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Hitchhiking on the neuronal highway: Mechanisms of transsynaptic specificity

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Abstract

Transsynaptic viral tracers are an invaluable neuroanatomical tool to define neuronal circuit connectivity across single or multiple synapses. There are variants that label either inputs or outputs of defined starter populations, most of which are based on the herpes and rabies viruses. However, we still have an incomplete understanding of the factors influencing specificity of neuron-neuron transmission and labeling of inputs vs. outputs. This article will thus touch on three topics: First, how specific are the directional transmission patterns of these viruses? Second, what are the properties that confer synaptic specificity of viral transmission? Lastly, what can we learn from this specificity, and can we use it to devise better transsynaptic tracers?

Introduction

Elucidation of the connectome is a necessary first step towards defining the function of the brain. Viral methods in particular have been instrumental in illuminating the complicated connectivity of intermingled neuronal populations (Nassi et al., 2015). By spreading between neurons through synaptic connections, they have enabled high-resolution mapping of connectivity throughout both the central and peripheral nervous systems. However, each viral tracing method currently in use has limitations, including multi-synaptic spread, non-exclusive synaptic spread, and/or rapid cellular toxicity. Thus, neuroscientists would greatly benefit from improved viral vectors 1) whose spread can be spatially and temporally controlled, 2) that are non-toxic, and 3) can be used in long-term behavioral experiments. The principle barriers preventing development of improved tracing systems are our insufficient understanding of the basic biology underlying viral transsynaptic transmission, the major factor(s) that causes cellular toxicity, and the balance of labeling efficiency and cellular toxicity.

The intention of this article is not to provide a comprehensive review of neurotropic virus biology. In-depth reviews of viral transmission in the nervous system (Koyuncu et al., 2013), direct cell-cell viral spread (Sattentau, 2008), inflammatory responses to central nervous system infections (Dahm et al., 2016), and viruses as neuroanatomical tracers (Nassi et al.,

2015) can be found elsewhere. In this article, the alphaherpesviruses herpes simplex virus (HSV) and pseudorabies virus (PRV), as well as the rhabdoviruses rabies virus (RABV) and vesicular stomatitis virus (VSV) will be used as examples to illustrate potential mechanisms of direction-specific viral transmission. I will then explore how these viruses label connected neuronal circuits while largely avoiding other cells such as glia.

Mechanisms of directional transport

Alphaherpesvirinae: the original transsynaptic viral tracers

The initial observation of HSV transneuronal transmission was reported in the early 1920s, when an HSV isolate was obtained from the lip of a human patient. The virus was then introduced into the eyes of rabbits through the cornea, and each time the infection produced encephalitis through transmission via the innervating nerves (Goodpasture, 1925). The introduction of HSV as a neuroanatomical tool was brought about by the concurrent development of various retrograde and anterograde small molecule/protein tracers in the 1970s (Bak et al., 1977; Cook and Stevens, 1973). These studies found that HSV could transmit between neurons along anatomically-connected pathways.

A critical question for any neuroanatomical tracer is to define if it transmits to inputs (retrograde) or outputs (anterograde) of the initially infected neurons. Studies have found that though most strains show bidirectional transsynaptic transmission, the direction of transmission of HSV (Zemanick et al., 1991) and PRV (Card et al., 1998) is strain-dependent. For example, the PRV Becker strain can transmit in both the anterograde and retrograde directions, and the Bartha strain is thought to transmit exclusively in the retrograde direction (Bartha, 1961). The Bartha strain contains a large deletion in 4 viral genes (Lomniczi et al., 1987), including gE, gI, Us9, and Us2. Each of these genes codes for either a transmembrane (gI, gE, Us9) or membrane-associated protein (Us2). Us2 is a viral tegument protein that is highly conserved across alphaherpesviruses. Though its function has not been well elucidated, it has been shown to interact with ubiquitin and to be associated with the cell membrane (Kang et al., 2013). However, as Us2 deletions have no effect on viral release or anterograde transsynaptic transmission (Ch’ng and Enquist, 2005), it is unlikely to play a major role in directional transsynaptic specificity.

Us9 is a type II membrane protein present on the HSV envelope. The first studies of Us9 mutants found that viral capsid proteins are still detectable in axons, but glycoproteins are not (Tomishima and Enquist, 2001). However, other studies have found that capsid proteins are not present in the axon in Us9-deletion viruses (Lyman et al., 2007). Interestingly, the association of Us9 with lipid rafts also appears to be critical for viral particle localization to the axon (Lyman et al., 2008). Incorporation within these rafts appears to be necessary for interaction with the molecular motor kinesin-3 protein KIF1a that mediates anterograde axonal transport (Kramer et al., 2012; Lyman et al., 2008), an interaction that requires gE/gI (Kratchmarov et al., 2013).

gE and gI are surface glycoproteins that form a complex on the cell surface. Mutations in gE or gI specifically prevent cell-cell spread of virus with no effect on production of infectious virus or the efficacy of cellular entry, while deletions or mutations in other viral

glycoproteins such as gB or the gH-gL complex prevent spread by both extracellular and cell-cell routes (Balan et al., 1994; Dingwell Ks et al., 1994). Therefore, gE and gI appear to mediate spread specifically through cell-cell contacts. gE and gI mutants also showed deficits in putative anterograde transsynaptic spread *in vivo*, suggesting that cell-cell spread, but not extracellular viral release, is required for viral transmission in neural circuits.

gE, gI, and Us9 are necessary for anterograde transsynaptic transmission, as deletion from the bi-directional PRV Becker strain was sufficient to recapitulate the anterograde transneuronal deficiency of the PRV Bartha strain. These genes are also sufficient to support anterograde transneuronal transmission, as supplying the gE, gI, and Us9 genes to the Bartha strain was sufficient to enable anterograde transsynaptic transmission. Interestingly, individual mutants in gE, gI, or Us9 were all partially deficient in anterograde transneuronal transport as shown by a delay in spread kinetics, but none of these mutants completely abolished anterograde transneuronal spread (Ch'ng and Enquist, 2005), suggesting that they work together but no one gene is absolutely required.

gE and gI accumulate within the trans-Golgi network (TGN) prior to sorting to the cell surface, and this accumulation is necessary for the proper sorting of glycoproteins and ultimately virions to the cell surface (Farnsworth and Johnson, 2006). gE, gI, and Us9 all contain TGN sorting motifs in their C-terminal domains that are critical for HSV/PRV function/anterograde spread (Brideau et al., 2000, 1998; Dingwell et al., 1995; Snyder et al., 2008). However, these proteins do not appear to be important once the virus is within axons, suggesting that they may be responsible for sorting the viral proteins/particles to the correct cellular motors prior to anterograde transport (DuRaine et al., 2017). There is evidence that this accumulation in the TGN leads to the recruitment of viral tegument proteins, which may in turn lead to preferential envelopment at those TGN sites (Johnson and Baines, 2011). These results together suggest that gE, gI, and Us9 all play a role in directing anterograde transsynaptic transmission of HSV/PRV. One potential mechanism is that viruses are assembled while sorting through the TGN, where the proteins were sorted by virtue of C-terminal domain targeting motifs, and ultimately directing enveloped capsids to the proper kinesin motors for anterograde transport (Diefenbach et al., 2016; Kramer et al., 2012; Wolfstein et al., 2006).

Notably, there are two prominent models for how alphaherpesviruses are transported down neuronal axons to enable anterograde transsynaptic transmission. One is the married model, in which viral capsids and glycoproteins are assembled together in the cell body and are transported down the axon together as a unit (Antinone and Smith, 2006). The other is called the separate model, where the components are transported separately and assembled locally within axons (Penfold et al., 1994). Evidence for and against both of these models have been discussed elsewhere (Cunningham et al., 2013; Kratchmarov et al., 2012). For the purposes of this review, it is sufficient to note that specific herpes viral genes can affect anterograde transsynaptic spread.

Another potential clue about the source of directional specificity may come from the HSV strain H129, which was originally isolated from the brain of a patient in 1977 who developed encephalitis (Dix et al., 1983). This strain has been thought to transmit

exclusively in the anterograde direction. However, to date little is known about what provides the H129 strain with preferential anterograde transmission. A recent study that sequenced this variant identified amino acid differences in a variety of proteins compared with a laboratory strain, but did not identify any proteins that drive anterograde transsynaptic specificity (Szpara et al., 2010). This could be, in part, due to the fact that the absolute anterograde specificity of H129 may be somewhat overstated. A series of careful observations of viral spread have demonstrated delayed but reliable retrograde transsynaptic transmission (Archin and Atherton, 2002a, 2002b; Card and Enquist, 2014; Wojaczynski et al., 2015), in addition to anterograde transmission.

RABV

RABV was the second virus to be regularly employed in a laboratory setting for transsynaptic tracing. The original RABV studies used a replication-competent virus to map multiple nodes of connected circuits in primates (Kelly and Strick, 2000, 2003). However, one significant limitation to this type of work is that RABV infection is lethal to humans, and appropriate containment to ensure safety is therefore critical. In contrast, PRV usually does not infect humans, though a recent report suggest that it may occur in rare cases (Ai et al., 2018).

The development of monosynaptically-restricted variants has enabled much broader use of RABV-based tracing methods by neuroscience labs (Wickersham et al., 2007b). By deleting the RABV glycoprotein from the viral genome, the virus cannot spread on its own. However, resupplying the glycoprotein to targeted cell types enables transsynaptic transmission to direct inputs of these defined cells. As this virus cannot spread on its own, it is designated as biosafety level 2 and is thus much more accessible to neuroscience labs.

Accumulated anatomical evidence suggests that RABV transsynaptic transmission throughout the central nervous system is in the retrograde direction (Ugolini, 2011). However, little is known about how this specificity, particularly the lack of anterograde transmission, is achieved. In general, relatively little is known about the biology of RABV transmission, especially compared to the alphaherpesviruses. Three receptors have been identified for RABV: the nicotinic acetylcholine receptor (Lentz et al., 1982), p75NTR (Tuffereau et al., 1998) and NCAM (Thoulouze et al., 1998). However, it is unclear which, if any, of these receptors is engaged *in vivo,* as deletion of p75NTR (Tuffereau et al., 2007) or NCAM (Thoulouze et al., 1998) does not block RABV infection. In addition, the acetylcholine receptors may be involved with infection at the neuromuscular junction, and may actually serve to mediate toxic effects of the glycoprotein (Hueffer et al., 2017).

Though little is known about what imparts RABV with apparent retrograde specificity in transsynaptic transmission, when RABV was engineered to encode the glycoprotein from VSV, the transmission pattern in the brain was similar to that of wild-type VSV (Yan et al., 2002) (discussed below). This suggests that the direction of RABV transsynaptic viral transmission can be altered by modification of the viral glycoprotein. Although clear evidence of anterograde transsynaptic transmission of RABV has not been reported in the central nervous system, putative anterograde transsynaptic transmission has been noted in the peripheral sensory dorsal root ganglion (DRG) in two separate studies (Tsiang et al.,

1989; Zampieri et al., 2014), as well as in DRG cultures (Bauer et al., 2014). Interestingly, in the latter study, they observed that the glycoprotein was necessary for trafficking of the viral particle along axons, as the viral capsids were not present in axons when the glycoprotein was not expressed. It is not clear why anterograde transsynaptic transmission has been reported for DRG neurons in the periphery and not in neurons in the central nervous system; it could be because these DRG neurons are pseudo-unipolar and thus lack traditional dendrites. The sorting of proteins and release of virus may thus be substantially different in these peripheral neurons relative to those in the central nervous system.

RABV was the first virus whose transsynaptic spread was validated electrophysiologically (Wickersham et al., 2007b) to spread predominantly through synaptic connections. 9 out of 11 paired recordings demonstrated that monosynaptic RABV transmitted to directly-connected inputs via retrograde transmission, and 0 out of 9 recordings from non-labeled nearby neurons showed connectivity. Given that it was impossible to unambiguously define potential starter cell-input pairs, this may be an underestimate of the true synaptic preference of RABV (Wickersham et al., 2007b).

VSV

VSV provides an ideal reductionist system to study transsynaptic transmission. VSV is a preferred model system for studying the biology of *Mononegavirales,* which includes RABV, Ebola, and measles viruses. A notable advantage of VSV is the modest genome size: whereas HSV/PRV have a relatively large genome (152kb/150kb) with over 80 open reading frames, VSV and RABV have only 5 open reading frames and a much smaller genome (~11 kb). Therefore, identification of factors influencing viral transmission should be more straightforward. Initial observations of VSV spread in the brain were consistent with anterograde transneuronal transmission (Lundh, 1990). Though some later studies suggested VSV spread was not transsynaptic (van den Pol et al., 2002), anterograde transsynaptic spread of VSV was confirmed a few years later (Beier et al., 2011). Further, we showed that replacing the VSV glycoprotein VSV-G with RABV-G enabled retrograde viral transsynaptic transmission, and replacing with LCMV-G enabled anterograde transsynaptic spread (Beier et al., 2011; Beier et al., 2013).

If swapping the glycoprotein alone is sufficient to redirect transsynaptic transmission, this suggests that 1) The VSV virion itself is capable of transmitting in either direction, and 2) there must be some instructive signal within the glycoprotein that dictates direction of functional transmission. Though VSV-G has been found to be sorted to either the somatodendritic or axonal compartments (Dotti and Simons, 1990; Nakata and Hirokawa, 2003), the majority of evidence to date points to somatodendritic localization. Thus, these results are at face value inconsistent with the hypothesis that VSV-G targeting alone may be sufficient to explain the preferential anterograde transsynaptic spread *in vivo.* A lack of VSV-G-driven preference in directional viral release is consistent with another recent study which found that VSV-G localization to the basolateral compartment of polarized cells was not required for basolaterally-biased viral release (Drokhlyansky et al., 2015). In this case, virus with or without VSV-G was released from both apical and basolateral compartments,

though the virus maintained a basolateral preference in both cases. Similarly, studies in neurons have reported VSV release from axons as well as dendrites (Dotti et al., 1993).

Interestingly, VSV-G as well as the HSV gE and gI glycoproteins, which are critical for anterograde viral transmission, are sorted to the basolateral surface of polarized cells (Dingwell and Johnson, 1998). For VSV-G, the amino acid sequence YTDI, present in the C-terminus of the protein, has been implicated in basolateral targeting (Thomas and Roth, 1994; Trowbridge et al., 1993). A similar YXXΦ motif is conserved in gE proteins from HSV-1, 2, and PRV (McGeoch et al., 1985, 1987; Petrovskis et al., 1986), though a potential anterograde targeting function of this domain in gE has not been elucidated.

One issue that may explain discrepancies in viral transmission patterns is that different methods have been used to assay viral directional spread. Studies in polarized epithelial cells principally examine the location of viral proteins at different surfaces and the titers of viruses released from each surface. However, viral release from polarized epithelia into the extracellular fluid may not be the dominant mode of transmission between neurons *in vivo* (discussed later). In addition, the development of neuronal polarity *in vivo* differs between neuron types and developmental stages (Takano et al., 2015), suggesting that results from one cell type may not be applicable to all neuron types. Also, the apical-basolateral distinction that exists for polarized epithelial cells may not always hold for neurons (Jareb and Banker, 1998). Therefore, more work is needed to understand the directional transmission patterns of VSV *in vivo.*

As with RABV, the transsynaptic spread of VSV was confirmed electrophysiologically, with 5 out of 8 neurons recorded demonstrating monosynaptic anterograde spread of virus (Beier et al., 2011). Specificity was confirmed through showing that 0/10 non-labeled nearby neurons were connected. We postulated that the rapid cellular toxicity of VSV may have artificially reduced the apparent connectivity rate of VSV, as VSV is more cell-toxic than RABV.

Adeno-associated virus (AAV)

Recently, certain serotypes of AAV were postulated to transmit transsynaptically in the anterograde direction (Zingg et al., 2017). Electrophysiological evidence for transsynaptic specificity was supplied by optically-exciting AAV-infected, ChR2-expressing neurons in the primary visual cortex and conducting slice electrophysiology experiments from downstream labeled cells in the striatum. While 9/9 recorded striatal cells demonstrated post-synaptic responses consistent with monosynaptic transmission, the important control of recording from nearby, non-infected neurons (Beier et al., 2011; Wickersham et al., 2007b) was missing. Without this control, it is possible that because ChR2 was expressed in a large population of cortical projection neurons, stimulation of axons from neurons in this large volume of cortex would directly activate the majority of downstream striatal neurons. Thus, while these experiments do strongly suggest anterograde transneuronal transmission of a small fraction of AAV virions via transcytosis, non-synaptic release of these virions remains a possibility. Non-enveloped viruses are not known to transmit directly to juxtaposed cells (Sattentau, 2008), a likely mechanism of transsynaptic transmission of HSV/PRV, RABV, and VSV (discussed below). Thus, while an AAV-based anterograde transsynaptic tracer

would be a great non-toxic alternative to current transsynaptic viruses, more work is required to show its transsynaptic specificity and applicability to a variety of circuits.

Caveats and limitations to current transsynaptic viral methods

The pros and cons of the most commonly used viruses for transsynaptic labeling are listed in Table 1. One significant limitation of currently used transsynaptic viruses is their inherent toxicity, both at the level of the cells and the organism. HSV, VSV, and RABV can all infect and spread within humans, and work with HSV, PRV, RABV, and VSV must be done in at least biosafety level 2 containment. HSV, PRV, and RABV are lethal within days of infection in rodents, and VSV is lethal when administered directly within the central nervous system, and sometimes peripherally such as through the olfactory epithelium, depending on the age of the animal (Lundh et al., 1988). Cellular toxicity is also an issue; PRV can alter neuronal physiology and connectivity within just hours after infection (McCarthy et al., 2009), VSV triggers significant cellular toxicity by one day post-infection (Beier et al., 2011; van den Pol et al., 2009), and RABV triggers anatomical pathology observable after a week or two (Wickersham et al., 2007a). This toxicity is a major impediment towards more widespread usage of transsynaptic viruses to study neuronal circuit function, as the cell populations being studied may be altered by viral toxicity. To combat this issue, PRV variants with transient *in vivo* expression have been engineered (Oyibo et al., 2014; Wu et al., 2014). For RABV, multiple approaches have been employed; less toxic strains than the conventional vaccine strain B19 have been introduced (Reardon et al., 2016), the viral nucleocapsid protein has been destabilized using a conditional degron domain (Ciabatti et al., 2017), though there is debate about the identity of this virus (Matsuyama et al., 2019), and the polymerase gene was deleted along with the glycoprotein (Chatterjee et al., 2018). However, these viruses all have limitations, including slowed spread kinetics, current inability of helper AAV vectors to supply the L gene to enable monosynaptic spread *in vivo*, or significant remaining cellular toxicity. For VSV, some mutants with reduced toxicity are already available, such as a variant with a M51R mutation in the viral matrix protein (Ahmed and Lyles, 1997; Beier et al., 2011). However, this vector is still cell toxic.

Another less-reported limitation is that the specificity of most viral transsynaptic tracers is almost certainly overstated. The putative anterograde-specific HSV H129 strain does display retrograde transmission, albeit in a kinetically-delayed fashion (Wojaczynski et al., 2015). Although RABV is repeatedly mentioned to transmit exclusively in the retrograde direction, there have been multiple reports of its anterograde transsynaptic transmission along peripheral nerves (Bauer et al., 2014; Tsiang et al., 1989; Ugolini, 2011; Zampieri et al., 2014). In addition, although VSV transmission has been less thoroughly explored, it has been suggested to be non-pathway specific in dissemination through the olfactory system in one study (van den Pol et al., 2002), and specific in another (Beier et al., 2011). Electrophysiological evidence in support of direct synaptic connectivity exists for RABV (Wickersham et al., 2007b) and VSV (Beier et al., 2011), though this author is not aware of electrophysiological evidence of HSV/PRV transsynaptic specificity. In addition, HSV/PRV, RABV, and VSV can all invade axons passing through the site of initial viral inoculation. This will cause viral labeling of inputs not synaptically connected to target neurons, which may degrade the specificity of circuit tracing.

An often understated fact is that viral transmission is not always from neurons to other neurons. Glial infection has been observed for PRV-Bartha (Viney et al., 2007), HSV (Ohara et al., 2001) RABV (Marshel et al., 2010), and VSV (Lundh, 1990). It was noted for PRV-Becker that anterograde transneuronal transmission is not always from the axonal terminal (Tomishima and Enquist, 2002). Rather, viral transmission occurred to glia along the optic nerve. Given the start-stop and anterograde-retrograde trafficking of HSV along axons (Smith et al., 2004), it is likely that the virus can escape from axons and into nearby cells at the points in which transmission halts. However, these viruses may not replicate properly within glia: for example, PRV has a deficiency in capsid envelopment in astrocytes (Rinaman et al., 1993). Therefore, by effectively absorbing viral particles locally but preventing transmission of this virus further, glia may help to support the neuronal specificity of viral transmission. Additionally, viruses are grown in tissue culture, which do not have conventional neuronal synapses. Thus, these viruses do not need synapses to transmit from one cell to another. Yet, their remarkable specificity (even if not absolute) must be due to a property of neuronal or other brain cells, likely specificity in the uptake or budding processes.

When conducting transsynaptic tracing experiments, there are a few points to keep in mind. First, viral titer is critical. Titers may be assessed in different ways, for example through the number of copies of viral genomic DNA, or through functional titer, where infection events are quantified in a viral dilution series. This is important as the ratio of functional to total viral particles in a preparation may vary drastically for different viruses, and even in different preparations of a given virus (Klasse, 2015). Assuming viral titering in tissue culture is performed on a suitable cell line, functional titer is likely a better estimate of how well viruses will perform *in vivo.* Note that neither viral transduction nor transmission may be a linear function of input virions (Nathanson et al., 2009), though there has been little systematic analysis of this possibility. In addition, for both replication-competent and incompetent viruses, it is ideal to analyze multiple time points postinoculation. Viruses may label different numbers or types of connections over time, and inferences regarding circuit connectivity can be made through a comparative temporal analysis. However, different types of virus and strains of the same virus have different tropisms and spread kinetics, so cross-comparison can be difficult. In addition, transsynaptic viruses likely only label a small fraction of connections to any particular starter neuron (Beier et al., 2013; Marshel et al., 2010). Given that only a small percentage of inputs to a given starter cell are labeled, and viral transmission may be activity-dependent (Beier et al., 2017), it is important to take a conservative approach to interpreting transsynaptic tracing data.

In all of these analyses, it is critical to note that transsynaptic spread is assayed by functional viral expression in a secondary pre- or post-synaptic cell. Therefore, the readout is the successful completion of the entire viral replication/spread/infection cycle. A virus considered to be a preferentially anterograde transsynaptic vector may have enhanced anterograde transmission and/or defective retrograde transmission. For example, if a virus can be efficiently trafficked tc axons and not dendrites in starter neurons and thus is released from the axonal but not dendritic compartment, then anterograde but not retrograde viral transsynaptic spread would be observed. A similar anterograde bias would be observed for a

virus that is released from both pre- and post-synaptic sites but is not efficient in retrograde axonal transport in pre-synaptic cells.

An important consideration not given sufficient emphasis in the literature is that only longdistance viral transmission and not local spread is typically analyzed to assess directional transsynaptic specificity. At the injection site, this is often due to the difficulty of dissociating local viral spread from neurons infected by the initial inoculum. In addition, viruses may egress from the cell soma or be released non-specifically by cell lysis (McCarthy et al., 2009). However, the practical consequence is that viruses are only deemed to be retrograde transsynaptic tracers if they can label inputs far from the injection site, and anterograde transsynaptic tracers if they can label outputs far from the injection site. Thus, selective transmission to local microcircuits would be missed. For example, the author has not observed clear evidence of retrograde transmission of VSV when injected into the lateral geniculate nucleus of the thalamus (and assessing infection of retinal ganglion cells) or the striatum (assessing cortical infection), a result consistent with previous studies (van den Pol et al., 2002). However, we did not assess the potential for local retrograde transsynaptic transmission, as we could not discern it from infection via the initial inoculum or long-distance spread from another brain site. In addition, when testing the directional specificity of VSV pseudotyped with LCMV-G, we searched for single starter neurons (yellow) surrounded by patches of secondarily-infected neurons (green), and recorded from those green neurons as these would be the most likely cells labeled through transsynaptic spread. Thus, we were assessing the capability for local anterograde transsynaptic spread. The requirements to travel a short axonal segment a few μm in length may be substantially different than traversing an axon that is mm or even cm long. This property should not be specific to VSV; indeed, in PRV gE mutants, viral components could still be visualized in the proximal axonal segment but not in the distal axon, suggesting that the requirements for transport to these sites differ (Ch'ng and Enquist, 2005). More research is required to understand the cellular and molecular properties that dictate biases in transsynaptic spread.

Directional tracing summary

For each virus or viral family – HSV/PRV/RABV/VSV, the glycoprotein appears to be the key component that dictates the direction of viral transsynaptic transmission. So what factors can influence the direction of spread? PRV glycoproteins are normally located throughout the axon, but are found only in the cell body and proximal segment of the axon in mutants lacking anterograde spread, such as PRV-Bartha (Ch'ng and Enquist, 2005). VSV-G is thought to be localized predominantly in the somatodendritic compartment (Doth and Simons, 1990), and the localization of the RABV glycoprotein in neurons, to this author's knowledge, has not been reported. Thus, it is still an open question. The answer likely lies in some combination of internal glycoprotein sorting and the ability to retrogradely traffic along axons in secondarily-infected neurons. For PRV, different glycoproteins appear to mediate infection from the inoculum and through cell-cell transmission (Ch'ng et al., 2007), but RABV and VSV only have one glycoprotein mediating both processes. Thus, these systems may provide a more straightforward solution to this question.

It is clear from HSV/PRV and VSV transsynaptic experiments that the viral capsid is capable of transmitting from either dendrites or axons, with the functional outcome depending on the glycoproteins present. So how is it possible that directional specificity can be achieved? In the case of PRV, given the effect of the gE/gI/Us9 deletion present in the Bartha strain, retrograde transneuronal transmission may be the “default state” by which the virus transmits in the absence of anterograde trafficking of viral proteins. Indeed, this is supported by the observation that most neurotropic viruses can spread retrogradely through circuits but only a select few have shown anterograde transmission (Koyuncu et al., 2013). PRV-Bartha capsids are still competent to bud from both the cell body and axonal compartments of the neuron, as assessed by normal anterograde transmission when gE/gI/Us9 are restored to the PRV Bartha strain (Ch’ng and Enquist, 2005).

While gE/gI/Us9 proteins appear be involved with trafficking of HSV/PRV proteins to the axon, in the case of VSV trafficking of viral components together along the axon seems unlikely. Indeed, viral components appear to be localized to separate microdomains within the cell (Swinteck and Lyles, 2008). It is, however, possible that components of VSV independently get transported to the dendritic or axonal compartments and assemble there. It is also possible that each viral protein takes a similar path, and these proteins interact in the TGN or en route to the axon, though it is generally believed that for VSV, viral components are transported separately and thus capsids first interact with the glycoprotein at the plasma membrane. In support of this idea, we used multiple different glycoproteins with VSV, including those from RABV (RABV-G) and lymphocytic choriomeningitis virus (LCMV-G), and tested the direction of transneuronal viral transport. None glycoproteins should have specific interactions with any component of VSV, yet specific retrograde (RABV-G) or anterograde (LCMV-G) transmission patterns were observed (Beier et al., 2011; Beier et al., 2013).

Another potential contributor to directional specificity may be the transsynaptic transmission process itself. For example, viral receptors may be located at only one pole or the other, and therefore enable transmission only in one direction. While no convincing evidence has emerged to date to support this mechanism playing a major role *in vivo*, it nonetheless may be involved in the direction selectivity of viral transmission.

Transsynaptic transport is a general phenomenon dictated by direct cell-cell transmission

The canonical mode of viral transmission involves the synthesis of intracellular virions, release of virus from infected cells into the extracellular space, binding of these virions to extracellular receptors, and entry/uncoating in these cells. However, many viruses can transmit directly between cells without ever being released into the extracellular space (Sattentau, 2008). While each mode of spread has its advantages and disadvantages, direct cell-cell transmission reduces both the amount viral surface exposed and the time that surface is exposed to the innate and adaptive immune responses. Thus, this provides a significant advantage in evading immune detection, which is essential for gaining access to the central nervous system and bypassing the body’s natural defense mechanisms.

While alphaherpesviruses can be released from cells and spread through extracellular media, this is likely not the dominant mode of transmission between neurons. When the

glycoprotein gD is deleted, spread of free PRV virions was prevented, but there was no effect on transneuronal spread (Babic et al., 1993; Mulder et al., 1996; Peeters et al., 1993; Rauh and Mettenleiter, 1991). gE and gI also appear to be responsible for cell-cell spread, as virions lacking gE or gI cannot spread from cell-cell but show no defects in viral production nor the rate of cell binding and entry (Balan et al., 1994; Dingwell et al., 1994). Interestingly, gE-gI accumulate specifically at cell junctions of epithelial cells, suggesting that gE-gI bind to junction proteins (Dingwell and Johnson, 1998). This process also recruits virions to junctions and away from apical surfaces (Johnson et al., 2001). Cell-cell transmission is thought to be highly efficient: in contrast to extracellular routes of infection, only a small number of particles are necessary for PRV cell-cell transmission (Taylor et al., 2012).

For RABV, cell-cell transmission was postulated as early as the 1970s (Charlton and Casey, 1979; Iwasaki and Clark, 1975; Murphy et al., 1973). It was noted that there was simultaneous uptake in presynaptic terminals of viral particles budding from postsynaptic neurons (Charlton and Casey, 1979), suggesting direct neuron-neuron transmission of virus. VSV has also been observed to transmit from cell to cell. When it does so, the apex of the virus can be seen being engulfed in clathrin-coated pits in the neighboring cell before the virus has been completely released from the initial cell (Vassalli et al., 1986). We also postulated cell-cell transmission as a mechanism of VSV transmission through brain cells, for example through the rostral migratory stream (Beier et al., 2011).

Beyond these often-used tracers, cell-cell transmission occurs for other less explored neurotropic viruses. The Borna virus (BDV), like RABV and VSV, is a member of the order *Mononegavirales* and can cause the neurological Borna disease. Transmission of BDV has been traced centripetally and transsynaptically after olfactory, ophthalmic, or intraperitoneal inoculation (Carbone et al., 1987; Morales et al., 1988). BDV is trafficked retrogradely through the same endocytic pathway as RABV (Charlier et al., 2016; Lalli and Schiavo, 2002). Despite the fact that viral spread between cells could clearly be observed over time, very little virus was found in the supernatant of BDV-infected cells (Bajramovic et al., 2003; Gosztonyi et al., 1993). BDV-G is localized mostly in the cell body, consistent with ER retention signals (Gonzalez-Dunia et al., 1997), and on neurite terminals (Bajramovic et al., 2003). Later work also showed that BDV-G interacts with a 78 kDa chaperone protein BiP, which co-localizes with the pre-synaptic marker PSD-95 (Honda et al., 2009). The localization of the viral glycoprotein specifically to neurite termini, with the nucleoprotein present throughout neurites (Bajramovic et al., 2003), suggests that budding of infectious virions occurs at neurite terminals where the glycoprotein is present, which happens to be near membrane appositions to nearby cells, namely synapses. This spread pattern would therefore be consistent with retrograde transsynaptic transmission between connected neurons.

A similar mode of transmission has been proposed for the measles virus (MV), which has been observed to spread between neurons in a specific, polarized fashion (Ehrengruber et al., 2002). In individuals suffering from the neurological disease subacute sclerosing panencephalitis (SSPE) caused by MV, despite the fact that measles protein and RNA can be detected within cells, little infectious virus can be recovered (Katz, 1995; Payne et al., 1969).

This runs in contrast to infections in non-neuronal tissues, where high titers of virus can be collected. In addition, the canonical MV receptor CD46 is not necessary for spread in neurons (Lawrence et al., 2000; McQuaid et al., 1998), suggesting that the virus uses a different mode of entry into neurons than for other cells. The authors suggested that MV viral assembly is abnormal in neurons and thus an alternate, cell-cell spread mechanism dominates.

Conclusion/future directions

The most likely explanation for transsynaptic transmission is that viruses are preferentially released from sites of budding and viral assembly, which likely occur at areas of rapid activity and associated with cargo transport. In neurons, these sites are the synapses. It may be that viral glycoproteins tend to be localized preferentially to synaptic sites, or that the sites of assembly of complete virions tend to be at synapses, or that the assembly and/or release of virus at non-synaptic sites produce non-productive infections. As the cellular machinery is designed to rapidly deliver cargo to and from synaptic terminals, virus is likely preferentially released from pre- or post-synaptic terminals, directly facing the opposing synaptic terminal. While the size of the chemical synapse is roughly 20 nm, the length of the wild-type RABV, HSV, or VSV particle is about 200 nm (Cureton et al., 2009; Savtchenko and Rusakov, 2007; Zhou et al., 2000). Thus, the budding virus would abut the opposing membrane long before completing exocytosis from the initial cell. Given the preference of RABV and VSV to bud in a direction perpendicular to the cell membrane (Orenstein et al., 1975; Wirblich et al., 2008), even at tripartite synapses in which glial cells are located very near to the synaptic cleft, the virus likely never physically encounters glial cells.

What appears as transsynaptic specificity likely is a combination of preferential release from cellular sites rich in transport and exocytosis machinery and the close apposition of synaptic membranes. Understanding how viruses transmit between neurons should provide valuable information both about basic mechanisms of viral transmission as well as the rational design of methods for analyzing neuronal connections.

The principle areas of optimization for future transsynaptic tracers are 1) Non-toxic, high efficiency tracers, 2) Controllable direction of spread, 3) Multiple, orthogonal tracing systems, 4) Spread across a controlled number of synaptic connections. Towards goal #1, efforts are ongoing in multiple labs to develop non-toxic transsynaptic tracers, thus far with modest success (Chatterjee et al., 2018; Ciabatti et al., 2017; Matsuyama et al., 2019). As discussed above, currently existing variants designed to reduce toxicity all have certain limitations. For goal #2, an anterograde monosynaptic tracer has been a major goal for the community for at least a decade. Though the first monosynaptic anterograde tracer was published in 2011 (Beier et al., 2011), this virus has thus far not been extensively employed *in vivo.* A monosynaptically-restricted HSV also was recently reported (Zeng et al., 2017), which is based on the conditional deletion of the essential thymidine kinase (TK) gene of HSV. However, both of these viruses are still highly cell-toxic, and the original infection of the monosynaptic HSV cannot at this point be targeted specifically to defined cell types, rather relying on the expression of Cre to initiate spread. Ideally, next generation tracers can be readily modulated to control the direction of viral transmission, depending on the desire

to label inputs, outputs, or both. For goal #3, the need for multiple orthogonal tracers is necessitated by superinfection exclusion, also known as homologous or heterologous interference, a well-characterized phenomenon whereby an initial infection with a virus prevents subsequent infection of the same cell by the same or a closely related viral strain (Bennett, 1953). This can prevent multiple viruses (for example, two recombinant RABV expressing different fluorophores) from infecting the same cell. However, this is not universally true, as multiple isogenic PRV variants with different fluorophores can infect the same cells (Hogue et al., 2018). Lastly, for goal #4, monosynaptically-restricted transsynaptic viruses are currently the most useful to the majority of neuroscientists as they can map the direct inputs or outputs located throughout the brain to a target population. However, definition of di- and tri-synaptic connections may be possible through the use of multiple viruses and multiple viral receptors (e.g., TVA, TVB) (Young, 1998) to restrict viral infection to targeted cell types.

Development of enhanced viral transsynaptic tracers will certainly play a vital role in continued efforts to further elucidate the functional connectome of the brain. Improved tracing systems will enable the integration of viral anatomical tracing with behavioral and electrophysiological analyses to investigate the function of specific connected pathways. They may also help to understand how inputs and outputs of defined populations work together to orchestrate complicated behavioral responses. The lessons learned from transsynaptic viruses will surely be invaluable as we continue the quest to elucidate the functional organization of the brain.

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Highlights

- Discuss what is known and unknown about the mechanisms of transsynaptic specificity
- Examine how viruses can label either inputs and/or outputs of starter neurons
- Highlight the pros and cons of different viral transsynaptic tracers
- Explore future directions for developing improved viral transsynaptic methods

Table 1:

Commonly used transsynaptic viruses.

Virus	Strengths	Limitations
PRV-Bartha	Retrograde-only virus. Can spread across multiple connections (e.g., current versions are not monosynaptic-restricted). Deletion of genomic segment promoting retrograde-only spread enables comparative studies to identify sources of directional specificity. Cre-dependent versions exist to initiate tracing from Cre-expressing cells. Multiple viruses with different fluorophores can infect the same cells. DNA genome enables interaction with recombinase technologies.	Rapidly cell toxic and causes cell lysis, infecting nearby non-synaptically connected neurons. No monosynaptically-restricted version yet available. Large genome difficult to manipulate.
HSV-129	Rapid gene expression in infected cells. Recent variants enable spread to initiate from recombinase-expressing cells. Mostly used as a polysynaptic virus, but recent variants can be used monosynaptically. DNA genome enables interaction with recombinase technologies.	Rapidly cell toxic and causes cell lysis, infecting nearby non-synaptically connected neurons. Exclusive anterograde specificity recently called into question. Large genome difficult to manipulate.
RABV	Retrograde transsynaptic only. Can be monosynaptic or polysynaptic. Small genome is easy to manipulate. Least toxic transsynaptic virus in use.	Only retrograde transmission is possible. Polysynaptic version is lethal in humans. RNA genome prohibits recombinase-induced modification in the brain. Though genome is small, rescue of virus from cDNA is low throughput.
VSV	Rapid gene expression in infected cells (~4-8 hours). Can be monosynaptic or polysynaptic, and can transmit retrogradely or anterogradely across synaptic connections. Small genome is easy to manipulate. RNA genome prohibits recombinase-induced modification in the brain.	Highly cell-toxic. Most recombinants do not transmit efficiently *in vivo*. Rescue of virus from cDNA is low throughput. Not as often used as PRV, HSV, or RABV.
AAV	Non-toxic, enables long-term gene expression in infected neurons. DNA genome enables interaction with recombinase technologies. AAVs containing a wide variety of transgenes are publicly available. Easy to purchase and grow.	Very high titers of virus required to enable transmission. Further validation needed to demonstrate transsynaptic specificity.