosphoprotein, structural), and L (RNA-dependent RNA polymerase that replicates the genome). The rabies viral genome can only be recognized and replicated by L as an RNA-N complex. Rabies glycoprotein G coats the outer surface of the rabies virus and is essential for transducing neurons and retrograde transmission.

Biology of Recombinant Viruses: AAVs, Retro/Lentiviruses, and Rabies dG

The viral recombinant technology offers a versatile method for modification and production of viral vectors. Each viral genome is divided into several plasmids that are reconstituted via co-transfection in a host cell for viral particle production or packaging (Gray et al., 2011; Osakada & Callaway, 2013; Salmon & Trono, 2007). The resulting recombinant viral particles are typically non-propagating (lacking necessary genes for reproduction) or are pseudotyped/serotyped to target a restricted population of cells.

AAV, retro/lentivirus, and rabies dG transfer vectors are vehicles that artificially carry genes of interest (fluorescent markers, sensors, modulators, etc.) into cells. They can be manipulated with molecular cloning techniques to deliver the desired sequence of promoters and genes. To reconstitute a functional virus, a separate plasmid carries the capsid and/or envelope genes that are responsible for AAV serotypes or retro/lenti/rabies pseudotypes. Other necessary genes for production of infectious viral particles are contributed from a third or a fourth plasmid. The division of the viral genome in recombinant technology is designed to facilitate viral genome manipulation and to enhance safety features for viral applications in research.

Recombinant AAVs

Natural AAVs are non-enveloped parvoviruses with a single-stranded DNA genome. They transduce dividing and non-dividing mammalian cells and are often found as contaminants

in human and primate blood and tissue samples. Natural AAV2 is the only human virus capable of targeted integration in human chromosome 19 (Hirata, Chamberlain, Dong, & Russell, 2002). However, all genes necessary for integration have been removed from the recombinant AAV.

Recombinant AAVs are reconstituted by co-transfection of three plasmids. The AAV transfer vector/plasmid carries the gene of interest flanked by Inverted Terminal Repeats (ITRs). Genetic sequences between the ITRs are packaged and delivered to the target cells by AAV particles. The second plasmid necessary for reconstitution of AAVs is the helper plasmid. It delivers VA RNAs (Virus Associated non-coding RNAs), E2A, and E4OEF6 genes that are transiently expressed in the packaging cell line for AAV production. Rep/Cap plasmid encodes genetic sequences for replication of the AAV genome and capsid structural proteins. An AAV that is packaged with Rep2 and Cap9 genes is designated as AAV2/9. Since most AAVs are packaged with Rep2, AAV2/9 is often shortened to AAV9. Cap genes dictate the AAV serotype and its tropism. None of the genes on the helper and Rep/Cap plasmid are packaged into AAV particles. Therefore, the resulting recombinant AAV is infectious yet non-propagating.

Due to the limited capacity of AAVs, genetic sequences between ITRs should be designed economically. Delivered genes separated by self-cleaving sequences (e.g. P2A, T2A) can be expressed from the same promoter to save space. Also, AAV transfer plasmids often carry genes that optimize expression such as Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE) and/or growth hormone polyadenylation signal (human hGHpA, bovine bGHpA, or mouse mGHpA). DNA sequences carrying the WPRE sequence form tertiary structures that promote gene expression and boost viral titers in packaging cell lines (Klein et al., 2006). Polyadenylation signals also stabilize and improve titer and expression in neurons (Choi et al., 2014). Such elements can be excised from transfer vectors to accommodate a larger gene of interest at the cost of lowered expression.

In cultured neurons, recombinant AAV expression is apparent after 3–4 days. *Ex vivo* mouse brain slice cultures express genes delivered by AAVs after 7–10 days; and *in vivo* expression, following stereotactic injection of mouse brain with AAVs, may take 3–4 weeks for optimal expression. A myriad of AAV transfer vector plasmids, carrying optogenetics tools and sensors, are available from non-profit plasmid repositories such as Addgene. AAV transfer vectors carrying genes of interest and neurobiology research components can be packaged with any Rep/Cap plasmid to express different serotypes.

Recombinant lentiviruses

All members of Retroviridae family of viruses including retroviruses and lentiviruses deliver their genome as single-stranded RNAs. The Lentiviral genome consists of *gag*, *pro*, *pol* and *env* genes that encode capsid proteins, reverse transcriptase, protease, integrase and the envelope glycoproteins (Salmon & Trono, 2007). Primary targets of natural lentiviruses (e.g. Human Immunodeficiency Virus, HIV) are immune cells. Each Viral particle also carries a reverse transcriptase that converts the viral single-stranded RNA genome into DNA. The viral DNA is then transferred onto the nucleus through nuclear pores and is randomly integrated into the host chromosomes.

Gamma-retroviruses (often referred to as retroviruses) cannot penetrate the nuclear envelope and only transduce cells during division when the nuclear envelope breaks apart. For example, a recombinant Gamma-retrovirus carrying a fluorescent gene only transduces and expresses fluorescence in dividing cells. This limitation has been explored to specifically target, identify, and manipulate dividing stem/progenitor cells in mammalian brains (Jessberger, Toni, Clemenson, Ray, & Gage, 2008). Since lentiviruses trigger a lessened immune response compared to retroviruses and are capable of infecting both dividing and non-dividing cells, the use of recombinant lentiviruses is more prevalent for delivery of genes to non-dividing cells such as neurons (Sakuma et al., 2012). Lentiviruses are not as efficient as AAVs for *in vivo* gene delivery and their delivered genes are expressed less robustly.

Recombinant retro/lentiviruses are also designed to be infectious but non-propagating. During the development of recombinant lentiviral vectors, the viral genome was divided among multiple plasmids and the machinery necessary for viral replication was removed from the viral particles (Salmon & Trono, 2007). Each subsequent generation of lentiviral vectors was designed to enhance safety features for use in research laboratories.

The 2nd generation packaging system dispenses several accessory genes and splits the lentiviral genome into three plasmids to reduce the potential for recombination and production of replication-competent lentiviruses. The 2nd generation packaging system employs three plasmids: (1) lentiviral transfer plasmid; (2) helper packaging plasmid; and (3) the envelope plasmid. The helper packaging plasmid contains *gag*, *pol*, *rev*, *tat* and accessory genes. The envelope protein delivers the retro or lentiviral Env or desired pseudotyping proteins. Transfer vectors deliver the desired genes flanked by LTRs. The maximum genetic material carried between the LTRs should not exceed 9 kb (Kumar, Keller, Makalou, & Sutton, 2001).

The 3rd generation packaging system eliminates the need for *tat* gene and moves *rev* onto a 4th plasmid. The 4th generation moves the *gag* and *pol* genes onto separate plasmids to further reduce the chance of recombination (Schlimgen et al., 2016). The 2nd generation packaging system can be used to produce lentiviruses using 2nd, 3rd, or higher generation lentiviral transfer vectors. Higher generations offer higher safety features but at the cost of lowered yields since host cells are transfected with multiple plasmids to reconstitute the viral genome, and therefore, not all cells will contain all the necessary components for packaging the viruses.

The 2nd generation retroviral packaging system is similar in steps and material to the lentiviral 2nd generation system. However, the retroviral yield and titer (approximately 10^5 transducing units or TU/ml) are approximately 10 times lower than a typical lentiviral titer (approximately 10^6 TU/ml).

The recombinant integrase deficient lentiviruses (IDLVs) are infectious viruses that lack the necessary machinery for genome integration. Therefore, they do not cause insertional mutations. Since genes delivered by IDLVs are diluted down and disappear as cells divide, they are ideal for transient expression of CRISPR-Cas9 components or recombinases in

hard-to-transfect cells. In non-dividing cells such as neurons, IDLVs have lasting expressions similar to integrating lentiviruses but do not integrate into the chromosomes. IDLVs can only be packaged with 3rd generation transfer vectors, since the deficient integrase gene is available on a helper packaging plasmid that lacks *tat* and *rev* (Liu et al., 2014).

In the lentiviral transfer vector plasmid, LTRs flank the desired transgene sequence under the control of an exogenous promoter. Delivered genes are used to identify cell types, overexpress or knockdown genes, or deliver CRISPR-Cas9 components for gene editing. Genes delivered by lentiviruses are expressed within 24–48 hours after transduction.

Recombinant rabies dG

Rabies viruses are neurotropic members of *Rhabdoviridae* with a single negative-strand RNA genome. They are highly toxic and cause severe illness as they spread through the nervous system. Recombinant SADB19 and CVS-N2c rabies are the most commonly used strains for trans-synaptic gene delivery in retrograde studies (Reardon et al., 2016). To make rabies virus safe for use in research laboratories, the surface glycoprotein G has been deleted in the genome of recombinant viruses. Rabies G protein plays a predominant role in retrograde transfer of virus among neural circuitry. When used for pseudotyping, rabies G promotes retrograde transmission of other types of enveloped proteins such as lentiviruses as well (Mazarakis et al., 2001). Rabies G is the sole exposed protein on the surface of the virus and is necessary for trans-synaptic transfer of virus.

Avian envelope protein (e.g. Env A, Env B, etc.) is often used to pseudotype rabies dG. The resulting virus is incapable of infecting neurons, propagation, or retrograde transfer in mammalian cells. The corresponding avian protein receptor (e.g TVA, TVB, etc.) and glycoprotein G are delivered by a secondary virus (typically an AAV) and expressed as transgenes in mammalian neurons. Therefore, rabies dG pseudotyped with Env A selectively infects TVA-positive neurons. Trans-synaptic transmission occurs when glycoprotein G is co-expressed in TVA-positive neurons. The retrograde transmission is limited to one synaptic jump since the second infected neuron does not contain glycoprotein G. Furthermore, rabies transmission is restricted to synaptic junctions and does not spread to adjacent neurons (Callaway & Luo, 2015).

Rabies has a simple genome that consists of 5 genes: the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the glycoprotein (G), and the RNA-dependent RNA polymerase (L). Viral genome RNA and N form a ribonucleoprotein (RNP) that serves as a template for replication and transcription by L. In recombinant rabies virus, the genome is contained on a plasmid and the gene of interest is inserted in place of G – hence recombinant rabies dG.

SADB19 and CVS-N2c rabies dG viruses are also highly toxic and cause damage to neurons within a week or two after infection. Additional modifications to SADB19 rabies dG genome have given rise to less toxic viruses for prolonged expression. Rabies dGL (deletion of G and L genes) and self-inactivating rabies viruses (SiR dG) are promising new tools for studying neural network organizations (Chatterjee et al., 2018; Ciabatti, Gonzalez-Rueda,

Mariotti, Morgese, & Tripodi, 2017). Rabies L protein is essential for viral genome transcription and protein production. Without L, rabies viruses do not replicate, but in the presence of G, small amounts of dGL virus travels retrogradely to infect projection neurons (Chatterjee et al., 2018). Neurons infected with SADB19 dGL appear intact and functional for many months. The Self-inactivating (SiR dG) rabies genome is engineered to contain a PEST protein sequence that targets viral protein for destruction (Ciabatti et al., 2017). Degradation of viral proteins removes toxicity and allows for long-term expression of genes for neuroanatomical studies. Since SADB19 dGL and SiR are less efficient in retrograde transmission than rabies dG, they are ideal for delivery of genes that are effective in trace amounts such as recombinases (e.g. Cre, or Flp).

CVS-N2c rabies dG is an alternative to SADB19 rabies dG that offers increased efficiency in transduction, trans-synaptic transmission with lowered toxicity (Reardon et al., 2016). This strain of rabies is propagated in Neuro2A cells, a mouse neuroblastoma cell line, that stably expresses viral components necessary for packaging the virus. The G glycoproteins between CVS-N2c and SADB19 strains differ. Therefore, packaging plasmids and secondary viruses (typically AAVs) delivering avian receptor proteins (e.g. TVA) and the rabies G protein are not interchangeable between the two strains. Packaging of pseudotyped CVS-N2c rabies dG requires months and yields much lower titers. However, the CVS-N2c virus is extremely effective in transducing neurons at low titers.

Viral Tropism: Serotypes and Pseudotypes

Serotyping and pseudotyping are means of controlling and refining viral targets. Changes to viral tropism is often achieved by manipulation of viral surface proteins.

AAV Serotypes

The surface of non-enveloped viruses such as AAVs are coated by capsid proteins. The interaction between AAV capsid and the host cell surface proteins determines what type of cells are infected by AAVs. Serotypes are variations in the amino acid sequences of capsid proteins and they dictate viral avidity and tropism. Selection of appropriate serotype optimizes targeting and volume of transduction (Table 1). For example, serotypes 5, 8, and 9 will robustly transduce the striatum of the mouse brain. Hippocampal neurons show preference for transduction with AAV9 and optimal expression is reached with AAV6, 8, and 9 in the cortex of the mouse brain (Aschauer, Kreuz, & Rumpel, 2013; Cearley et al., 2008; Cearley & Wolfe, 2006).

Variations on AAV serotypes occur either naturally or deliberately by introducing mutations in capsid proteins (M & Schaffer, 2015). Techniques such as Cre-recombination-based AAV targeted evolution (CREATE) help create engineered capsids with desirable cell/tissue targeting capabilities (Chan et al., 2017; Deverman et al., 2016; Kotterman & Schaffer, 2014; Kotterman, Vazin, & Schaffer, 2015). In the CREATE method, PCR is used to introduce sequence variation in a portion of the capsid gene. Next, the generated capsid library is used to package an AAV transfer vector carrying a fluorescent gene - often flanked by recombinase sensitive sequences. Then, the resulting AAV library with mixed capsids/ engineered serotypes is delivered to Cre-mice and allowed to express for a few weeks.

Fluorescent AAVs from the desired target tissue are then collected and analyzed to determine the genetic sequence of capsid proteins and engineered serotypes. Dr. Gradinaru's team used the above technique in a recombinase dependent system to identify PHP.B, PHP.eB and PHP.S serotypes (Deverman et al., 2016). Engineered capsids PHP.eB and PHP.S can be administrated intravenously and cross the blood-brain barrier to transduce the entire central or peripheral nervous system, respectively (Chan et al., 2017).

AAVs are typically delivered by stereotactic injections into the mouse brain. Engineered serotypes such as PHP.eB and PHP.S can be administrated intravenously to minimize damage caused by the site of injection. Most research exploring AAV serotype expression in mouse brain is conducted in C57BL/6J. Of note, recent studies suggest that the PHP.B serotype capabilities are limited to C57BL/6J and do not extend to the BALB/cJ strain of mice (Hordeaux et al., 2018). Each engineered serotype should be assessed for expression individually.

Pseudotyping retro/lentiviruses and rabies dG

Pseudotyping can only be performed on enveloped viruses such as retro/lentiviruses and rabies viruses. During propagation, the envelope membrane is assembled from the host plasma membrane. Therefore, any overexpressed protein on the surface of the host plasma membrane becomes a part of the budding enveloped virus and facilitates pseudotyping.

Retro and lentiviruses are often pseudotyped for targeted gene delivery in neurobiology research. Unmodified envelopes and capsid proteins of retro and lentiviruses preferentially target immune cells.

The most common protein used for pseudotyping lentiviruses is the Vesicular Stomatitis Virus Glycoprotein (VSV-G). It broadens the range of lentiviral targets to include almost all mammalian cells. Other pseudotyping proteins, such as rabies glycoprotein, target lentiviruses specifically to neuronal cells and enable them to traverse in the retrograde direction. Avian sarcoma leukosis virus envelope proteins (Env A, Env B, Env C, and Env E) and their paired avian derived receptors (TVA, TVB, etc.) target lentiviruses to specific neurons within a population (Matsuyama et al., 2015). The avian receptor is often delivered by a secondary virus (such as an AAV) and expressed conditionally (e.g. recombinase dependent) on the surface of target neurons.

Env A pseudotyped lentiviruses are effective molecular tools to detect activation of neurons in response to stimuli in order to understand how behavior, perception, and activity maps onto specific brain regions. When a neuron becomes activated, levels of its intracellular Ca^{2+} rises transiently and the expression of immediate-early genes (IGE) such as *Fos* are initiated. Lentiviruses delivering fluorescent markers expressed from Fos promoters are often used to label activated neurons. In the technique Capturing Activated Neural Ensembles (CANE), TVA is expressed from an endogenous *Fos* locus, thus it is only present in activated neurons. When an EnvA-pseudotyped lentivirus is injected into the region of interest in the brain, only neurons with Fos and TVA expression are infected by the Env A-pseudotyped virus. A lentivirus carrying recombinases (Cre, Flp, etc.) or other fluorophore reporter genes permanently labels Fos-TVA-positive activated neurons after stimulation (Sakurai et al.,

2016). Using this technique, activated TVA positive neurons can be labeled or functionally manipulated via targeted transduction by Env A-lentiviruses that deliver labels, GECIs, or optogenetics. The CANE system enables researchers to specifically target activated neurons (Sakurai et al., 2016).

Anterograde/Retrograde Transfer of Viruses

AAVs are ideal vectors for expression of small genetic sequences such as calcium imaging tools and optogenetics. Several naturally occurring AAV serotypes have enhanced trans-synaptic anterograde properties such as serotypes 1, 2, and 9 (Zingg et al., 2017) and the designer RETRO serotype travels retrograde (Tervo, Hwang et al. 2016). AAVs with anterograde and retrograde distributions are valuable tools for identification of neural projections and network connectivity. For example, anterograde axonal tracing with AAV 1 and 9 has revealed that a subpopulation of superior colliculus neurons receive cortical collicular projections from the auditory and visual cortex (Zingg et al., 2017). Serotypes 1 and 5 have the most promising retrograde activity among natural serotypes (Rothermel, Brunert, Zabawa, Diaz-Quesada, & Wachowiak, 2013). However, their tropism dictates the level of expression in various sections of a mammalian brain and significantly affects their retrograde transmission. Serotype RETRO has a broad retrograde functionality in the central nervous system with robust gene expression (Tervo et al., 2016).

Anterograde transport occurs when neuronal dendrites are transduced, and the cell machinery transports the virus toward the axon. For retrograde tracing, axons are transduced, and the virus is transported toward the soma of the neuron. Since recombinant AAVs are non-propagating viruses, they do not spread from neuron-to-neuron as efficiently as rabies and herpes simplex viruses. Anterograde and retrograde transport occurs at the site of AAV exposure for all infected neurons. Therefore, presence of a mixed population of neurons at the site of injection results in all neural projections from the area to be targeted by anterograde or retrograde transport of AAVs. For example, dopamine D1 and D2 receptor-containing neurons that are localized in a similar region of brain (e.g. striatum), will both become transduced when exposed to AAVs with anterograde or retrograde serotypes. Therefore, neural projections from both populations become labeled and express the delivered gene (Gerfen et al., 1990). AAVs are ideal for identification of neural projections from a region of brain rather than specific cell types.

Pseudotyped rabies deleted glycoprotein virus (Rabies dG) offers the versatility of targeting and monosynaptic transmission from a specific population/type of neurons (Callaway & Luo, 2015). As described previously, rabies dG pseudotyped with Env A exclusively transduces TVA-positive neurons. In this two-step procedure, TVA is typically delivered by an AAV and expressed conditionally (recombinase technology, type-specific or inducible promoters, etc.) in target neurons. After TVA expression (2–4 weeks post-delivery by an AAV), the Env A pseudotyped rabies dG is administrated to transduce TVA-positive neurons. For retrograde transmission, the AAV delivering TVA must also deliver the strain specific rabies G protein.

As an alternative to using recombinant pseudotyped rabies dG viruses, lentiviruses are also utilized as neural tracers when pseudotyped with rabies virus glycoprotein (Rabies G) (Mazarakis et al., 2001) or the chimera of both VSV-G and Rabies G (Kato, Kobayashi, et al., 2011; Kato, Kuramochi, et al., 2011). Lentiviruses are less toxic and take less time to produce than recombinant pseudotyped rabies viruses.

Recombinant Virus Usage

Recombinant viruses are powerful vectors for delivering genetic materials to neurons. Viral transfer plasmids encoding molecular tools and genes of interest are routinely designed, constructed, and tendered for use.

Lentiviral and AAV vectors are both used to carry genetically-encoded calcium indicators (GECIs) such as GFP based GCaMPs and the related G-GECO1 variants (T. W. Chen et al., 2013; Zhao et al., 2011). The stable expression of GECIs allows researchers to monitor long-term Ca^{2+} oscillations by microscopy *in vitro* and *in vivo*. In addition to GECIs, development of optogenetic tools has advanced means of manipulating neuronal function. An optogenetic tool is a light-sensitive protein, that can be delivered to neurons via viral vectors, and affects neuronal function following exposure to light with a specific wavelength (Rost et al., 2017). Channelrhodopsin-2 (ChR2) and Natronomonas halorhodopsin (eNpHR) are examples of key optogenetic tools created by Deisseroth's laboratory (Deisseroth et al., 2006). Optogenetics technology offers spatial, temporal, and targeted control over neuronal function.

Neuronal-specific promoters such as synapsin 1 (Syn), neuron-specific enolase (NSE), calcium/calmodulin-dependent protein kinase II alpha (CaMKIIα, activated in excitatory glutamatergic neurons), and glutamate decarboxylase 67 (GAD67, activated in inhibitory GABAergic neurons) are often used in viral transfer vectors to selectively express genes in neurons.

The further regulation of gene expression can be achieved by use of recombinase (e.g. Cre, Flp, Dre) transgenics and recombinase sensitive sequences in viral transfer vectors (e.g. gene of interest flanked by lox-P sequence) (Atasoy, Aponte, Su, & Sternson, 2008; Sauer & Henderson, 1988). Unlike AAVs, retros, and lentiviruses, the rabies virus is maintained as an RNA-protein complex in the cytoplasm of cells and its genes are expressed via a specialized viral polymerase. Therefore, neuron-specific promoters or recombinase recognition sites cannot be introduced onto recombinant rabies transfer vectors/plasmids.

NOTE: All recombinant viruses described in this overview are non-propagating and safe for use in Biosafety Level 2 laboratories. Please consult and follow your institute's biosafety guidelines for safe handling and disposal of viruses. Also, use personal protective clothing and gear to protect yourself and your coworkers.

ACKNOWLEDGEMENT

This research was supported by the Intramural Research Program of the National Institute of Health (NIH), National Institute of Environmental Health Sciences (NIEHS). We are grateful to Dr. Steve Wu, and Dr. Robert Petrovich for critical reading of the manuscript and helpful advice. We would also like to acknowledge and thank

Dr. Charles Gerfen for intellectual input and initiating the concept for this paper. At the NIEHS, we would like to thank Dr. Jerrel Yakel, Dr. Bernd Gloss, Dr. Guohong Cui, Dr. Zhenglin Gu, Ms. Pattie lamb, and Ms. Amy Papaneri for intellectual and technical contributions.

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Table 1.

AAV serotype expression in mouse brain.

Transduction efficiency of AAV serotypes in mouse brain summarized from published studies (Aschauer et al., 2013; Cearley et al., 2008; Cearley & Wolfe, 2006; Tervo et al., 2016).

	Region of Brain		
AAV Serotype	**Striatum**	**Hippocampus**	**Cortex**
1	+	+	+
2	+	–	–
5	+++	++	–
6	+	–	+++
7	++	+++	+++
8	+++	+	++
9	+++	+++	+++
DJ	+++	+++	+++
PHP.B	+++	+++	+++
PHP.eB	++++	++++	++++
hu.48R3	+	++	++
hu.11	+	++	++
hu.32	+++	+++	++
hu.37	++	++	+++
pi.2	+	+++	++
rh.8	++	++	++
rh.10	+++	++	+
RETRO	+++	+++	+++