

Invasion of the Nervous System

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Abstract

In vertebrates, the nervous system (NS) is composed of a peripheral collection of neurons (the peripheral nervous system, PNS), a central set found in the brain and spinal cord (the central nervous system, CNS). The NS is protected by rather complicated multi-layer barriers that allow access to nutrients and facilitate contact with the peripheral tissues, but block entry of pathogens and toxins. Virus infections usually begin in peripheral tissues and if these barriers are weakened, they can spread into the PNS and more rarely into the CNS. Most viral infections of the NS are opportunistic or accidental pathogens that gain access via the bloodstream (e.g., HIV and various arboviruses). But a few have evolved to enter the NS efficiently by invading neurons directly and by exploiting neuronal cell biology (e.g., rhabdoviruses and alphaherpesviruses). Most NS infections are devastating and difficult to manage. Remarkably, the alphaherpesviruses (α -HVs) establish life-long quiescent infections in the PNS, with rare but often serious CNS pathology. In this review, we will focus on how α -HVs gain access to and spread in the NS, with particular emphasis on bidirectional transport and spread within and between neurons and neural circuits, which is regulated by complex viral-host protein interactions. Finally,

we will describe the wide use of α -HVs as tools to study nerve connectivity and function in animal models.

Introduction

Alphaherpesviruses (α -HVs) are large, enveloped viruses with double-stranded, linear DNA genomes that are tightly packed in an icosahedral protein shell termed capsid (Dai and Zhou, 2018). The virions contain a characteristic layer between the envelope and the capsid, termed tegument, that is composed of several viral and host proteins as well as some mRNAs (Kramer et al., 2011; Lippe, 2014; Sciortino et al., 2001). The tegument layer is divided into the capsid-associated inner and the envelope-associated outer layers (Owen et al., 2015). α -HV infections (e.g. herpes simplex virus 1 and 2 [HSV-1 and HSV-2] and varicella zoster virus [VZV]) are among the most common viral infections in the world (Looker et al., 2015a; Looker et al., 2015b; Moffat et al., 2007). Many alphaherpesviruses are species specific, meaning that they primarily infect a single host (e.g., VZV infects humans; bovine herpesvirus type-1 [BHV-1], cattle; Marek's disease virus [MDV], chickens). However, there are also examples of α -HVs with a broader host range. Pseudorabies virus (PRV) is a swine α -HV that can cause zoonotic infections in most mammals except higher primates. HSV-1 is a human α -HV but can also infect many small laboratory animals. Both PRV and HSV-1 grow well in tissue culture yielding high titer cell-free virus particles, and their genomes have been cloned into bacterial artificial chromosomes (BAC) amenable to generate new recombinants. As a result, these two viruses have been studied widely as prototype α -HVs in cultured cells and in animal models.

The life cycle of an α -HV involves a productive infection cycle in mucosal epithelial cells followed by a life-long quiescent (often called latent) state in the peripheral nervous system (PNS) neurons that innervate the infected mucosal tissue. Generally, mucosal surfaces (e.g., nasopharyngeal cavity, genitals, and eyes) are common sites of primary infection. The productive infection proceeds in most susceptible and permissive cell types, in a well characterized cascade fashion where viral immediate early (IE) proteins interact with host cell proteins

and activate transcription of early (E) viral genes (Honest and Roizman, 1974). These E proteins are required to create the optimal environment for viral DNA replication and subsequent synthesis of late (L) viral proteins, the structural components of the virions (Honest and Roizman, 1974; Roizman and Whitley, 2013). This cascade of viral gene expression results in the rapid production of large numbers of progeny virions ready to spread to other tissues and hosts. Because epithelial tissues are highly innervated by sensory neurons, progeny viral particles typically first enter the axons of these neurons. Next, viral particles move efficiently along axons and reach the neuronal cell bodies (i.e., soma), which are tightly packed within the structures called the ganglia where they are typically linked by synapses. The ganglia of the PNS contain neurons either responsible for processing sensory information (sensory neurons) or regulating the function of internal organs (autonomic neurons). Sensory neurons within dorsal root ganglia (DRG), trigeminal ganglia (TG) and sacral ganglia are the primary sites of quiescent HSV infections (Croen et al., 1988; Mitchell et al., 2003). However, neurons of autonomic sympathetic ganglia that innervate peripheral tissues such as superior cervical ganglia (SCG) also harbor quiescent viral genomes after infection of peripheral tissues (Cheung, 1989; Enquist et al., 2002; Jones, 2003; Richter et al., 2009).

Neurons are terminally differentiated and extremely polarized. The neuronal cell body contains the nucleus with all the cellular transcription machinery, but neurons also have highly specialized extensions of their cell body cytoplasm called dendrites and axons (Figure 1). Dendrites can be branched and make connections (synapses) with axons from other neurons. Single axons can extend as long as meters, which is many thousands of times the diameter of a typical cell. The maintenance and the communication of distal axons with their cell bodies require specialized signal transduction, intracellular sorting, and trafficking systems. As obligate intracellular parasites, viruses are dependent on these cellular functions that often are cell-type specific.

After the productive infection in epithelia, when the viral progeny invade axons of the peripheral neurons, long-distance transport of viral particles to the cell

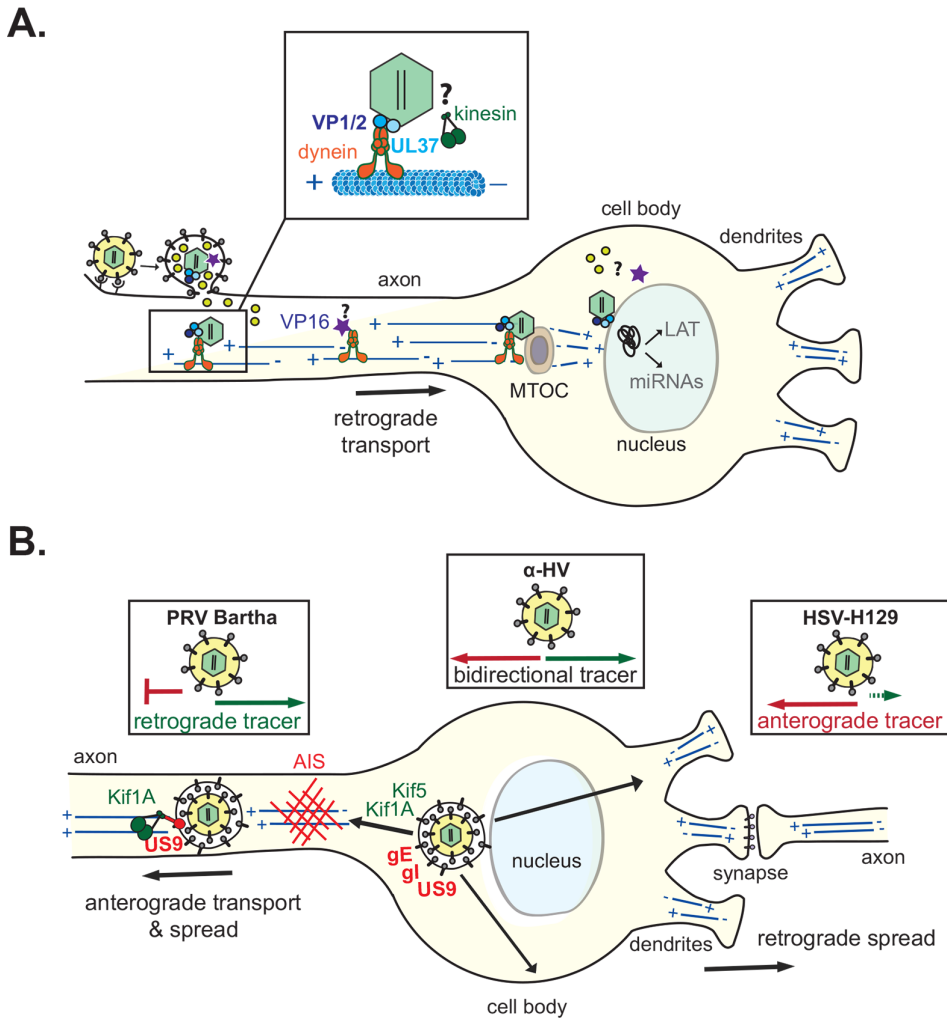


Figure 1. α -HV entry and spread in the nervous system. (A) α -HV particles enter neurons by direct fusion of their virion envelopes with the plasma membrane. Capsids with inner tegument proteins (e.g. VP1/2, UL37 and US3, blue circles) engage microtubule motors (dyneins and kinesins) for retrograde transport toward the neuronal nucleus. Microtubules in axons are unipolar; minus-ends are directed toward the microtubule organizing center, MTOC in the cell body. Somato-dendritic microtubules have mixed polarity. The interaction of UL37 and VP1/2 may regulate the recruitment of dynein vs. kinesin motors by VP1/2 protein. The outer tegument proteins (yellow circles) including the transactivator protein VP16 (purple star) are not co-transported with the capsid-dynein complexes. This might bias the infection mode in the neuronal nucleus toward latency that is characterized by the limited expression of the viral genome (the latency associated transcript (LAT) and micro RNAs are expressed). **(B)** Kinesin motors (e.g. Kif1A and Kif5) mediate transport of α -HV virions contained within a vesicle to the cell periphery (highlighted with arrows). Interaction of viral envelope proteins (e.g. US9, gE and gI) with specific kinesin

motors is required to pass the axon initial segment (AIS) to sort the virion carrying vesicles into the axon. Kif1A is represented in the axon interacting with PRV envelope protein US9, though other kinesin motors may be involved in different stages of herpesvirus egress. α -HVs have bidirectional spread capacity in the nervous system (both anterograde and retrograde). PRV Bartha (lacking gE, gI, US9 genes) virions can only spread retrogradely between synaptically connected neurons. HSV-1 H129 strain spreads predominantly in the anterograde direction. Viral genomes do not encode genes for microtubule motor proteins. Therefore, nucleocapsids that enter axons or cell bodies must recruit the host cell dynein complex for efficient retrograde transport of the capsid complex (capsids plus inner tegument proteins) to the nucleus. The inner tegument proteins that remain associated and co-transport with the nucleocapsids are VP1/2 (aka UL36), UL37, and US3 (Granzow et al., 2005; Luxton et al., 2005). They mediate the loading of viral 'cargo' onto cellular dynein complexes both in neuronal and non-neuronal cells (Antinone and Smith, 2010; Buch et al., 2017; Coller and Smith, 2008; Luxton et al., 2005; Zaichick et al., 2013). This interaction represents a potential bottleneck in the establishment of neuronal infection because only a finite number of dynein motors and adapters are found in axons.

body nucleus - termed retrograde - occurs on microtubules and relies on the interaction of viral inner tegument proteins with the cellular motor dynein (Zaichick et al., 2013). By contrast, the outer tegument proteins are not co-transported toward the nucleus, presumably because they are not associated with capsids post-entry (Antinone et al., 2006; Luxton et al., 2005; Smith, 2012). After the capsids surrounded by the inner tegument proteins reach the nucleus, the genomes are released into the nucleus and the specialized quiescent, or latent, mode of infection initiates in the native host. During this latent phase, α -HV genomes are silenced: they are circularized and decorated with silencing nucleosomes containing histone modifications that allow transcription of only a small segment of the viral genome, particularly the latency-associated transcript (LAT) (Jarman et al., 1999; Jones, 2003; Kennedy et al., 2015).

To be sustained in a host population, the silenced viral genomes must retain the capacity to re-initiate the productive cycle (i.e., reactivate). Reactivation is usually triggered when the immunocompetent host experiences stress such as physical trauma, sunburn or fever (Cliffe and Wilson, 2016; Roizman and Whitley, 2013; Wilson and Mohr, 2012). When stress signaling pathways are activated, transcription of viral genes and replication of the viral genome

occurs, and new viral particles are produced. These move back from the cell body nucleus along the axon in the anterograde direction (the opposite direction relative to that during initial infection) to the site of the original infection to ensure the spread of infection to other hosts (Smith, 2012). Surprisingly, despite the general global effect of stress on host cells, the threshold for reactivation of latently infected PNS neurons is high such that reactivation does not occur in all the neurons that harbor silenced viral genomes. Reactivation may be more likely to occur in neurons within the same ganglia that contain more copies of the silenced viral genome (Sawtell et al., 1998). Even within a single PNS nucleus, not all silenced genomes reactivate at the same time (Catez et al., 2012; Lomonte, 2017; Maroui et al., 2016; Sawtell, 1998). Currently, host and viral factors that affect the maintenance of latency and subsequent reactivation are not completely understood.

In people with healthy immune systems, reactivations are typically well controlled, and symptoms are mild, with local cold sores, genital lesions, or shingles blisters being typical manifestations. Asymptomatic reactivation and shedding of HSV-1 (Miller and Danaher, 2008) and VZV (Amlie-Lefond and Gilden, 2016; Gilden et al., 2016) are also known. However, in rare cases or in immunocompromised hosts, reactivations may result in less well contained lesions leading to oral or genital ulcerations, or spread to other locations such as eyes causing keratitis or blindness (depending on the ganglia where latency is established), and even CNS, causing encephalitis (Gnann and Whitley, 2017; Tsatsos et al., 2016). HSV-2 reactivations tend to be more frequent and do not always present as lesions. Such asymptomatic shedding of HSV-2 is common (Bertke et al., 2013; Groves, 2016). A rare but serious complication of HSV-2 (or HSV-1) shedding in the genital tract is neonatal HSV infections, which are estimated to occur in 10 of 100,000 live births globally (Looker et al., 2017). Neonatal HSV infections can spread to the CNS or other organs causing disseminated disease, which is fatal in approximately 60% of untreated cases (Corey and Wald, 2009). In rare cases, reactivating virus particles spread to the CNS, causing seizures and focal necrosis (Gilden et al., 2016; Whitley, 2015). Control of latency, reactivation, and subsequent spread

of infection is affected by many cell intrinsic, tissue-specific and systemic factors that are challenging to dissect.

The mammalian nervous system, despite its vital importance in host survival, is well exploited by α -HVs. In this review, we will focus on how α -HV particles efficiently invade the nervous system and spread among the connected neurons, and how these properties not only lead to neuropathology, but also make α -HVs useful tools for studying the nervous system circuitry and function (Table 1).

I. Invasion of the NS: Intraneuronal Spread

Entry into axons and retrograde transport toward the neuronal nucleus

Most neurotropic virions enter neurons by endocytosis, using cellular receptors that are concentrated at nerve terminals. However, α -HV virions generally enter neurons by direct fusion of their lipid envelopes with the plasma membrane (Connolly et al., 2011; Heldwein and Krummenacher, 2008), although some examples of α -HV endocytosis have been shown (Mues et al., 2015). Nectin-1, a member of the immunoglobulin (Ig) superfamily, is a receptor for HSV and PRV virions on many cells including neurons (Kopp et al., 2009; Shukla et al., 2000; Spear et al., 2000). Binding of the conserved viral glycoprotein D (gD) on the virion surface to nectin-1 initiates the entry process (Geraghty et al., 2000; Shukla et al., 2000). The concerted action of the glycoprotein complex gH/gL and the viral fusogen gB is required for the efficient fusion of the viral envelope with the cellular membrane (Heldwein and Krummenacher, 2008). Upon membrane fusion, the capsid with inner tegument proteins is deposited into the axonal cytosol, ready for subsequent retrograde microtubule-dependent intracellular transport toward the neuronal nucleus (Figure 1A).

Microtubule networks, composed of α -tubulin and β -tubulin heterodimers, are arrays of polar cylinders that provide tracks for the long-distance transport of endocytic and exocytic vesicles, organelles, and chromosomes (Desai and Mitchison, 1997). Microtubules have a dynamic 'plus-end' that can grow and shrink rapidly while the 'minus-end' is often tethered to a microtubule-

organizing center (MTOC) and is relatively stable (Luders and Stearns, 2007). Microtubules in axons have uniform polarity, and minus-ends are always directed toward the MTOC in the cell body (Conde and Caceres, 2009). On the other hand, microtubules in somato-dendritic regions of neurons have mixed polarity. Despite being nucleated by the MTOC, many of the axonal microtubules are no longer connected to the MTOC. They are distributed along the axon to form discontinuous, overlapping bundles.

Directional transport along microtubules is mediated by two classes of molecular motors (Hirokawa et al., 2010; Vale, 2003). Plus-end-directed motors are known as kinesins and they are involved in transport to the cell periphery (Kikkawa et al., 1995; Okada et al., 1995). Conventional kinesin is a heterotetramer composed of two heavy chains and two light chains. These motors are highly processive and relatively powerful, capable of moving large cargo complexes. Minus-end transport is mediated by cytoplasmic dynein that directs traffic from the periphery to the cell center (Kardon and Vale, 2009). This complex motor protein consists of two heavy chains, two intermediate chains, and multiple light intermediate and light chains. Cytoplasmic dynein function is mainly regulated by dynactin and Lis1/nudE complexes (DeSantis et al., 2017; Dohner et al., 2002; McKenney et al., 2011; Reck-Peterson et al., 2018).

Live-cell imaging experiments examining the retrograde transport of monomeric red-fluorescent protein-tagged PRV capsids and green-fluorescent protein (GFP)-tagged tegument proteins in sensory neurons revealed that outer tegument proteins, such as UL47 (VP13/14), UL48 (VP16), and UL49 (VP22), were lost during viral entry and did not move along with capsids to the nucleus (Antinone et al., 2006; Antinone and Smith, 2010; Luxton et al., 2005). In contrast, the inner tegument proteins UL36 and UL37 remained associated with capsids as they entered neurons, trafficked to the nucleus, and subsequently docked at the nuclear rim. UL36 is the largest herpesvirus protein that directly interacts with the capsid protein UL25 and tegument protein UL37 (Bottcher et al., 2008; Collier et al., 2007; Klupp et al., 2002; Lee

et al., 2009). Zaichick et al., showed that a proline-rich domain near the carboxy terminus of UL36 mediates the interaction of incoming PRV capsids with dynein/dynactin (Zaichick et al., 2013). In a different set of experiments using isolated HSV-1 capsids with varying tegument protein compositions, inner tegument proteins recruited dynein motors onto incoming HSV-1 particles. Radtke et al. demonstrated that individual HSV-1 capsids with these inner tegument proteins can also recruit kinesin motors (Radtke et al., 2010). Indeed, bi-directional motility of incoming capsid in axons was detected, albeit much less frequently than the dominating retrograde motion (Scherer et al., 2016; Smith et al., 2004). This raises the question: how is the processive retrograde transport of capsids achieved early in infection if both kinesin and dynein motors are simultaneously recruited by inner tegument proteins?

Recently, UL37 has been identified as the viral protein promoting the retrograde-biased motion of both PRV and HSV-1 incoming capsids (Richards et al., 2017). Mutations in the R2 region of UL37 abrogated nervous system invasion by both PRV and HSV-1 in animal models without affecting viral replication in epithelial cells. Since UL37 has not yet been reported to directly interact with the molecular motors, the interaction of UL37 and UL36 may regulate the recruitment of dynein motors by UL36 protein (Klupp et al., 2002; Richards et al., 2017) (Figure 1A). The fact that the UL37 R2 mutation blocks neuroinvasion by two different α -HVs while not affecting viral propagation in epithelial cells, and that robust replication of this mutant in peripheral tissues allowed mice to mount an adaptive immune response against HSV-1, further prompted the idea of using this mutant as a live-attenuated vaccine candidate for HSV-1 and HSV-2 (Richards et al., 2017). Moreover, the progeny of this mutant virus is unable to invade the nervous system, reducing the possibility of latency and reactivation.

Since α -HVs are pantropic and can infect various cell types with mixed or unipolar microtubule polarity, perhaps, the inner tegument proteins evolved the ability to recruit both minus- and plus-end directed motors. These motors may engage in a tug-of-war to determine the direction of net transport. Alternatively,

their action may be regulated by host or viral kinases (e.g., US3) or other viral tegument proteins (e.g., UL36 and UL37). The dual recruitment of kinesin and dynein motors and regulation of their activity might also coordinate the nuclear delivery of viral capsids from MTOC to the nuclear pore complexes in the soma (Dohner et al., 2002) (Figure 1A).

Local Control of Retrograde Infection

How do viral particles reach the neuronal cell bodies to establish productive infection after axonal invasion? The answer involves events in the initial virion-host interaction and in the way viral proteins engage cellular machineries. For example, when isolated axons in compartmented neuronal chambers are infected with a large number of PRV virions, translation of local axonal mRNAs is stimulated. This leads to local synthesis of several axonal proteins (Koyuncu et al., 2013b). When axonal protein synthesis is inhibited by cycloheximide or emetine, the efficiency of the retrograde capsid transport is reduced. Some of these newly made neuronal proteins, including a dynein regulator, Lis1, are indeed crucial for efficient virus transport toward the cell bodies (Koyuncu et al., 2013b).

Induction of new protein synthesis in neuronal axons and dendrites has been a focus of research and debate in neurobiology since the early 2000s (Jung et al., 2012). It is now known that in PNS neurons, axonal protein synthesis is essential for growth cone navigation, damage repair, and communication with the distant cell body. Some of the proteins synthesized locally engage signaling molecules transported to the cell body where they act as messengers to alarm cell bodies in case of distal axonal injury (Hanz et al., 2003; Perry et al., 2012).

Interestingly, when isolated axons are infected with a large number of PRV virions, a mechanism similar to the axonal damage response is triggered. Both injury and infection activate local protein synthesis and fast transport of signaling molecules toward neuronal nuclei. In the case of PRV axonal infection, such action leads to more efficient transport and subsequent productive infection in cultured neurons (Koyuncu et al., 2013b). If the infecting

viral particle amount is below a certain threshold (below 10^3 plaque forming units [pfu] for PRV), stimulation of local protein synthesis and retrograde injury signaling is poor resulting in silenced (quiescent) infection in the neuronal nuclei (Koyuncu et al., 2015).

As there is a minimum threshold for efficient retrograde transport in axons and initiation of productive infection in the soma, there is also a limit in the number of viral particles that can be transported in axons due to the availability of host proteins. If too many particles are invading axons (e.g. 10^6 pfu and above), only a fraction of the viral capsids will reach the neuronal nuclei to start infection (Koyuncu et al., 2015). When isolated axons of SCG neurons in compartmented chambers were co-infected with 10^4 pfu PRV and 10^6 pfu of UV-inactivated-PRV, the competition for the host machinery and excess amounts of inactivated particles interfered with the retrograde transport of replication-competent PRV and led to a quiescent infection in the neuronal nuclei. (Koyuncu et al., 2017; Koyuncu et al., 2015). These experiments clearly show that the number of viral particles infecting axons and reaching the neuronal soma directly affects whether the infection will be productive or quiescent.

In a natural α -HV infection, axons innervating the peripheral epithelia are exposed to the inflammatory milieu of infected epithelial cells even before the onset of neuronal invasion by the progeny virions. Because PNS neurons do not divide and are irreplaceable, they must have a high threshold for apoptosis or other modes of cell death that nonneuronal cells often use for antiviral defense. How does this complicated environment rich in cytokines affect axonal infection, particle transport, and productive infection thresholds? Song et al. investigated these questions by treating isolated axons in compartmented chambers with type I (α/β) or II (γ) interferon (IFN) before infecting them with PRV or HSV-1 (Song et al., 2016). These studies revealed that when axons were treated with either IFN β or IFN γ , the efficiency of PRV and HSV-1 axonal transport was reduced by two different modes of local interferon responses in axons. Exposure of axons to IFN γ induced transcription of antiviral genes in

the distant neuronal nuclei to further block viral replication. By contrast, treatment of axons with IFN β did not activate transcription of antiviral genes in the cell bodies but instead activated STAT1 phosphorylation in axons by an unknown kinase (Song et al., 2016). These local antiviral responses in axons limited but did not completely block capsid transport to the neuronal nucleus. Since the productive infection of SCG neurons requires that a high number of capsids reach the cell body, the local cytokine response in axons may contribute to the establishment of a latent infection in the ganglia cell bodies by interfering with particle transport resulting in a lower number of capsids reaching the nuclei.

Besides affecting retrograde transport in axons, both type I and type II IFNs limit replication and spread of α -HVs in many cells by inducing the transcription of a multitude of interferon-stimulated genes (ISGs) (Schoggins, 2014). In the sensory ganglia of latently infected mice, interferons and other cytokines secreted by infiltrating T-cells, contribute to the establishment and maintenance of latency (Enquist and Leib, 2017; Leib et al., 1999; Mikloska and Cunningham, 2001; St Leger and Hendricks, 2011). Using porcine dissociated TG neurons cultured in a two-compartment setup, De Regge et al. demonstrated that pretreatment of neuronal cell bodies with IFN α suppressed productive infection of axonally infecting α -HV leading to a stably silenced infection similar to latency (De Regge et al., 2010). Not only do IFN β and IFN γ promote establishment of latency, but they also block HSV-1 reactivation by interfering with an early step in the process (Linderman et al., 2017). Interference is an early event because both types of interferon fail to block reactivation when they are introduced at a later phase. Recently, type III interferons (interferon lambda; IFN λ) have been shown to restrict both HSV-1 and HSV-2 replication by inducing the JAK/STAT pathway and ISG expression (Li et al., 2011; Li et al., 2017).

Productive vs. Quiescent: Decision-making step in the neuronal nucleus

Axonal biology and long-distance transport of capsids influence the infection mode of α -HVs in neurons. Several bottlenecks (e.g. long-distance transport in

axons) or thresholds (e.g., enough virus particles to induce injury signaling, protein synthesis and to engage retrograde transport machinery in axons) must be overcome to reach and establish infection in the neuronal nucleus (Koyuncu et al., 2018). This long sequence of events leading to the viral latency in the neuronal nucleus is challenging to dissect due to the difficulty of monitoring early events. The combined use of cell culture models along with *in vivo* animal models revealed that the decision to either replicate lytically or enter latency depends on outer tegument proteins, particularly on the fate of the VP16 (aka UL48) protein. VP16 interacts with host transcription factors to activate viral gene expression and thus to initiate productive infection (Campbell et al., 1984; Wilson et al., 1993; Wysocka and Herr, 2003). However, being an outer tegument protein, VP16 is not co-transported with capsids to the neuronal nucleus. Only a few retrogradely trafficking HSV-1 capsids co-localized with VP16 in axons, and no co-transport was observed during retrograde PRV capsid transport (Antinone et al., 2006; Antinone and Smith, 2010). It is still unclear whether VP16 in the tegument undergoes retrograde transport in axons, alone or with other outer tegument proteins, or not (Figure 1A). Nevertheless, if outer tegument proteins are transported separately from the viral capsids in axons, their asynchronous arrival at the cell bodies could bias the infection mode toward latency (Hafezi et al., 2012; Roizman and Sears, 1987).

By using an organotypic model established by culturing chicken trigeminal ganglia explants (TGE) in a double chamber where the two compartments are separated by a cloning cylinder ring, Hafezi et al., tested the long-standing hypothesis of separate long-distance transport of capsids and outer tegument proteins in axons leading to a quiescent infection (Hafezi et al., 2012). When the ganglionic compartment was infected with HSV-1, a productive infection was observed. However, when axons were infected with HSV-1, a quiescent infection was established in a small number of neurons. These neurons expressed the latency associate transcript (LAT) characteristic of a latent infection. These data clearly showed that axonal delivery of α -HV particles,

favors establishment of a quiescent state of infection in the ganglia (Hafezi et al., 2012; Roizman and Sears, 1987).

By using SCG neurons grown in compartmented chambers that have three compartments (tri-chambers), Koyuncu et al. showed that axonally infecting low dose PRV also establishes quiescent infections that can be reactivated (Koyuncu et al., 2015). Using this model, they further uncovered two separate pathways that can trigger a shift in the mode of neuronal infection from silenced to productive: a slow, stress-mediated pathway and a fast, viral tegument-mediated pathway (Koyuncu et al., 2017). The stress-mediated pathway involves elevated cyclic AMP (cAMP), which subsequently activates protein kinase A (PKA) in the neuronal soma. If PKA was activated in the neuronal cell bodies at the same time that axons were infected with PRV at a pfu that normally results in a quiescent infection, viral genomes were not silenced. This effect was dependent on the activity of PKA and the stress signaling kinase, cJun N-terminal kinase (JNK) (Koyuncu et al., 2017). Remarkably, exposure of cell bodies to viral light particles that contain viral tegument proteins, but lack capsids, was sufficient to rapidly bypass the silencing of axonally infecting PRV genomes, which was not dependent on active PKA or JNK. This finding suggested that tegument-stimulated escape from silencing did not involve a stress activated escape pathway (Koyuncu et al., 2018; Koyuncu et al., 2017).

Anterograde axonal sorting and transport

α -HVs are unique in their capacity to spread in a bidirectional fashion between chains of synaptically connected neurons (Figure 1B). Both post-entry retrograde transport and post-replication anterograde transport, are highly regulated processes relying on different host microtubule-dependent motors rather than on free diffusion (Enquist, 2012; Koyuncu et al., 2013a; Miranda-Saksena et al., 2018; Radtke et al., 2010; Smith, 2012). While viral replication and capsid assembly occur in the nucleus, final tegument acquisition, followed by secondary envelopment at trans-Golgi-like membranes, yields mature virions in cytoplasmic vesicles (Bigalke and Heldwein, 2016; Ibáñez et al.,

2018; Smith, 2012). The direction taken by virion and virion components after neuronal infection, as well as the degree of spread within and between connected neurons, dictates the difference between a latent, reactivating PNS infection and a lethal CNS infection.

Post-replication anterograde transport occurs after episodic reactivation from latency. It is important to separate two distinct events that dictate direction of spread: sorting into axons and subsequent anterograde transport of viral cargo to axonal egress sites. For virion or virion components to be sorted into axons, they need to cross the axon initial segment (AIS). The AIS is a mesh of actin filaments that act like a selective gate, preventing physiological cargo such as transport vesicles from entering the distal axon in a non-specific fashion. Certain microtubule-dependent motors belonging to the kinesin-1 and kinesin-3 families are able to penetrate the AIS and they may provide axonal sorting specificity (Letierrier, 2018).

A controversy exists as to whether α -HVs assemble mature virions in vesicles in the cell body before axonal sorting and transport (married model), or if virion components and subassemblies are sorted independently and then transported to distant sites such as axon termini to complete assembly prior to egress (separate model) (Buch et al., 2017). For PRV, sufficient evidence supports the married model. For HSV-1, some data supports married, separated, and even a mixed model combining the elements of the first two models; however, recent research presents stronger evidence for the married model for HSV-1 as well. This debate has been detailed elsewhere (Antinone et al., 2010; Kratchmarov et al., 2012; Miranda-Saksena et al., 2000; Miranda-Saksena et al., 2009; Negatsch et al., 2010; Snyder et al., 2006; Taylor et al., 2012; Wisner et al., 2011). Regardless of the precise site of virion assembly in neurons, α -HVs display a highly regulated mechanism to sort and transport virion components in axons, facilitating the spread from presynaptic to postsynaptic neurons (Card et al., 1990; Holland et al., 1999; Salinas et al., 2010; Smith, 2012; Smith et al., 2001) (Figure 1B).

For HSV-1 and PRV, three conserved viral proteins located in the viral envelope, gI, gE, and US9, are involved in axonal sorting. Although the mechanism of axonal sorting is not fully understood, gI, gE and US9 could potentially change the balance of microtubule-dependent motor recruitment to increase the yield of viral sorting into axons. Mutant viruses lacking these proteins are unable to sort viral cargo into axons, and infection cannot spread to post-synaptically connected neurons (Awasthi and Friedman, 2016; Brideau et al., 2000; DuRaine et al., 2017; Howard et al., 2013; Husak et al., 2000; Kratchmarov et al., 2013; Polcicova et al., 2005; Snyder et al., 2008). In PRV, US9-null mutants have a strong defect in axonal sorting of virion components (Brideau et al., 2000; Lyman et al., 2007), but these mutants have no defects either in retrograde axonal transport or during infection of non-polarized cells. More recently, Daniel et al., reaffirmed that US9 is necessary for sorting viral particles into axons, but dispensable for subsequent axonal transport of particles from the proximal axon to terminals (Daniel et al., 2015). Kramer et al., showed that PRV US9 is co-transported with a microtubule-dependent motor from the kinesin-3 family, called KIF1A, which is involved in axonal sorting and transport of physiological cargo in uninfected neurons. Moreover, they showed that overexpression or disruption of KIF1A function increases or decreases capsid transport, respectively (Kramer et al., 2012). Interestingly, KIF1A was detected to be specifically targeted by the viral anterograde sorting complex for accelerated proteasomal degradation in the late phase of PRV infection (Huang et al., 2020). For HSV-1 and HSV-2, the specific roles of gI, gE and US9 in the mechanism of axonal sorting, transport and anterograde spread are still under investigation. For HSV-1, Diefenbach et al., reported that the basic domain of US9 can recruit KIF1A to facilitate egress from neurons (Diefenbach et al., 2016). Another related non-essential protein called UL56, which like US9 is a type-II membrane protein, has been shown to be a virulence factor in α -HVs (Berkowitz et al., 1994; Daniel et al., 2016). While in HSV-2, UL56 appears to associate with KIF1A (Koshizuka et al., 2005), in PRV, unlike US9, UL56 does not contribute to axonal sorting (Daniel et al., 2016). More recently, DuRaine et al., reported that motor proteins from the kinesin-1 family such as KIF5A, -5B, and -5C but not kinesin-3 proteins KIF1A and

KIF1B, promote anterograde transport of HSV-1 in axons (DuRaine et al., 2018). It is plausible to speculate that KIF1A could be essential for axonal sorting of viral components and KIF5 could play a role in axonal transport of virions. For VZV, gI and gE are critical neurovirulence factors and gI, in particular, is essential for viral spread in DRG and facilitating axonal localization of structural virion components (Christensen et al., 2013). The mechanism of axonal sorting and anterograde transport of α -HVs is not fully understood and still under ongoing investigation. It is clear that US9, gI and gE are important effector proteins but their specific role and relevance might be different among species.

II. Interneuronal spread

Neuroinvasion, neurovirulence and disease phenotype

We define a neuroinvasive virus as one with the capacity to invade the nervous system after infection in the periphery of the host. Many viruses can infect and spread within neurons after direct inoculation in the CNS, but these viruses often are not naturally neuroinvasive. By this definition, α -HVs are neuroinvasive. Although in the natural host they usually cause mild symptoms or are asymptomatic, they can cause serious disease if spread of infection is not contained by host defenses.

Neurovirulence is the degree of pathogenesis observed in the host nervous system after neuroinvasion. To be neurovirulent, a virus has to be neuroinvasive. Neurovirulence can range from extreme morbidity and mortality to more subtle effects on neuronal function and host behavior. The degree of neurovirulence depends on the interaction of viral gene products with host defensive actions. Mutant viruses that are neuroinvasive but have reduced virulence, not only help define critical viral gene products promoting pathogenesis but can also be used to study the nervous system (e.g., as neuronal circuit tracers), as we will discuss in section III. Several viral gene products define the neurovirulence range of α -HV strains in animal models, from highly attenuated to extremely neurovirulent and lethal. The identification of viral genes affecting neuroinvasion and neurovirulence properties of α -HVs

has been studied primarily in various susceptible animal models using different routes of infection (Card and Enquist, 1995; Enquist et al., 1998). These models have been essential to dissect the interplay between virus replication and spread and the host's defenses.

Attenuated mutant viruses have been used as vaccines candidates, gene delivery vectors for gene therapy, and for oncolytic virotherapy. There is an extensive list of neuroinvasion and neurovirulence genes encoded by α -HVs including but not limited to RL1 (ICP34.5), UL34 (thymidine kinase), UL36 (VP1/2), UL37, UL41 (VHS), UL56, and US12 (ICP47) (Brandt et al., 2003; Chou et al., 1990; Enquist et al., 1998; Kolb et al., 2016; Lee et al., 2009; Richards et al., 2017; Rosen-Wolff et al., 1991).

Paradoxically, α -HV infections in natural hosts are normally highly neuroinvasive but with low neurovirulence. Under some conditions or in different host species, neurovirulence can be extreme. Effective intrinsic and innate immune responses are paramount to contain viral spread to the site of primary infection. If the infection is not contained quickly, it can spread to capillary beds and lymphatic organs with the risk of further viral dissemination. Cellular pattern recognition receptors (PRR) such as Toll-like receptors (TLR) and pathogen-associated molecular patterns (PAMPs), can readily detect viral components after cell attachment and entry, triggering the production of many antiviral proteins and cytokines such as IFN- α/β (Shayakhmetov et al., 2010). The action of these cytokines in cells that have appropriate receptors usually causes cell death.

If this intrinsic response in epithelial cells is insufficient to limit viral replication, the innate immune response will be amplified leading to the production of more cytokines, complement, natural killer cells (NK), neutrophils and additional granulocytes. There is a fine balance between an appropriate immune response for efficient control of viral replication and spread, and an uncontrolled innate response that eventually can cause more damage than the acute viral infection, often described as a "cytokine storm" (Enquist et al., 1998;

Koyuncu et al., 2013a). Both the virus and the natural host have co-evolved to balance offensive and defensive actions. The fact that α -HVs can establish life-long, reactivatable infections, demonstrate that they establish sophisticated antiviral responses that can be both tissue specific and time-dependent. At least five viral proteins can modulate the host immune response to favor a proviral rather than an antiviral state: ICP0 for example, can target interferon-inducible protein 16 (IFI16), a host viral DNA sensor for degradation. Viral proteins ICP34.5, ICP27, VHS, and US3, can directly or indirectly inhibit the host proinflammatory nuclear factor kappa-light-chain-enhancer of activated B cell (NF- κ B) antiviral response (Knipe, 2015; Ma and He, 2014).

Two Modes of PRV Pathogenesis and the Role of Immune Response

α -HVs are pantropic and neuroinvasive (Goodpasture and Teague, 1923a, b). Once in the nervous system, infection spreads among synaptically connected neurons over long distances. The extent and direction of such spread determines the degree of α -HV pathogenesis. PRV has served as a model for studying the interneuronal spread of infection and pathogenic consequences of α -HV infections in animal models. Virulent PRV infection (e.g., PRV Becker) produces a severe peripheral neuropathy leading to pruritus and self-mutilation (known as the "mad itch"), as well as loss of motor coordination and ataxia. Infection of non-natural hosts with virulent PRV is lethal (Laval and Enquist, 2020; Pomeranz et al., 2005). PRV Bartha is a vaccine strain obtained by extensive passaging in the laboratory and is highly attenuated, producing minimal symptoms (Lomniczi et al., 1984; Mettenleiter et al., 1985).

Brittle, Reynolds and Enquist, employed a mouse flank infection model in which the infection is initiated in epithelial tissue, followed by spread of infection to the PNS, with the potential for further progression into the CNS. This model facilitated comparison of the spread and pathogenesis of the virulent PRV strain with the attenuated PRV strain, Bartha (Brittle et al., 2004). The PRV Bartha genome harbors a deletion in the unique short (US) region that removes the gE, gI and US9 genes- all required for sorting virions into

axons (Bartha, 1961; Szpara et al., 2011). Virulent PRV infection of a single site on the mouse flank results in frantic scratching and biting of the skin at the inoculation site, as well as the corresponding dermatome (pruritus). These mice progressively became weaker and died in approximately 3 days with no detectable behavioral abnormalities indicative of CNS pathology. In contrast, mice inoculated with PRV Bartha showed virtually no symptoms (Brittle et al., 2004). These animals survived nearly three times longer than those infected with wild-type PRV. Interestingly, they neither showed any signs of pruritus, nor did they develop skin lesions, despite viral replication in the skin and corresponding ganglia. However, late in infection (6 days), PRV Bartha-infected mice developed severe neurological abnormalities such as ataxia and died approximately in 10 days with abundant virus in brain tissue. This model, which emulates a naturally occurring α -HV infection route, identified two distinct modes of pathogenesis and lethality depending on the virulence (the extent of disease) and invasiveness (the spread of infection) of the infecting virus strain. Importantly, these differences between PRV Becker and PRV Bartha reflected the functions of specific PRV-encoded genes essential for pathogenesis. However, the specific pathophysiological and immunological processes leading to the development of the characteristic neuropathic itch and the quick death of the PRV Becker infected animals remained unclear.

To elucidate the mechanism of pruritus, Granstedt et al., used an *ex-vivo* live-cell imaging of infected submandibular ganglia (SMG) and showed that PRV Becker infection, but not PRV Bartha infection, travels back-and-forth from the salivary glands and the PNS neurons innervating the gland (Granstedt et al., 2013a; Granstedt et al., 2013b). This 'round-trip' infection leads to a massive infection of peripheral neurons, which may be responsible for the severe peripheral neuropathy induced by virulent PRV but not attenuated strains (e.g. PRV Bartha). Furthermore, PRV Becker-infected neurons exhibited increased action potential (AP) rates and also became electrically coupled, which means firing synchronously (McCarthy et al., 2009). When neurons were infected with a PRV Becker mutant that could not express glycoprotein B (gB; required for membrane fusion during viral entry and cell-

cell spread), AP rates were not increased. Changes in electrical connectivity were not observed, and membrane fusion between adjacent neurons (syncytia) did not occur. These results suggest that the infection-induced elevated activity and electrical coupling result from virally induced membrane fusion and this may underlie neurological symptoms observed during infection, in particular pruritus. Granstedt et al., further showed that PRV Bartha and PRV US9 null mutants do not induce increased firing in this model.

In primary SCG neuronal cultures, as well as in differentiated PC12 cell cultures, PRV infection leads to a significant increase in intracellular Ca^{2+} accumulation during the late phase of infection (Kramer and Enquist, 2012). At least two viral membrane proteins are responsible for the elevated spontaneous activity of neurons and intracellular Ca^{2+} increase both *in vitro* and *in vivo*: gB and US9. gB mediates membrane fusion during viral entry as well as fusion of infected cells leading to syncytia, and membrane protein US9 (together with glycoproteins gE and gI) promotes axonal sorting of virus particles and glycoproteins into axons (Curanovic and Enquist, 2009; Granstedt et al., 2013b; Kramer and Enquist, 2012; Lyman et al., 2007; McCarthy et al., 2009). These findings lead to a model where viral membrane fusion proteins are transported into the axons of infected neurons, form fusion pores between adjacent non-myelinated axons, and cause the neurons to become electrically coupled. This leads to elevated and synchronous activity propagating through the neuronal network. Whether the observed increase in calcium levels or coupled electrical activity is required for efficient viral spread, is still under investigation.

The reason for the rapid death of virulent PRV infected animals has been explored by Laval et al., These authors studied the local and systemic inflammatory response during PRV infection using a mouse footpad inoculation model, where the infection spreads from PNS to CNS via the sciatic nerve, which enables a more accurate assessment of the kinetics of the immunopathological processes associated with pruritus (Laval et al., 2018). In this model, PRV Becker induced severe pruritus in the foot, and animals

became moribund at 82 hours post infection. The authors found epithelial and neuronal cell necrosis, and inflammation with a massive neutrophil infiltration in the footpad and DRGs. Elevated plasma levels of circulating pro-inflammatory cytokines (IL6 and G-CSF) and chemokines (Gro-1 and MCP-1) were detected in Becker-infected mice. Specifically, IL6 and G-CSF levels were increased in several tissues, including the footpad, PNS, CNS and heart at 82 hours post infection. High plasma levels of C-reactive protein (CRP) confirmed the acute inflammatory response to PRV-Becker infection. Surprisingly, the increase in IL6 and G-CSF occurred before viral replication in DRG. Efficient replication and spread in the footpad were sufficient to induce cytokine production by DRG (Laval et al., 2019). By contrast, mice inoculated with PRV-Bartha in this model, did not show any signs of pruritus. Infection did not induce a specific and lethal systemic inflammatory response and, instead, spread further into the brain (Laval et al., 2018). These data clearly demonstrate the impact of inflammation in the clinical manifestations of PRV infection.

The importance of these observations is two-fold: The wild-type strain of PRV induces heavy inflammation in the non-native host which leads to the death of the animal even before the viral particles travel to the CNS. The attenuated strain does not induce inflammation in the non-native host which gives virus particles enough time to reach the CNS. However, the route of neuroinvasion is different from the wild-type virus since the attenuated strain cannot spread in the anterograde direction. Most probably, virus particles reach brain tissue via motor circuits in this case. In the native hosts, α -HVs do not induce a cytokine storm, and their replication, particularly in the PNS is quite well controlled by the immune system. Even though progeny virus particles may reach the CNS after reactivation episodes, in healthy immunocompetent hosts, the innate immune system of the CNS neurons do not let productive infection of these particles.

Neonatal Herpes simplex encephalitis (HSE)

As noted above, HSV-1 and HSV-2 are highly neuroinvasive but rarely neurovirulent. Nonetheless, rare cases of herpes simplex encephalitis (HSE)

occur in humans. The incidence of HSE in infants ranges from 1/500,000 to 5.9/100,000 live births, depending on the cited study (Kropp et al., 2006; Pinninti and Kimberlin, 2018; Whitley, 2006). The routes that HSV uses to invade the brain and cause HSE are not fully understood. HSE can occur either after acute primary infection or after reactivation from latency in individuals previously infected with the virus (Whitley, 2015). Neonatal patients are the most affected by HSV infections, presumably due to immature immune systems, with incidence of systemic infection ranging from 1/3,000 to 1/20,000 births (Kimberlin, 2004). When viral infection affects the CNS, mortality rates are approximately 50% (Kropp et al., 2006; Looker et al., 2017; Pinninti and Kimberlin, 2018; Whitley, 2006).

Neonatal HSV-2 infections are more common than those caused by HSV-1. Vertical infections typically occur during vaginal delivery but may also happen by transplacental transmission or during breastfeeding. Parents or other individuals with orolabial lesions can also infect newborns, and nosocomial transmission from infected hospital workers can occur as well. Strikingly, between 20% and 30% of pregnant women are seropositive for HSV-2, and 2% of neonates will show neonatal disease if mothers are shedding virus at the moment of birth. It is not well known why neonatal transmission is higher in mothers that are acutely infected during pregnancy as opposed to mothers with viral reactivation. It is possible that maternal antibodies cross the placenta and prevent, in some cases, the disease in the newborns. To further complicate the challenge of viral detection in pregnant women and prevention of neonatal transmission, more than 60% of mothers infected during pregnancy show no evident symptoms of genital infection (Kimberlin, 2004). The mortality rates of neonatal HSV infection can be as high as 80%, depending on the type of disease.

Newborns with localized disease can be classified into two groups. The group with the more severe symptoms exhibits CNS infection and HSE with or without infection of the skin, eyes, and/or mouth (SEM). The group with lesser symptoms only exhibits SEM lesions without evidence of CNS invasion, HSE

or disseminated disease. Before the antiviral era, most neonates with SEM, progressed to disseminated disease of CNS and/or visceral organs with high morbidity and mortality rates. With antiviral therapy, morbidity rates were reduced from 38% to 2-12% when neonates were treated promptly during the initial SEM phase (Kimberlin, 2004). Finally, newborns with disseminated infection exhibit viral invasion of visceral organs and disseminated intravascular coagulation with or without SEM and CNS disease. Untreated disseminated infection cases have mortality rates as high as 85% (Kimberlin, 2004; Kropp et al., 2006; Poole and Kimberlin, 2018). If left untreated, 60% of patients that survive will have severe and chronic neurological damage, and even with antiviral treatment, mortality is as high as 30%. Prompt parenteral antiviral treatment can decrease mortality rates by 50% (Pinninti and Kimberlin, 2018).

Although acyclovir is the treatment of choice for neonatal HSE, multiple antiviral drugs have shown relative efficacy to treat α -HV infection. All the drugs currently used, except for one called Foscarnet, are nucleoside analogs that are incorporated into the nascent viral DNA, preventing the attachment of additional nucleosides, causing chain termination and therefore reducing viral replication. This group of antivirals includes vidarabine, trifluridine, acyclovir, valacyclovir, ganciclovir, valganciclovir, and cidofovir. Foscarnet on the other hand is a pyrophosphate that inhibits the viral DNA polymerase and can be used in cases of acyclovir resistance (Pinninti and Kimberlin, 2018; Poole and Kimberlin, 2018).

HSE due to inborn mutations affecting the intrinsic immune system

The outcome and severity of α -HV infection in the CNS depends on viral and host gene products (Kropp et al., 2006; Pinninti and Kimberlin, 2018). There is increasing evidence that some cases of human HSE are caused by inborn mutations affecting the host intrinsic immune response. One noteworthy example is the increased incidence of HSE after primary infection with HSV-1 in children with dominant-negative mutations leading to Toll-like receptor 3 (TLR3) deficiency and impaired TLR-dependent IFN- α/β production (Zhang et

al., 2007). Importantly, these patients show lesions restricted only to the frontal and temporal lobes, suggesting that this specific mutation affects the capacity of neurons and oligodendrocytes in the forebrain to control and repress HSV-1 replication and spread in these otherwise healthy children (Casrouge et al., 2006; Lafaille et al., 2012). Interestingly, this particular deficiency in the intrinsic immune response seems to be specific in restricting HSV-1 from spreading further in the CNS since TLR3-deficient mice respond normally and are resistant to infection by several other viruses such as phlebovirus, West Nile virus, and influenza A virus (Casrouge et al., 2006; Guo et al., 2011; Guo et al., 2015; Herman et al., 2012; Lafaille et al., 2012; Ott et al., 2017; Perez de Diego et al., 2010; Zhang et al., 2013).

Although there is a strong correlation between TLR3-dependent IFN- α/β deficiency and HSE incidence in children and adults, HSE occurs in patients without these mutations, further complicating the etiology of this rare but exceedingly serious outcome. Signaling molecules such as ICAM-1 (an intercellular adhesion molecule) and ITGA5 (integrin alpha 5) increase in response to HSV-1 infection in otherwise healthy individuals but not in HSE patients, suggesting a TLR3-independent pathway influencing HSE (Engel et al., 2015; Perez de Diego et al., 2013; Sironi et al., 2017).

More recently, the Casanova laboratory has expanded the study of HSE cases unrelated to TLR3 deficiencies causing forebrain infections. Roughly 5% of HSE cases occur due to brainstem infections, and in 50% of these cases no other CNS region is affected by the virus. Again, inborn mutations (in this case, in RNA lariat metabolism) are strongly associated with these instances of HSE (Zhang et al., 2018). Bi-allelic mutations in DBR1 were identified in several unrelated patients with brainstem infections due to HSV-1, influenza or norovirus. DBR1 is the only identified RNA lariat debranching enzyme, which is expressed at high levels in spinal cord and brainstem. The accumulation of intron lariats could impair cell-intrinsic responses and virus recognition resulting in increased susceptibility to viral infection by cells in the brainstem. Apparently, full DBR1 activity is needed for protective antiviral immunity in the

brainstem, and not all neurons are equally susceptible to a productive α -HV infection. While cortical neurons are constitutively more resistant to HSV-1 infection even in the absence of antiviral signals, trigeminal ganglia neurons are more prone to get infected because they require a preemptive activation of antiviral immunity via TLR3 or IFN- α/β receptors (Zimmer et al., 2018).

III. α -HVs as Neural Circuit Tracers in Animal Models

α -HVs, especially HSV-1, HSV-2 and PRV, have been used for roughly 50 years to define and manipulate synaptically connected circuits in the nervous system of animal models. This powerful technique relies on the fact that when α -HVs infect neurons and produce infectious progeny, this progeny spread only through chains of synaptically connected neurons (a neuronal circuit), in a process often referred to as transsynaptic spread (Figure 1B). Other viral species such as vesicular stomatitis virus (VSV), rabies virus (RABV), mouse hepatitis virus, and betanodavirus can also spread transsynaptically (Ikenaga et al., 2002; Lanciego and Wouterlood, 2020; Maturana et al., 2020; Nassi et al., 2015; Pomeranz et al., 2005). The use of recombinant viruses as transneuronal tracers of neuronal circuitry is now a widely used approach to define the synaptic organization of neural networks (Table 1). The main advantage is that virus replication and spread delivers a self-amplifying marker of neural connectivity.

The capacity of a virus infection to spread predominantly among chains of synaptically connected neurons is remarkable. Several hundred published manuscripts have demonstrated that α -HVs are unable to spread inter-axonally to physically adjacent but non-connected neural circuits. Moreover, viral spread through the nervous system will only happen via intact and synaptically connected circuits (Card and Enquist, 1995, 2014; Pomeranz et al., 2005). The infected neurons apparently do not release infectious progeny randomly, but only at or near sites of synaptic contact. It is unclear whether viral particles actually spread through a synapse. Careful analysis suggests that spread occurs between sites of close membrane apposition such as synapses or glial-

neuronal contacts (Card and Enquist, 1995; Card et al., 1993; Card et al., 1990; Enquist et al., 1994; Levine et al., 1998).

Non-viral tracers have been used for more than 100 years. One of the first successful attempts was made by the Spanish neuroscientist and physician Santiago Ramon y Cajal, that stained and visualized entire neurons with their processes. Ramon y Cajal applied a staining method developed by the Italian physician Camilo Golgi that used silver nitrate and potassium dichromate to impregnate fixed brain slices. He described in great detail in more than 3000 drawings of brain structures, the shape and complexity of the nervous system (Bro and Haycock, 1977; Haycock and Bro, 1975). Ramon y Cajal suggested that the brain works by constantly changing connections between cells, a finding that was disputed by the scientific community at the time. In his memoir, he wrote: "*I expressed the surprise, which I experienced upon seeing with my own eyes the wonderful revelatory powers of the chrome-silver reaction and the absence of any excitement in the scientific world aroused by its discovery*" (Katz-Sidlow, 1998). Santiago Ramon y Cajal and Camilo Golgi received the Nobel Prize in Physiology or Medicine in 1906 for their seminal studies of the structure of the nervous system. Although Ramon y Cajal's findings with the Golgi staining method allowed important morphological advancements, it did not permit transneuronal tracing and demonstration of functional cell connections. Several decades later, scientists reported on the use of many conventional tracers including but not limited to small proteins such as horseradish peroxidase and albumin; inorganic fluorescent molecules such as fast blue, diamidino yellow and fluoro-gold; dextrans such as fluoro-ruby and biotinylated dextran amine; lectins such as wheat germ agglutinin and phaseolus vulgaris-leucoagglutinin; beads like latex microspheres; bacterial toxins such as tetanus and cholera B subunit; trophins like nerve growth factor and brain derived neurotropic factor; amino acids like tritiated-proline, tritiated-leucine and biocytin; and fluorescent long-chain dialkyl-carbocyanines such as Dil and DiO. For additional information on conventional or non-viral tracers see (Boldogkoi et al., 2004; Nassi et al., 2015; Pomeranz et al., 2005). These molecules allowed the basic understanding of nerve damage and regeneration

as well as the concept of anterograde and retrograde transport in neurons (Cowan and Cuénod, 1975). Non-viral tracers are still widely used but share some important limitations. Only some of them can cross synapses, and even if they do, they will only label first-order of connected neurons (monosynaptic only, not polysynaptic). Since they do not self-propagate or amplify in the nerve circuit, they become diluted quickly as a function of time and distance from the site of injection. Consequently, they cannot inform on the polysynaptic architecture of neurons committed to a specific function or task. Fortunately for neuroanatomists and neurobiologists, nature has already evolved a great tool with the neurotropic viruses. Nearly a century ago, Goodpasture and Teague (Goodpasture, 1925; Goodpasture and Teague, 1923b) were the first to hypothesize that infectious virions are taken up by nerve endings and then retrogradely transported to the neuronal cell bodies. Fifty years later, neuroscientists were able to show that HSV-1 could spread by transneuronal transfer between and within chains of synaptically connected neurons (Cook and Stevens, 1973; Kristensson et al., 1974; Kristensson et al., 1971; Kristensson et al., 1982; Kristensson et al., 1978).

It was not until the 1980's that α -HVs become widely used for polysynaptic circuit tracing (Card and Enquist, 1995; Card et al., 1991; Dolivo et al., 1979; Hogue et al., 2015; Ugolini et al., 1987). HSV-1, HSV-2 and PRV have similar behavior as circuit tracers. They are able to infect CNS and PNS neurons, including sensory, motor, sympathetic and parasympathetic neurons, in several mammal species. They can complete their replication cycles in less than 12 hours in susceptible and permissive cells, with PRV replicating faster than HSV (Kaplan and Vatter, 1959). Both viruses infect a broad range of animal models including mouse, rat, guinea pig, hamster, gerbil, rabbit and chicken embryo. Moreover, PRV can also infect swine (the natural host), dogs, cats, cattle, sheep, and some species of birds. Unlike HSV, PRV does not infect higher primates such as apes, chimpanzees and humans (Gustafson and Kanitz, 1975; Olander et al., 1966; Saunders and Gustafson, 1964). In Table 1, we provide a comprehensive list of HSV-1 and PRV mutants that express fluorescent reporter proteins used for circuit tracing studies. The polysynaptic

tracing properties of α -HVs in susceptible animals are influenced by several factors that will be briefly described below. For detailed descriptions, see (Card and Enquist, 2014; Card et al., 1999; Card et al., 1993; Card et al., 1990; Card et al., 1991; Enquist et al., 1998; Enquist et al., 1994; Wojaczynski et al., 2015).

α -HVs infections typically initiate at the periphery in mucosal epithelia from where they invade the termini of sensory neurons to undergo retrograde transport towards cell bodies. In cell bodies α -HVs can establish lifelong latency and eventually undergo reactivation and anterograde transport of newly made particles towards sites of egress, most likely back out towards the periphery (Mangold et al., 2020).

The most commonly studied laboratory strains of PRV are Becker, Bartha, Kaplan, NIA-2 and NIA-3. HSV-1 laboratory strains include MacIntyre, F, 17syn +, KOS, SC16, H129, FMC, and KJ502 while HSV-2 strains include 186 and G (Enquist et al., 1998; Norgren and Lehman, 1998; Szpara et al., 2014). Most of these α -HVs strains exhibit a wild-type phenotype and will spread in a bidirectional fashion through synaptically connected circuitry. They can also be highly neurovirulent and therefore are not optimal transsynaptic tracers.

The most effective α -HVs tracing strains are neuroinvasive mutants with reduced neurovirulence and unidirectional transsynaptic spread properties as opposed to the wild-type bidirectional spread phenotype (Table 1). Perhaps one of the better and more characterized tracing α -HVs is the attenuated vaccine strain Bartha of PRV (Bartha, 1961). Bartha was obtained after extensive passaging of a virulent PRV isolate obtained from pigs in cultured chicken cells and chicken embryos. The PRV Bartha genome was sequenced by Szpara et al. and compared to the sequence of virulent PRV strains (Szpara et al., 2011). The PRV Bartha genome contains several mutations in genes UL21, UL44 (gC), UL10 (gM), and a small 3.5 kb deletion in the US region of the viral genome resulting in the deletion of US8 (gE) and US9 and disruption of US2 and US7 (gI) that may contribute to the decreased neurovirulence. PRV

Bartha allow longer survival times than a wild-type PRV strain in the infected animal. In the laboratory, PRV Bartha grows to titers above most wild-type PRV strains and is more thermostable. Additionally, it can readily spread through the PNS and the CNS of animals. More importantly, Bartha selectively spread in the retrograde direction through neural circuits, making it an excellent transsynaptic tracing tool (Dolivo, 1980).

Another noteworthy and widely used α -HV polysynaptic tracer is HSV-1 strain H129. This is the only known virus to move preferentially in the anterograde direction through neural circuits (Wojaczynski et al., 2015). Zemanick et al. showed that H129 was, indeed, transported preferentially in the anterograde direction after injection into the motor cortex of cebus monkeys (Zemanick et al., 1991). Although the genome of H129 was fully sequenced by Szpara et al., the precise relationship between the genotype and the mutant phenotype has not been established (Szpara et al., 2010). Genome sequence analysis determined that more than 50% of genes in H129 genome, contain missense or silent mutations, but no deletions or other null mutations. The retrograde spread defect is most likely caused by multiple genes leading to differences in somatodendritic sorting and egress, or the dendritic versus axonal partition of viral progeny (Wojaczynski et al., 2015).

Over the last twenty years, several fluorescently labelled α -HV tracers have been constructed. Most of them replicate and express gene markers in a constitutive fashion, while others can either replicate and or express transgenes in a conditional fashion, allowing for monosynaptic and polysynaptic tracing studies (Card et al., 2011b; DeFalco et al., 2001; Li et al., 2019; Lo and Anderson, 2011; Wu et al., 2014) (see Table 1 for more details).

Besides choosing the viral strain that better suits the tracing circuit of interest, it is critical to consider the total amount of infectious virus inoculated. The quantity is often called the "titer" and is typically expressed in plaque-forming units per milliliter (PFU). The titer in PFUs is determined using standard tissue culture cells. Sufficient PFUs must be inoculated to achieve replication and

transsynaptic spread in the infected animal. The optimal amounts of virus vary depending on the viral strain, animal species and age, and the circuit of interest. The quantity of virions injected will obviously affect the survival time of the infected animal. A virion is defined as an infectious particle. A parameter often ignored is the "particle to PFU" ratio, which can vary between different viral mutants and even among different stocks of the same recombinant virus. It is always a good idea to inject sufficient PFUs so that at least 50% of the animals are infected (the infectious dose 50 or ID₅₀), assuming that the ID₅₀ has been determined for the strain or mutant used (Card et al., 1995; Card and Enquist, 2014).

The number of synapses and extent of the circuit of study will influence the infection outcome in animal models. The density of neuron terminals at or near the site of inoculation will affect efficiency of virion uptake and possibly the spread kinetics (Card et al., 1999). Conversely different neuronal types can also show disparate permissivity and susceptibility to viral infection as shown by Zimmer et al., (Zimmer et al., 2018). The length of the axons will affect the temporal kinetics of spread and anatomical barriers such as the extracellular matrix at the tissue or organ level might also delay or prevent viral invasion.

Finally, it is important to consider that the spread of infection and subsequent labeling of synaptically connected neurons occur in a sequential fashion. Different neuronal circuits can show differences in the spread kinetics defining first-, second-, and third-order neurons. First order neurons will show signs of cytotoxicity before second or higher order neurons are infected. Furthermore, reactive nonneuronal responses are also faithful indicators of the temporal progression of viral spread through a CNS circuit (e.g. reactive glia surrounding an infected neuron are a clear indication of a late stage infection, while the absence of reactive gliosis is indicative of an earlier stage of infection) (Card and Enquist, 2014; Card et al., 1990; Enquist and Card, 2003; Levine et al., 1998; Wojaczynski et al., 2015).

IV. Discussion

Most acute and persistent viral infections often begin at epithelial or endothelial cell surfaces. Remarkably, many α -HVs efficiently enter the PNS of their natural hosts and establish a quiescent infection with little or no CNS pathogenesis. Their initial peripheral infection stimulates a well-controlled intrinsic and innate immune response, as well as a long-lived adaptive immune response. The α -HV genomes remain quiescent in PNS neurons for the life of their hosts, only occasionally reactivating to produce virions that can re-infect peripheral tissues and spread to other hosts. Perhaps the pro-survival, cytolysis-resistant nature of mature neurons, facilitates in part the establishment of such persistent and reactivatable infections.

PRV and HSV infect most if not all known primary sensory neurons, sympathetic and parasympathetic neurons, and motor neurons in a broad range of susceptible mammalian animal models. The directional spread of viral infection in the NS of the host is an essential aspect of the viral life cycle and pathogenesis. α -HVs such as HSV and PRV can spread efficiently within synaptically connected neural circuits and therefore have been used extensively to define the architecture of the mammalian nervous system (Table 1). Wild-type PRV and HSV-1 will spread in a bidirectional retrograde and anterograde fashion to both presynaptic and postsynaptic connected neurons (Figure 1B). Replicating PRV and HSV behave as self-amplifying tracers that will faithfully spread to higher-order neurons without decay or dilution of the reporter transgene expressed by the virus.

The interplay between viral and neuronal proteins, starting from the invasion of the nervous system leading to latent infections in the PNS ganglia, is complex and difficult to dissect using common cell culture techniques and currently available animal models. Therefore, many questions still remain concerning the temporal dynamics of how genome silencing in the neuronal nucleus is established in the first place, what controls reactivation cycles, and what happens to neurons after reactivation episodes. Another poorly understood issue is whether reactivating virus particles reach the CNS tissue to establish

latent infections and whether subsequent reactivations in the CNS can lead to neuroinflammation predisposing the tissue for other neurodegenerative diseases such as Alzheimer's disease. Detailed molecular investigation of α -HV infected primary PNS and CNS neurons is required to understand fundamentals of herpesvirus induced neurodegeneration.

Table 1. List of commonly used fluorescently labeled α -HV tracing recombinants.

The list includes 29 viral recombinants based on PRV Becker, PRV Bartha and HSV-1 H129. PRV Becker is a wild-type highly virulent laboratory strain that undergoes retrograde and anterograde transport in neural circuits. We only include one attenuated IE180 null and US9 null Becker recombinant that is unable to replicate and spread. PRV Bartha is an attenuated vaccine strain widely used for retrograde transneuronal tracing. Bartha infected animals live several days longer without significant symptoms compared to PRV Becker. HSV-1 strain H129 is widely used for anterograde transneuronal tracing. Transsynaptic spread of H129 is significantly more efficient in the anterograde direction than in the retrograde direction. For each viral recombinant we provide the name, strain (Becker, Bartha, H129), a brief description of the recombinant characteristics, the spread phenotype (retrograde "R", anterograde "A", anterograde mainly "A mainly", conditional spread "Cond"), and the corresponding reference.

Virus	Strain	Description	Spread	Ref
PRV-151	Becker	CMV-EGFP reporter gene cassette inserted into the gG locus	R & A	(Demmin et al., 2001)
PRV-180	Becker	mRFP-UL35 (capsid) fusion protein	R & A	(del Rio et al., 2005)
PRV-GS443	Becker	EGFP-UL35 (capsid) fusion protein	R & A	(Smith et al., 2001)
PRV-950	Becker	mTurquoise2-UL35 (capsid) fusion protein	R & A	(Hogue et al., 2014)
PRV-959	Becker	mNeonGreen-UL35 (capsid) fusion protein	R & A	(Koyuncu et al., 2015)
PRV-960	Becker	mCherry-UL35 (capsid) fusion protein	R & A	(Hogue et al., 2015)
PRV-181	Becker	mRFP-UL35 (capsid) and GFP-VP22 (tegument) fusion proteins	R & A	(del Rio et al., 2005)
PRV-823	Becker	Us3 null mutant, expressing mRFP-UL35 (capsid) fusion protein. Less virulent than Becker but not as attenuated at PRV Bartha	R & A	(Olsen et al., 2006)
PRV-833	Becker	US3 null mutant, expressing mRFP- UL35 (capsid) fusion protein and eGFP-VP22 (tegument) fusion protein. Less virulent than Becker but not as attenuated at PRV Bartha	R & A	(Olsen et al., 2006)

PRV IE180-null-hSyn-Cre	Becker	IE180 and US9 null mutant with hSyn-Cre reporter gene cassette replacing the US9 locus. There will be long-term constitutive Cre expression but no viral replication or spread will occur unless trans-complemented with IE180	Cond R	(Oyibo et al., 2014; Wu et al., 2014)
PRV-152	Bartha	CMV-EGFP reporter gene cassette inserted into the gG locus	R	(Demmin et al., 2001; Smith et al., 2000)
PRV-290	Bartha	CMV-mTurquoise2 reporter gene cassette inserted into the gG locus	R	(Hogue et al., 2018)
PRV-614	Bartha	CMV-mRFP reporter gene cassette inserted into the gG locus	R	(Banfield et al., 2003)
PRV-Ip298	Bartha	mCherry reporter gene cassette fused to UL23 (TK) ,	R	(Schneeberger et al., 2019)
PRV-263	Bartha	CMV-Brainbow 1.0L reporter gene cassette inserted into the gG locus. Default expression of tdTomato reporter unless a Cre-expressing cell is infected where there is expression EYFP and mCerulean	R	(Kobiler et al., 2010)
PRV-267	Bartha	CMV-Cre reporter gene cassette inserted into the gG locus	R	(Card et al., 2011a)
PRV-2001	Bartha	UL23 (TK) null mutant with CMV-LoxP-STOP-LoxP-Tau-EGFP-IRES-TK reporter gene cassette inserted into the gG locus. Viral replication and EGFP expression is conditional upon infecting Cre-expressing cells	Cond R	(DeFalco et al., 2001)
PRV-Introvert-GFP	Bartha	UL23 (TK) null mutant with TK1-intron-LoxN-Lox2272-intron-GFP1-2A-TK2-intron-LoxN-Lox2272-intron-GFP2 reporter gene cassette inserted into the TK locus and driven by the native TK promoter. Viral replication and EGFP expression is conditional upon infecting Cre-expressing cells	Cond R	(Pomeranz et al., 2017)

PRV-Introvert-mCherry	Bartha	UL23 (TK) mutant with TK1-intron-LoxN-Lox2272-intron-mCherry1-2A-TK2-intron-LoxN-Lox2272-intron-mCherry2 reporter gene cassette inserted into the TK locus and driven by the native TK promoter. Viral replication and mCherry expression is conditional upon infecting Cre-expressing cells	Cond R	(Pomeranz et al., 2017)
PRV-B180	Bartha	UL23 (TK) mutant with DIO-CMV-TK-GFP reporter gene cassette inserted into the gG locus. Viral replication and EGFP expression is conditional upon infecting Cre-expressing cells	Cond R	(Hanchate et al., 2020)
HSV-414	H129	CMV-EYFP reporter gene cassette inserted into the UL37/UL38 locus	A mainly	(Wojaczynski et al., 2015)
HSV-424	H129	mRFP-UL35 (capsid) fusion protein	A mainly	(Wojaczynski et al., 2015)
HSV-772	H129	CMV-EGFP reporter gene cassette inserted into the UL26/UL27 locus	A mainly	(Wojaczynski et al., 2015)
HSV-774	H129	mRFP-UL35 (capsid) fusion protein, and CMV-EGFP reporter gene cassette inserted into the UL26/UL27 locus	A mainly	(Engel)
HSV-373	H129	CMV-mCherry reporter gene cassette inserted into the UL26/UL27 locus	A mainly	(Engel)
HSV-768	H129	CMV-Brainbow 1.0L reporter gene cassette inserted into the UL26/UL27 locus. Default expression of tdTomato reporter unless a Cre-expressing cell is infected where there is expression EYFP and mCerulean	A mainly	(Engel)
HSV-floxed	H129	CMV-LoxP-EGFP-pA-LoxP-tdTomato-pA reporter gene cassette inserted into the UL26/UL27 locus. Default expression of EGFP unless a Cre-expressing cell is infected where there is expression of tdTomato	A mainly	(McGovern et al., 2015)

HSV-ΔTK-TT	H129	UL23 (TK) null mutant with CAG-LoxP-STOP-LoxP-tdTomato-2A-TK reporter gene cassette inserted into the TK locus. Replication, spread and tdTomato expression is conditioned to infecting a Cre-expressing cell	Cond A mainly	(Lo and Anderson, 2011)
H129-ΔTK-tdT	H129	UL23 (TK) null mutant with CMV-tdTomato reporter gene inserted into the TK locus. Constitutive expression of tdTomato but no viral spread unless trans-complemented with TK	Cond A mainly	(Zeng et al., 2017)

V. Web Resources

The Center for Neuroanatomy with Neurotropic Viruses (CNNV), is a national resource supported by the NIH to develop improved transneuronal tracing technology and make it available to investigators. The CNNV offers a comprehensive collection of viral recombinants that can be found in the following link: <http://www.cnnv.pitt.edu/resources.htm>

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