Alphaherpesvirus Latency and Reactivation with a Focus on Herpes Simplex Virus

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Abstract

We are at an interesting time in the understanding of alpha herpesvirus latency and reactivation and their implications to human disease. Conceptual advances have come from both animal and neuronal culture models. This review focuses on the concept that the tegument protein and viral transactivator VP16 plays a major role in the transition from latency to the lytic cycle. During acute infection, regulation of VP16 transactivation balances spread in the nervous system, establishment of latent infections and virulence. Reactivation is dependent on this transactivator to drive entry into the lytic cycle. In vivo de novo expression of VP16 protein is mediated by sequences conferring pre-immediate early transcription embedded in the normally leaky late promoter. In vitro, alternate mechanisms regulating VP16 expression in the context of latency have come from the SCG neuron culture model and include the concepts that (i) generalized transcriptional derepression of the

viral genome and sequestration of VP16 in the cytoplasm for ~48 hours (Phase I) precedes and is required for VP16-dependent reactivation (Phase II); and (ii) a histone methyl/phospho switch during Phase I is required for Phase II reactivation. The challenge to the field is reconciling these data into a unified model of virus reactivation.

*The greatest enemy of **knowledge** is not ignorance, it is the illusion of **knowledge**.-- Daniel J. Boorstin.*

The task of compiling this review was uncomfortably humbling, as if cataloging the stars in the universe. While not completely dark, our night sky is missing a multitude of studies which are among the many points of light contributing to our field. This article is a focused review in which we discuss from the vantage point of our expertise, just a handful of concepts that have or are emerging. A lookback at some of the pioneering work that grounds our field is also included.

Introduction

The mechanisms of latency and reactivation of the human alpha herpesviruses, herpes simplex virus 1 and 2 (HSV1, HSV2) and varicella zoster virus (VZV), remain major unsolved mysteries. Each year, recurrent HSV disease contributes to ~200 million new HSV infections (worldwide, extrapolated from US infection rates) (Azwa and Barton, 2009; Pepose et al., 2006), two-thirds of new sexually acquired HIV infections (Glynn et al., 2009), ~10 million cases of eye disease (Pepose et al., 2006), ~150,000 cases of encephalitis (Stone and Hawkins, 2007), and devastating neonatal disease (Looker et al., 2017; Roberts, 2009). Herpesvirus infections are also linked to type II diabetes (Sun et al., 2005), cardiovascular disease (Mukamal et al., 2004; Visser and Vercellotti, 1993), and now strongly implicated in Alzheimer's disease (Fulop et al., 2018; Itzhaki, 2018; Itzhaki and Wozniak, 2008; Lathe et al., 2019). The ubiquity of HSV results from the ability of the virus to establish latent infections that periodically reactivate and transmit the infection to new hosts (Ahmed and Stevens, 1990; Knipe, 2007; Roizman et al.).

The availability of animal models that support HSV latency and reactivation, and the work of many laboratories over many years, have contributed to a collective understanding of the HSV life cycle. Infection occurs at the body surface, usually a mucosal epithelium, where the virus replicates, spreads, and gains access to the innervating neuron axonal endings. At this point, nucleocapsids deposited into the axonal cytosol use retrograde axonal transport to reach neuronal cell bodies that are housed within peripheral ganglia (a collection of sensory or autonomic nerve cell bodies innervating specific tissues). The trigeminal ganglia (TG), which innervate the oral mucosa and other regions of the face, is the typical site of HSV1 latent infection, whereas the sacral ganglia (SG) innervates the genital mucosa and serves as the predominant site of latent HSV2 infection.

Overview

Our current understanding of HSV latency and reactivation arises out of a blend of human studies and findings from various animal and neuronal culture models. It is important to recognize the limitations of these models when summing up what we know. Importantly, new technologies and approaches with increased sensitivity have driven new waves of data acquisition which requires assimilation into what we think we know about an extremely complex process. There is increasing evidence for the concept that HSV1 latency/ reactivation can be a risk factor in the development of neurodegenerative disorders. Also arising from the human model, the concept of "continuous" ganglionic reactivation has emerged as an explanation for the unexpectedly high frequency of viral DNA in human genital swabs in some patients in the absence of detectable infectious virus.

This article focuses on an unexpected observation that the tegument protein and viral transactivator, VP16, plays a major role in the transition from latent into the lytic cycle and is required for balancing the latent/lytic transition (Figure 1). During acute infection, regulation of VP16 transactivation balances spread into the nervous system, establishment of latency, and virulence. In

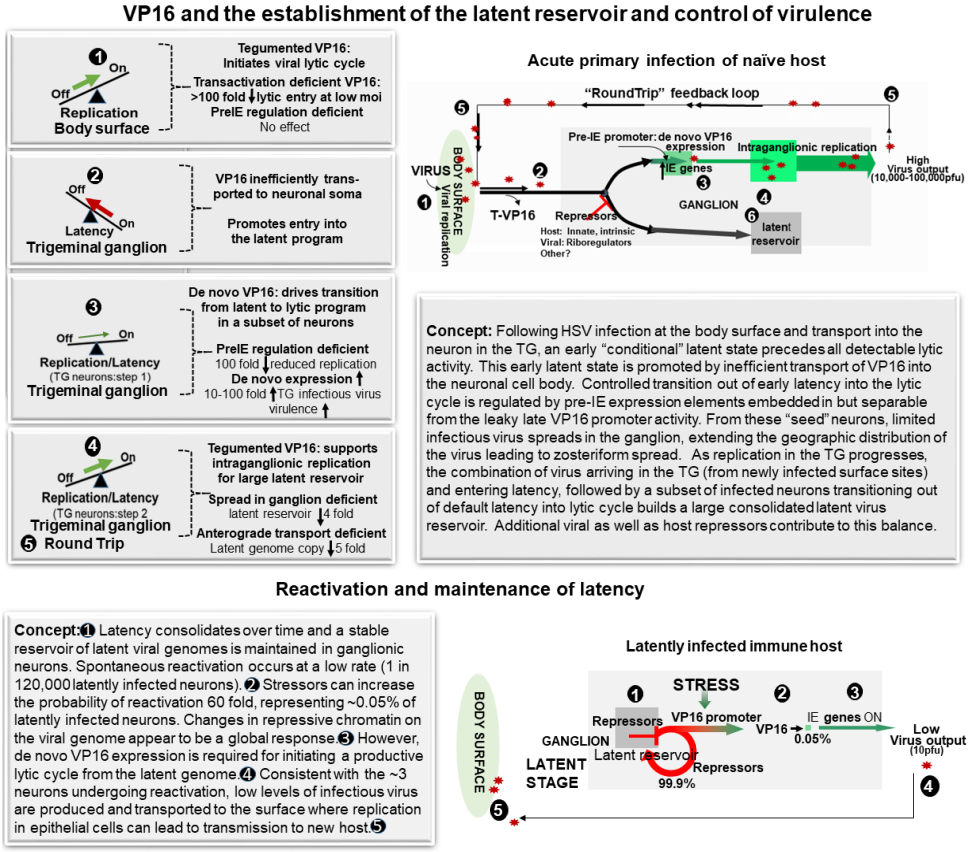

Figure 1. Regulation of VP16 function balances latent/lytic programs. ***Entering the neuron:*** The VP16 tegument protein, which is a potent transactivator of viral immediate-early gene expression, is not available to perform this function in neurons infected via axonal endings because of inefficient transport of VP16 via axon to the cell body during acute infection. The viral DNA enters the nucleus where it forms a circular episome and becomes associated with nucleosomes, which promotes the latent transcriptional program.

turn, reactivation is dependent on this transactivator to drive entry into the lytic cycle. A potential mechanism for regulating VP16 expression in the context of *in vitro* latency has come from the superior cervical ganglion (SCG) neuron culture model (Cliffe et al., 2015; Kim et al., 2012). The concepts that have

emerged from this work are as follows: (i) generalized derepression of the viral genome (Phase I) precedes VP16-dependent reactivation and is required for it (Phase II), and (ii) a histone methyl/phospho switch during Phase I is required for Phase II reactivation. Notably, in vitro neuronal models of latency vary in this regard and some do not display this dual phase reactivation mechanism (Edwards and Bloom, 2019). It will be important to reconcile the findings in diverse in vitro and in vivo models of alpha herpesvirus latency and reactivation to develop testable hypotheses and determine relevant regulatory molecular mechanisms. To frame the present, we start by discussing the early seminal findings.

Creating the latent reservoir: The latent reservoir (which is essential for viral reactivation) is established upon HSV1 reaching neuronal nuclei. The number of latently infected neurons and the number viral genomes residing within each neuron are important parameters that contribute to the potential of future reactivation events. Although latency is the default outcome of neuronal infection in some neurons, HSV1 enters the lytic cycle during acute infection initiated via preIE expression of VP16. This regulated entry into the lytic cycle in the ganglionic neuron modulates virulence while supporting the viral replication that ultimately increases the size of the latent reservoir in the ganglia which occurs during this acute stage of infection. Virus produced in the ganglion is transported back to the body surface, resulting in a positive feedback loop and zosteriform spread. Exit from this "default latency" pathway is balanced by negative factors such as virus and host riboregulators including microRNAs that target viral genes, other repressors such as intrinsic immune functions (promyelocytic leukemia bodies (PML)) and positive factors (*de novo* VP16, multiple viral genome copies).

Preserving the latent reservoir: During latent infection, modification in the chromatin associated with the viral genome evolves into an increasingly repressive state. Despite this repression, there is a low level of transcription from the genome related to lytic genes. The significance of this transcription is not known but could reflect a repressive mechanism working in tandem with

the LATs and microRNAs. Replicating virus and viral proteins are not detected during latency in the TG. Long-term studies in the mouse and rabbit reveal the latent reservoir appears to be stable. Expression from the LAT locus is important to maintain reactivation competent latent infections.

Reactivation. Stress results in *de novo* expression of VP16 which coordinates activation of the viral immediate-early (IE) genes. A productive lytic cycle ensues in 0.05% of latently infected neurons per event although more generalized changes in the chromatin associated with the latent genomes occurs. Thus, HSV has devised a complex regulatory strategy that maintains the vast majority of its latent genomes in latency, while allowing the release into a productive lytic cycle in an extremely rare number of neurons. During reactivation, virus does not spread to neighboring neurons in the ganglia but is transported back the body surface where subsequent replication in epithelial cells amplifies virus output and facilitates transmission to new hosts. Spontaneous reactivation in sensory ganglia has been documented in mouse models. Adapted from (Thompson et al., 2009).

How 150 years of research have shaped our ideas about herpesvirus pathogenesis

The earliest scientific investigations on alpha herpesvirus latency were primarily clinical observations. Herpes, an ancient Greek word meaning "to creep", was used by Hippocrates to describe diverse skin diseases. The association between fever and blisters around the mouth and nose was recognized nearly 2500 years ago by the Roman physician Herodotus who described herpes febrilis (Wildy, 1973). The terms "fever blisters" and "cold sores" are still in use today. Through the ensuing millennia the recurrent nature of these types of diseases was appreciated.

Why do herpetic lesions recur? Are they from an infectious agent? What is the nature of the agent?

1863-1930s. Available Toolbox:

- Knowledge of transmission of infection and infectious agents including bacteria and "filterable agents" (the latter the earliest indication of viruses)
- Koch's famous postulates delivered in 1890 (Koch, 1890)
- Identification of a "neutralizing" substance in the blood that countered infections (Behring and Kitasato, 1890)
- Generation of these neutralizing substances, including those to herpes simplex
- Methods to store or serially passage herpes in animal brains (Perdrau, 1925)
- Light microscopy combined with histological staining to study tissue. Neurons distinguished from other cells used in conjunction with agents transmitted through nerves contributed to knowledge of brain structure and innervation from the periphery (Doer, 1920; Friedenwald, 1923; Goodpasture and Teague, 1923)

The association of herpetic lesions with the nervous system was appreciated as early as the mid-19th century (Von Barensprung, 1863). However, it was not until the beginning of the 20th century that clinicians and scientists began to appreciate a relationship between herpetic lesions on the body surface and the sensory nerve endings innervating the skin. In 1892 von Bokay observed that children often developed varicella after exposure to an adult suffering from zoster (von Bokay, 1909). In 1900 Head and Campbell deduced from the pattern of lesions on their patients that herpes zoster must be related to sensory ganglia. Their tour-de-force study (Head and Campbell, 1900), reprinted in part in 1997 (Head et al., 1997), was the first to show how herpetic viral diseases can be employed to help understand the anatomy of the nervous system, a practice that continues today (Sarno and Robison, 2018). Relying heavily on their work, Howard noted that human herpetic lesions of the face were associated with trigeminal ganglionitis and pneumonitis early in the 20th century (Howard, 1903). At about the same time Cushing reported that

some individuals treated for trigeminal neuralgia by surgical resection of the trigeminal ganglion and nerve roots developed herpetic lesions in areas innervated by the contralateral (opposite side) nerve but not on the ipsilateral (resected) side (Cushing, 1905), supporting the hypothesis that herpetic lesions were associated with stress or damage to the peripheral nervous system.

Vidal first demonstrated the infectious nature of herpes (Vidal, 1873), but of greater significance to those interested in latency were later studies involving transmission of herpetic stromal keratitis. At the end of World War I the transmission of human herpetic stromal keratitis to rabbit corneas by Gruter (Gruter, 1920; Kraupa, 1920) and Loewenstein (Loewenstein, 1919, 1920) and subsequently transmission back to a human (reviewed in (Holden, 1932)) cemented the idea that herpes was an infectious agent, and provided an animal model for study. During this same time, varicella was transmitted to naive children using vesicle fluid from children with varicella lesions (Kundratitz, 1925); however, attempts to infect laboratory animals failed (Rivers and Tillett, 1924). The lack of an animal model is a difficult challenge for the study of VZV pathogenesis and latency that persists to the present day (Mahalingam et al., 2019; Ouwendijk and Verjans, 2015). A creative approach to overcome the species specificity of VZV is the use of human tissue xenografts in mice with severe combined immunodeficiency (SCID). This model allows the analysis of VZV infection in differentiated human cells in the context appropriate tissue microenvironments, providing many insights into VZV pathogenesis (Moffat et al., 1995; Zerboni and Arvin, 2015; Zerboni et al., 2014). More recent studies using dissociated human TG have provided insight into VZV reactivation (Cohrs et al., 2017).

Several groups noted that herpes infection on the rabbit eye led to infection of the central nervous system (CNS) and changes in rabbit behavior (e.g. turning the head to a particular side), both suggesting a neural route of transmission (Doer, 1920; Friedenwald, 1923; Goodpasture and Teague, 1923). Goodpasture began a series of histological studies delineating herpes spread

within rabbits (Goodpasture, 1925a, b; Goodpasture and Teague, 1923) that culminated in the astounding and prescient conclusion that: "Following a primary infection, it seems quite probable that the virus remains in a latent state within the ganglia after the local lesion has healed. A second cutaneous eruption may occur as a result of injury…or the disturbed physiological states... which sets in activity a latent virus" (Goodpasture, 1929). Despite the seemingly overwhelming evidence of nerve involvement in herpes infection this idea was not universally accepted.

1930s-1980s. Toolbox additions include:

- The nature of viruses (Stanely, 1935)
- Tissue and virus culture as well as plaque assays for animal viruses (Dulbecco and Vogt, 1953)
- Ultra-low freezers for virus storage
- Electron microscopy
- Refined histological approaches, fixation methods and immunohistochemistry;
- Conditionally lethal viral mutants
- The "phage Church" and Lambda phage latency, DNA structure and code (Watson and Crick, 1953)
- The central dogma of DNA to RNA to protein
- Liquid DNA/RNA hybridizations
- Bacterial restriction-modification system (S.E. and M.L., 1952) and commercial restriction enzymes
- *In situ* hybridization for DNA and RNA (Gall and Pardue, 1969)
- Radiolabeling of proteins and nucleic acids and various separation methods including chromatography, electrophoretic gel systems, and blotting and probing (Southern, 1975)
- Cloning and cloning vectors (Bolivar et al., 1977)
- Engineered viral mutations
- Genetically modified and engineered animal models, especially mice
- Maxim and Gilbert, then Sanger sequencing

Good and Campbell employed Perdrau's rabbit model of herpetic encephalitis (Perdrau, 1925, 1938) to demonstrate that anaphylactic shock could "precipitate" herpetic encephalitis in previously infected rabbits pre-sensitized to egg albumin (Good and Campbell, 1948). Rabbits were infected with HSV and one to three months after they appeared to be disease free, anaphylactic shock was induced by exposure to egg albumin. Encephalitis occurred in 19/44 tests and virus was recovered from the brains of all rabbits that died and many that recovered. Virus was not detected in the brains of rabbits prior to anaphylaxis. Arguably this was the first well controlled evidence of latency and reactivation of HSV in the nervous system *in vivo*. We now know that intramuscular injection of HSV most likely resulted in virus replication in the skin at the site of injection and subsequent spread to the innervating sensory ganglia. Anaphylactic shock presumably caused virus reactivation within sensory neurons with subsequent spread to the brain resulting in fatal encephalitis but virus reactivation within brain neurons was not ruled out.

Schmidt and Rasmussen explored alternate methods to "precipitate" herpes leading them to favor HSV latency occurs in the skin. Of many methods tried, only intramuscular injections of adrenalin "precipitated" encephalitis (Schmidt and Rasmussen, 1960). Encephalomyelitis was "precipitated" in 60% of the rabbits given intramuscular adrenalin injections and, importantly, herpesvirus was detected in all six of these rabbit brains. A few years earlier at the Wisconsin meeting on Latency and Masking in Viral and Rickettsial Infections (Andrewes, 1957), six types of "latent" infections were described. Herpes was thought to be a unique agent because it could not be cultured from skin between episodes. Herpes latency was defined as the period of time in which skin was negative between outbreaks. Rasmussen speculated "that temporary vasoconstriction resulting from increased adrenalin output, could produce a local anoxia {e.g. local reducing conditions} in the skin and consequent 'reactivation' of residual herpesvirus" (Schmidt and Rasmussen, 1960). This hypothesis harkened back to the early observations of Perdrau. Early virologists stored their virus stocks as bits of infected rabbit brains in glycerin in ice boxes. Exclusion of air greatly increased the time such a stock remained

infectious. Perdrau discovered that oxidation destroyed herpesvirus activity, but the virus stocks could be "reactivated" by subsequent reduction (Perdrau, 1931).

Rasmussen replaced the commonly employed term "precipitate" with "reactivate" directing thought toward the concept that an active agent has been muted to a latent agent, pre-existing *in toto*, that is somehow reactivated (presumably by reducing conditions in vasoconstricted skin) to become infectious. Latent herpes was thought by most to be present in the skin and this was an early example of a "skin trigger" hypothesis: an idea that has recently regained some attention and is further discussed below.

The early neural work by Goodpasture was supported by the formation of cutaneous herpes lesions following surgery in the trigeminal nerve tract. For example Carton and Kilbourne noted that within a few days after section (axotomy) of the fifth cranial nerve, oral or facial lesions occurred in 90% of patients if the ganglion and nerve were not destroyed (Carton and Kilbourne, 1952). The existence of a dormant form of herpes in the TG was supported by the failure of several groups to isolate the infectious agent from human TG (Burnet and Williams, 1939; Carton and Kilbourne, 1952). However, observation of HSV lesions in skin following "blowout" fractures that destroyed the nerve tract innervating the site of recurrence suggested that the virus must have already been in the skin (Hoyt and Billson, 1976). We now know the complexity of innervation of the facial skin provides a plethora of alternate routes for virus transport to the skin.

HSV-2 is discovered and VZV is proposed to be a virus that goes latent in sensory ganglia

Plummer determined that there were two serotypes of herpes simplex virus (Plummer, 1964), with different biological properties (Plummer et al., 1968), and was the first to show reactivation of HSV in the peripheral nervous system (albeit in the absence of a critical control as detailed below) (Plummer et al., 1967). But, in the absence of a tractable animal model for varicella zoster virus

(VZV) infection, the idea that zoster was the result of reactivation of latent VZV arose instead from a long-term epidemiological study (Hope-Simpson, 1965). Formal proof that the same strain of VZV could cause both chickenpox and, subsequently, shingles in the same patient was obtained 20 years later (Straus et al., 1984)

While HSV had been isolated from skin, saliva, tears and mucosal surfaces (especially genital mucosa) by many different groups in the absence of frank lesions, the source of this virus was unknown (reviewed in (Stevens, 1975a, b)). Transplantation of facial skin from sites where lesions occurred to other parts of the body did not result in virus recurrence at those sites, and most efforts to isolate virus from such skin either directly or following explant into culture failed (reviewed in (Finlay and MacCallim, 1940; Hill, 1985)). Notably, in the absence of lesions, very low titers are usually found (Agyemang et al., 2018). And yet, viral DNA is detectable by PCR in genital swabs much more frequently than is infectious virus as discussed below. A potential role for extra-neuronal sites of HSV latency in these phenomena should not be discounted.

During the 1950s to late 1960s laboratory animal models of herpetic disease were further developed, notably in rabbits, guinea pigs, and mice. The use of these models has led to several seminal observations, including the discovery that the two serotypes of HSV (HSV1 and HSV2) caused different pathologies in mice (e.g. HSV-2 is much more virulent than HSV-1 and causes larger pox (plaques) on liver following intraperitoneal injection) (Plummer, 1964; Plummer et al., 1974; Plummer et al., 1968). In 1970 the first experimental evidence that HSV2 remained in neural tissues for many months was presented. HSV2 was isolated *in vivo* after adrenalin injection, and also by cultivation of trypsinized rabbit neural tissue with indicator cells that presumably resulted from reactivation *in vitro* (Plummer et al., 1970).

What is the cellular site of HSV latency and reactivation?

Cook and Stevens confirmed and extended the observation that HSV remained latent in neural tissues by developing a method to reactivate HSV-1

from dorsal root ganglia (DRG) of mice infected via the rear footpad. Following infection, virus could be isolated from feet, the PNS, and CNS with replication ceasing about 8 days post infection (dpi). Thirty dpi DRG were either excised and homogenized immediately, or explanted and co-cultivated on monolayers of susceptible cells. Infectious virus was not detected in DRG that were directly homogenized, but virus was produced by the great majority of explanted ganglia 7 to 14 days post explant. This report is often considered the first definitive proof that HSV resides within sensory ganglia in a non-infectious or latent state and is reactivated following the stress of axotomy and explant into culture (Stevens and Cook, 1971). Numerous groups subsequently applied this approach to diverse human tissues and detected reactivated HSV1 in sensory and autonomic ganglia (Baringer and Swoveland, 1973; Bastian et al., 1972; Plummer, 1973).

Stevens and colleagues went on to show that latent HSV1 could be reactivated only from central and peripheral neural tissues, including the adrenal medulla, following intravenous injection of virus to induce an artificial viremia. This solidified the idea that nervous tissues are the predominant sites of HSV1 latency (Cook and Stevens, 1976). This group also sought to demonstrate that the sensory neuron was the site of viral reactivation that led to skin lesions through the use of immunohistochemical and electron microscopic techniques (Cook et al., 1974), but their data cannot be unambiguously interpreted since tissue was examined at 48 hours and later following axotomy, a time now known to be confounded by secondary spread of reactivated virus within the explanted ganglia. The virus could have reactivated in cells other than neurons and then spread to them. The earliest solid evidence for virus reactivation in neurons was provided by the studies McLennan and Darby who employed temperature sensitive (ts) mutants in mice. At a core body temperature of 38.5 degrees, replication of the ts mutants was restricted and viral proteins in mice in which nerves were resected to induce reactivation *in vivo* were confined to neurons (McLennan and Darby, 1980). These neurons "appeared" to die.

Are any essential viral gene products required for the establishment or maintenance of latency?

Watson et al. and Lofgren et al. explored whether viral proteins essential for the viral lytic cycle were required for the establishment of latency by intra-cranially inoculating mice with ts viral mutants and asking if the mutants could later be reactivated from brain tissue at the permissive temperature *in vitro* (Lofgren et al., 1977; Watson et al., 1980). The major viral immediate early transactivator ICP4 was initially thought to be essential for latency establishment, a hypothesis disproved much later by Stevens (Sedarati et al., 1993). Therefore, even viral proteins essential for viral replication like ICP4 are not absolutely required for the establishment or maintenance of latency.

Is the latent HSV genome integrated into the host genome?

Alpha herpesvirus genomes are linear double-stranded DNA that circularize upon entry into the nucleus (Sheldrick and Berthelot, 1975). The physical state of the viral genome during latency was the subject of much speculation. Fraser and colleagues first demonstrated "endless" HSV DNA in latently infected mouse brain tissue by restriction endonuclease digestion and Southern blotting. Later, this and other groups confirmed that circularized genomes are also the predominant form found in latently-infected mouse TG (Efstathiou et al., 1986; Fraser et al., 1981; Rock and Fraser, 1983). We now know that the great majority of HSV and other human alpha herpesvirus latent genomes exist as extra-chromosomal circular episomes (Azarkh et al., 2010). While integration events cannot be ruled out, they are not thought to be biologically relevant. Curiously, pseudorabies virus (PRV), an alpha herpesvirus of swine, is reported to persist largely in a linear form along with some circularized genomes (Rziha et al., 1986).

Spontaneous reactivation from latency in mice

HSV is commonly stated to not spontaneously reactivate in the mouse. This misconception is argued against by the consistent levels of spontaneous reactivation that have been reported for over three decades. In the late 70s and early 80s, Hill and Blyth developed an informative model of *in vivo*

reactivation, recurrence, and recurrent disease (recrudescence) in mice. Infection of the mouse ear pinna resulted in latent infections in superior cervical ganglia (SCG) that could be reactivated by diverse stimuli including UV light, DMSO on the pinna, or stripping of the pinna with cellophane tape (Hill et al., 1975). In this model, virus could be routinely isolated from the pinna in about 10% of the animals on any given day, consistent with later estimates of spontaneous productive reactivation seen within mouse TG (Blyth et al., 1984; Blyth et al., 1981). They showed that some procedures to induce *in vivo* reactivation resulted in detectable virus within ganglia and in skin with no evidence of recurrent disease, whereas other procedures induced virus reactivation within ganglia and recurrent lesions that contained infectious virus. This led them to postulate that there were "ganglionic triggers" and "skin triggers", the latter leading to recurrent lesions with the possible involvement of prostaglandins (known to enhance the replication of HSV in cultured cells), which were found to be elevated in the skin selectively by "skin triggers" and not "ganglionic triggers" (Harbour et al., 1978; Harbour et al., 1977), for review see (Hill, 1985). This hypothesis may still be relevant today as it might explain why infectious virus, and more frequently viral DNA, can be detected in the absence of a frank lesion.

The development of a sensitive procedure to detect viral protein expression in the whole ganglion allowed extensive analysis of latently infected ganglia for the expression of viral proteins at a single cell level (Sawtell, 2003). Whole ganglion IHC (WGIHC) allowed for detection of single neurons positive for viral proteins. A comprehensive long-term study revealed that in mice, as in humans (Agyemang et al., 2018), the frequency of spontaneous HSV reactivation (based on detection of TG neurons positive for infectious virus and viral proteins) declined during the time period between 15 and 40 days and then remained stable over the 240 days examined (Sawtell, 2003). The frequency of spontaneous reactivation in the TG was similar to the frequency observed in SCG, which also corresponded to the frequency of spontaneously positive mouse ear pinnas as reported by Hill and colleagues (Blyth et al., 1981) (reviewed in (Hill, 1985)). Analyzing sectioned ganglia for HSV proteins,

Feldman et al. similarly found that 10% of mouse TG pairs contained neurons positive for HSV proteins and called this "molecular reactivation" (Feldman et al., 2002). Later, the same group determined that 10% of unstressed mice were positive for infectious virus in TG (Margolis et al., 2007) demonstrating that molecular reactivation is consistent with the well-recognized spontaneous reactivation rate of mice as previously reported by others. This rate is likely dependent upon the viral strain, latent reservoir, and mouse strain. The animal models noted for spontaneous reactivation, rabbit and guinea pig, also have a time-dependent reduction in the frequency of reactivation, with the highest rate of spontaneous reactivation observed being 20-35 dpi. In rabbits, reactivation is quantified by virus shedding in tear films. The source of this virus is not known but thought to be neurons in TG. In guinea pigs, vaginal lesions are scored as reactivation events. Viral DNA can be recovered from these lesions (Roizman et al., 2013). Thus, spontaneous reactivation of HSV occurs in diverse animal models including mice, rabbits and guinea pigs.

The timing of viral reactivation in neurons in vitro or in vivo following stress.

The kinetics of induced reactivation, both *in vivo* and in explanted ganglia, remained a major gap in our understanding of alpha herpesvirus infections. Although knowing when a triggering event occurred that resulted in spontaneous reactivation is not possible, an increased frequency of ocular shedding occurred in rabbits over a period of two weeks following a three-day procedure of iontophoresis of epinephrine into corneas to induce reactivation (presumably initiated in the innervating neurons) (Toma et al., 2008). Likewise reactivation in explanted ganglia was thought to take several days or even weeks (Stevens, 1975a). However, the rate of reactivation was faster in subsequent studies. While a variety of stimuli caused virus reactivation and spread to the mouse ear pinna in 3 to 5 days, Harbour et al. found that virus could be isolated from a few SCG as early as one day post treatment (Harbour et al., 1983). Furthermore, using a mouse model of *in vivo* HSV1 reactivation induced by a 10 minute hyperthermic stress (42.5 C), virus was detected in TGs of mice as early as 14 hrs with 70% of mice positive by 22 hrs (Sawtell and Thompson, 1992a) (reviewed in (Webre et al., 2012)).

The same timing of primary reactivation events is evident in axotomized and explanted TG with virus being detectable by 14 hours post explant. However, unlike the *in vivo* situation where virus does not spread from the original reactivating neurons, virus rapidly spreads within the explanted ganglia (Doll and Sawtell, 2017; Pesola et al., 2005; Sawtell and Thompson, 2004). The potential for reactivation to occur in explants within 14 hrs and the rapid spread of virus within the ganglion is inconsistent with conclusions drawn from earlier studies that reactivation requires several days to occur in explanted sensory ganglia. However, it should be noted that many factors could affect the levels of latency established in animal models and in turn the subsequent frequency and perhaps timing of viral reactivation in vitro and in vivo. These parameters include the method of inoculation, the virus inoculation titer, the strain of virus employed, and strain of animals employed.

What is the role of the immune system in maintaining latency?

As is the case today, the role of the host immune system in alpha herpesvirus latency and reactivation was of great interest to investigators. However, immunosuppression of latently infected mice failed to induce reactivation or produce recurrent disease (Blyth et al., 1981; Hurd and Robinson, 1977; Stevens and Cook, 1973), with only a low incidence of recurrence in hairless mice treated with the immunosuppressant drug, prednisone, being observed (Underwood and Weed, 1974). Conflicting with these findings is the more recent hypothesis that CD8 T-cells directed against a specific epitope on glycoprotein B may play a role in the maintenance of HSV1 latency (Bourne et al., 2018; Held and Derfuss, 2011; Knickelbein et al., 2008; Lahmidi et al., 2017; Liu et al., 2000; Treat et al., 2017). In contrast to HSV, depletion of CD4 cells induced reactivation of simian varicella virus in Rhesus Macaques (Traina-Dorge et al., 2019). The role of the host immune system in HSV and VZV latency and reactivation (Gershon et al., 2015) is a topic worthy of a its own review article and is not covered in further depth here.

In vitro primary neuronal quiescence/latency and the NGF depletion reactivation model

The application of cultured primary neuron models of HSV latency led to several new concepts in reactivation. Establishment of a quiescent/latent like state in cells including neurons requires the use of antiviral compounds, or the use of replication deficient viral mutants (Harris and Preston, 1991; O'Neill et al., 1972; Wilcox and Johnson, 1987). During the late 1980s Wilcox and colleagues developed an *in vitro* model of HSV latency in cultured primary neurons. Quiescent/latent infections were established by infecting the neurons at very low MOI and maintaining them in the presence of human IgG (which contains significant amounts of anti-HSV antibodies). The antibodies were removed after fourteen days and the cultures remained latently infected for at least five weeks. These autonomic neurons derived from SCG were dependent on nerve growth factor (NGF) for their survival and deprivation of NGF (by anti-NGF antibody depletion) resulted in reactivation of latent virus (Wilcox and Johnson, 1987; Wilcox et al., 1990). These cultures were interesting in that they mimicked some of what was known about latency *in vivo*, including expression of the LATs (stable LAT introns) in a subset of neurons (Doerig et al., 1991). However, they did not parallel the *in vivo* situation in all cases. For example, thymidine kinase negative mutants established latency and reactivated within these cultures (Wilcox et al., 1992), which is not the case for either *in vivo* reactivation in mice, or reactivation in explanted latently-infected mouse ganglia (Field et al., 1982; Field et al., 1979; Field and Wildy, 1978; Pyles and Thompson, 1994b). Reactivation in these cultures could be induced by cyclic AMP repressors and was dependent on caspase three (Hunsperger and Wilcox, 2003). This model was eventually abandoned in part because latent infections (identified by LATs expression) and reactivation was rare at the cellular level and it was not thought likely that widespread death of neurons caused by NGF deprivation would be relevant biologically except with a few exceptions such as nerve resection caused by "blowout" fractures or surgery (Hunsperger and Wilcox, 2003).

A resurgence of similar in vitro models of HSV latency are the topic of recent reviews (Bloom, 2016; Thellman and Triezenberg, 2017) and new in vitro models of VZV latency are under development (Baird et al., 2019; Depledge et al., 2018b; Laemmle et al., 2019). The ability to differentiate pluripotent human embryonic stem cells into neurons is being exploited to investigate latency and reactivation of HSV and VZV (for example see (D'Aiuto et al., 2019; Kurapati et al., 2017; Markus et al., 2015; Pourchet et al., 2017; Sadaoka et al., 2018)). Rodent fetal and adult neuronal culture models are also being used and are discussed further below.

Using molecular approaches to address fundamental questions

Discovery of the latency-associated transcripts (LATs) or latency related RNAs (LRRs)

There was great interest in determining whether any viral gene expression was required to maintain latency. The first report of abundant RNA transcription during HSV latency in neurons was published by Tenser and colleagues. However, the entire viral genome was employed as a probe and so the genomic location of this transcription was not known (Tenser et al., 1982). In 1986 two abstracts describing significant RNA transcription from the terminal repeat of HSV-1 during latency were submitted to the 11th annual International Herpesvirus Workshop. Stevens and colleagues described the transcription of ICP0 during latency but employed a double stranded probe. Rock and Nesburn also detected transcription but from the strand opposite of that of ICP0. Stevens et al. became aware of Rock's strand specific finding, confirmed that the transcription they detected was from the strand opposite of that of ICP0 and first described the latency associated transcripts (LATs) (Stevens et al., 1987). Arguably, credit for this discovery might be shared.

The discovery of the LATs led to speculation that they might encode a protein or a function important for the establishment, maintenance and/or reactivation of latent infections. However, the earliest reports suggested that the LATs played no role in the establishment or reactivation of HSV latency (Sedarati et al., 1989) (reviewed in (Fraser et al., 1992)). Subsequent early studies led to

confusion and controversy, largely due to the complex nature of transcription from this region that was not appreciated at the time. We now know that long, short, and micro non-coding RNAs from both DNA strands as well as unusually stable introns are generated from the LAT locus, and that most of these can be detected during both latency and during acute viral replication. Presumably expression of the LATs introns and miRNAs during the lytic cycle are the result of read through transcription late in the infection cycle. Activity from the LAT promoter is restricted during acute infection (Batchelor and O'Hare, 1990). Additionally, there are reports noting potential open reading frames (ORFs) in some of these RNAs (Bloom et al., 1996; Thomas et al., 1999; Thomas et al., 2002), but no latency related protein has yet been convincingly described. In contrast, the recently discovered VZV LAT RNA does encode a protein (Depledge et al., 2018a). The role of the LAT locus will be discussed in greater detail below.

Quantification of the number of genomes in the latently infected ganglion by the polymerase chain reaction (PCR)

Katz et al. first employed PCR to detect latent viral genomes, which laid the groundwork for the use of qPCR to address questions in HSV latency (Katz et al., 1990). These investigators confirmed the early finding of Stevens and colleagues that latent infections in sensory neurons could be established even with HSV mutants that did not replicate efficiently, including TK- and ICP4-deficient mutants, and added to this list mutants lacking ribonucleotide reductase, ICP27, and DNA polymerase (Katz et al., 1990; Lofgren et al., 1977; Watson et al., 1980). More startling was the high numbers of viral genomes Katz et al. detected in latently infected ganglia (Katz et al., 1990). While many groups had observed only a few to several hundred neurons expressing LATs, PCR detected hundreds of thousands to millions of viral genomes (reviewed in (Phelan et al., 2017)). This suggests that either neurons contain multiple copies of the viral genome or that many neurons harboring latent genomes do not express detectable LATs, or both. We now know that there are 1,000s of latently infected neurons in the TGs of mice and humans, each containing 10-100s of viral genomes and most of these do not express

detectable LAT stable introns. (Sawtell, 1997, 1998; Sawtell et al., 2001; Wang et al., 2005b).

How many genomes are in individual latently infected neurons? Latency at the single neuron level

While the qPCR method provided unprecedented sensitivity for detecting and quantifying viral genomes in latently infected TG, methods to examine viral DNA in single cells were needed to address outstanding questions. For example, how many neurons were latently infected, were other cells also harboring viral genomes, how many viral genomes were within individual latently infected neurons, and was this genome number uniform or variable? Towards that goal, an approach termed contextual analysis (CXA) was develop. TGs were perfusion-fixed to stop all metabolic processes and to preserve DNA, RNA and protein, and the cells were dissociated and purified on Percoll gradients. Following treatment with DNase linked to beads to remove DNA from the outside of the cells, the intracellular viral genome content of individual neurons and other cells was analyzed by qPCR. Viral genomes were detected in about 1/4th of the neurons, only in neurons and with copy numbers ranging from ~1 to >10,000 (Sawtell, 1997). Similar percentages of positive neurons and ranges of viral genome copies were later detected in human TG neurons using laser capture technologies combined with qPCR (Wang et al., 2005b). Other single-cell approaches addressing these questions were applied to VZV in human TG (LaGuardia et al., 1999; Levin et al., 2003).

The viral genome copy number in individual latently-infected neurons varies across HSV1 strains (Sawtell et al., 1998). Neurons latently infected with the virulent HSV1 strains McKrae or 17Syn+ contained an average of 81 ± 36 or 50 ± 12 genome copies, respectively. However, neurons latently infected with the avirulent strain KOS contained only 7 ± 2 viral genome copies, which may explain why it reactivates less well than the other strains (Sawtell et al., 1998). Yet, the percentage of latently infected neurons was similar with all three strains, varying between 26 and 32%. The reason for the low genome copy

number in KOS latently-infected TG neurons is not known but may be a reflection of the mutations in the KOS strain that disrupt the US9 gene TATA box, truncate US9 at aa58, and eliminate the native stop codon of pUS8A (Negatsch et al., 2011). pUS9 is required for efficient anterograde axonal transport (Howard et al., 2013), and pUS8A is a neurovirulence factor (Kato et al., 2016). Thus, the multiple "round trips" of virus from the body surface to nerve cells and back again, which contributes to the higher viral genome copy number latency (Thompson and Sawtell, 2000), may not occur with the HSV1 KOS strain and its derivatives.

Do any viral proteins enhance the establishment of latency?

A number of viral genes contribute to the efficiency with which latency is established. However, many of these are required for efficient lytic replication at body surfaces or in the nervous system. Surface replication efficiency is correlated with the number of latent sites established and, therefore, viral proteins important for lytic replication such as thymidine kinase appear to increase latent infections indirectly (Katz et al., 1990; Thompson and Sawtell, 2000). Other virally-encoded enzymatic functions such as dUTPase and uracil glycosylase enhanced establishment of latency and reactivation frequency likely by promoting replication within the trigeminal ganglia (TG) (Pyles et al., 1992; Pyles and Thompson, 1994a). The lack of these enzymes may also have led to a higher mutation frequency in the latent viral genomes that could exert a deleterious effect on reactivation from latency (Pyles and Thompson, 1994b). In aggregate these and similar studies on diverse viral proteins (i.e. TK, ribonucleotide reductase, virion host shut off, and various glycoproteins) demonstrate that no viral proteins are absolutely essential for the establishment of latency (Aggarwal et al., 2012; Diefenbach et al., 2008; Field et al., 1982; Field and Wildy, 1978; Izumi and Stevens, 1990; Jacobson et al., 1989; Johnson et al., 1986; Lam et al., 1996; Meignier et al., 1988; Smith, 2012; Strelow and Leib, 1995; Wang et al., 2005a; Wang et al., 2010). However, all viral proteins likely contribute to the efficiency with which latency is established indirectly by enhancing virus replication at body surfaces and in the nervous system.

Viral lytic gene transcripts are present in latently infected ganglia in the absence of detectable reactivation

In a landmark study, Kramer and Coen found transcripts for ICP4 and TK in latently infected mouse TG. Quantification of these transcripts revealed that they were present at extremely low levels. These transcripts did not necessarily initiate at recognized promoters and may have been the products of random transcriptional activity (Kramer and Coen, 1995). Thompson and Sawtell also reported RNAs related to ICP4, ICP0, ICP22, ICP27 and ICP47 in TG latently infected with HSV1. Transcription was found upstream of normal mRNA initiation sites. Furthermore, the ICP0 transcripts were not spliced as is the case during productive infection, again suggesting these might not be properly processed mRNAs, but rather random transcriptional events (Thompson and Sawtell, 2006). The presence of HSV related RNAs during latency has complicated the interpretation of results that utilize viral transcriptional activity as a marker of reactivation. This is especially true in vivo where reactivation occurs in only one or a very few neurons per ganglion and the neuronal distribution of the RNAs detected is not known. Sensitive whole ganglion approaches to detect viral transcription at the single neuron level during latency and in vivo reactivation are needed.

In depth considerations of latency models and mechanisms

The role of VP16 transactivation function in the switch between latent and lytic infection

Virion protein 16 (VP16, α-TIF, pUL48) is produced as a leaky late protein and is packaged into the viral tegument. Upon viral fusion into cultured cells and concomitant loss of the envelope, VP16 is released from the nucleocapsid/ tegument and is thought to complex with the host cell factor 1 (HCF-1) in the cytosol. The HCF-1/VP16 dimer is transported into the nucleus where it forms the trimeric VP16-induced complex (VIC) with Octamer-binding protein-1 (Oct-1). The VIC binds to the consensus sequences, TAATGARAT, which are present in the promoters of the five immediate early (IE) genes of HSV. The acidic carboxy terminal domain of VP16 is a strong transcriptional activator,

which then initiates the virus lytic gene transcription program by transactivating the IE genes (Ace et al., 1988; Campbell et al., 1984; Kristie and Roizman, 1987; Mackem and Roizman, 1982; O'Hare, 1993), reviewed in (Wysocka and Herr, 2003). The importance of VP16 in initiating the viral lytic cycle is revealed at low moi. In its absence, entry into the lytic cycle (plaquing efficiency) is reduced 100 to 1,000-fold (Ace et al., 1989; Smiley and Duncan, 1997).

Based on what is known about the critical role played by VP16 in initiation of the viral lytic cycle at low moi, its absence or presence would also be expected to play central roles in the establishment of, or reactivation from latency. Indeed, in cultured neurons, VP16 deposited into axons as part of the tegument complex is not transported retrograde to the neural soma with the nucleocapsid, and in the absence of its delivery to neuronal nuclei latency establishment may be favored (Aggarwal et al., 2012; Antinone and Smith, 2010). Importantly, cultured neurons infected via axons favors the entry of virus into a quiescent/latent state (Hafezi et al., 2012) and the addition of pseudorabies virus tegument proteins to the neuronal soma can shift the outcome to the lytic cycle (Koyuncu et al., 2017), a result similar to early studies of HSV light particles containing tegument proteins including VP16 (Dargan et al., 1995). As discussed further below, VP16 and its unique regulation as a pre immediate early gene in neurons orchestrates the choice between lytic and latent viral programs.

The generation of a transactivation-deficient mutant (VP16TF), in1814, provided the critical tool needed to test the role of VP16TF during latency and reactivation (Ace et al., 1989). This mutant contains a 12 bp insertion at aa379 in VP16 that retains the protein's essential contribution to virion structure but selectively disrupts the interaction of VP16 with Oct-1, thus preventing the formation of the VIC (Ace et al., 1988; Ace et al., 1989; Campbell et al., 1984; Wysocka and Herr, 2003). This and other VP16TF mutants are severely deficient in replication at low moi (McFarlane et al., 1992; Preston and McFarlane, 1998; Smiley and Duncan, 1997). Despite this, in1814 establishes latent infections in mice and subsequently reactivates with wild type kinetics

from latently-infected ganglia explanted into culture (Steiner et al., 1990). Moreover, attempts to artificially-induce the expression of VP16 *in vivo* did not disrupt the balance between latent and lytic infection (Sears et al., 1991). These two influential reports seemingly disproved the hypothesis that VP16 played an important role in regulating HSV latency and reactivation.

However, whether the VP16TF studies truly ruled out an initiating role of VP16 in reactivation requires a nuanced consideration of experimental design and interpretation. First, the method used to evaluate reactivation is a central factor. The most widely used approach to evaluate the ability of latent virus within a ganglion to produce infectious virus is to dissect the infected animal, sever the ganglionic neurons by axotomy, and explant the ganglion into culture (Stevens and Cook, 1971), sometimes also dissociating or mincing the ganglion prior to explantation (Blyth et al., 1981; Leib et al., 1989a; Nicholls and Blyth, 1989). Using this method, virus was detected five to ten days post explant, which was not different than the five to six days required for wildtype and rescued virus reactivation (Steiner et al., 1990). At the time, these studies and others led to the conclusion that VP16 transactivation was not involved in reactivation from latency, and as a consequence the IE protein ICP0 superseded VP16 as the major contender for this important role. However, whether explant-based reactivation accurately models *in vivo* reactivation is an important consideration (Sawtell and Thompson, 2004; Thompson et al., 2009).

Although there is general consensus that ICP0 is required for efficient reactivation from latency. *In vivo*, ICP0 is required for efficient viral replication and establishment of latency (Cai et al., 1993; Cai and Schaffer, 1992; Everett, 2000; Halford and Schaffer, 2001; Leib et al., 1989b), and ectopic expression of ICP0 can disrupt latency in explanted ganglion neurons (Halford et al., 2001). The intersection of latency establishment levels and reactivation efficiency have clouded the issue but it is clear that at equivalent levels of latency (number of neurons and copy number profile), ICP0 null mutants enter the lytic cycle (based on viral protein expression) but do not generate

detectable levels of infectious virus (Thompson and Sawtell, 2006). Thus, while ICP0 is essential for amplification of infectious virus production during reactivation *in vivo* this protein is not required, and appears to play no major direct role, in the initiation of reactivation *in vivo*.

Whether the contribution of ICP0 is to the initiation or progression of reactivation may seem to be a hairsplitting issue, but as discussed above the goal of these studies is to identify the interfaces between the critical host cell factors and their mechanisms of regulating essential viral functions. An early effort to achieve this used a viral mutant, ΔTFI, that was designed to gain insight into the role of cis-acting sequences in the ICP0 promoter that potentially regulate ICP0 expression during acute infection, establishment of latency, and reactivation (Davido and Leib, 1996). A deletion removed a number of sites in the proximal ICP0 promoter including Sp1, NF-kB, C/EBP, F2, and four TAATGARAT motifs. Strikingly, although there were reduced levels of ICP0 protein expression at early times post-infection in Vero cells and *in vivo*, and reduced levels of blepharitis in CD1 mice and death in SCID mice, neither establishment of latency nor explant reactivation were different from the wild type or the marker rescue viruses (Davido and Leib, 1996).

Our group was also focused on ICP0 and its role in reactivation, but we were utilizing an *in vivo* reactivation model triggered by hyperthermic stress to the intact animal (Sawtell and Thompson, 1992b). Whether reactivation *in vivo* was different than explant reactivation was of great interest. Remarkably, in1814 failed to reactivate and reactivation of ΔTFI was greatly reduced compared to wild type or marker rescue viruses *in vivo* (Thompson and Sawtell, 2006) (Thompson et al., 2009). Importantly, viral genome copy number and the number of latently infected neurons were not different, ruling out the possibility that the establishment of latency by in1814 and ΔTFI were reduced in this model. These studies revealed for the first time that reactivation in the *in vivo* context may have distinct constraints, which are relaxed in the explant setting.

These studies also suggested that the TAATGARAT motifs in the ICP0 promoter might be important in regulating reactivation *in vivo*. Specific restoration of a single TATA box proximal TAATGARAT element to the ICP0 promoter in ΔTFI restored wild type *in vivo* reactivation frequency, emphasizing the importance of VP16 interaction at TAATGARAT elements for the regulation of ICP0 expression during *in vivo* reactivation (Thompson and Sawtell, 2006). It was not yet clear if reactivation of the VP16TF mutant was actually blocked, or whether in1814 did reactivate and was not detected because of the ~100-fold reduced plaquing efficiency of the mutant (Ace et al., 1989). Even with wild type virus the levels of virus recovered from a reactivation event *in vivo* are low, and virus does not spread within the ganglion during in vivo reactivation as it does during explant induced reactivation (Doll and Sawtell, 2017). Thus, reactivation of in1814 in vivo may have occurred without being detected. This was tested by the inclusion of hexamethylene bisacetamide (HMBA), a cyto-differentiating agent used to complement the plaquing deficiency of in1814 (Ace et al., 1989), in the assays, reactivation in vivo (infectious virus production) was not detected in assays in the presence of HMBA.

We hypothesized that some yet unknown regulatory mechanism altered the normally leaky late expression of VP16 to that of a pre-immediate early gene in a neuron destined to reactivate, thereby permitting it to coordinate the expression of the five important IE genes and initiate the lytic cascade (Thompson et al., 2009). If this were the case, then in contrast to ΔTFI, in which viral proteins were expressed in neurons "starting" to reactivate, neurons expressing viral proteins in in1814 infected TG would be absent following stress. Using a whole ganglion approach that allows quantification of viral protein expression at the individual neuron level, the lack of neurons expressing viral proteins was confirmed (Thompson et al., 2009). This extended our understanding of the reactivation process now to include VP16 transactivation of ICP0 (and presumably the other IE genes as well but this has not been directly tested) as a key and absolutely required step in the earliest stages of viral protein expression from the latent genome. Of great

importance, mutations in the VP16 promoter, including a four base substitution in a G+C rich region scoring as a putative overlapping Egr-1/SP1 site ablated the ability of the virus to exit the latent state (Sawtell and Thompson, 2016; Thompson et al., 2009; Thompson and Sawtell, 2019). These findings are consistent with the hypotheses that both a functional ICP0 and VP16 transactivation were required for efficient virus production, but de novo pre-immediate early expression of VP16 is required to initiate viral gene expression during reactivation.

Additional supporting data comes from a series of refined viral mutants containing single or double amino acid changes in the core domain critical for VIC formation (Sawtell and Thompson, 2016; Sawtell et al., 2011), including the first viral mutant to specifically disrupt the HCF-1 interaction. The *in vivo* reactivation phenotypes observed for these mutants align with predictions based on in vitro biochemical analyses of amino acids critical for VIC formation (Stern et al., 1989; Wysocka and Herr, 2003). Together, there is now strong evidence that VP16 interactions with Oct-1 and HCF are critical for the earliest stages for reactivation *in vivo* (Figure 1). That the core domain mutant phenotype was a result of a deficit in VP16 transactivation was supported further by the *in vivo* reactivation impairment of a mutant in which the core domain was intact, but the acidic transactivation domain (TAD) was lacking (Thompson et al., 2009). Importantly, all of these mutants reactivate following ganglion explantation, emphasizing the importance of the reactivation model used and the associated neuronal metabolic state. Efforts to develop and characterize additional *in vivo* reactivation triggers to ask whether hyperthermic stress is representative of other stressors (i.e., do diverse stressors flow into a common interface on the viral genome) is in progress. In vivo reactivation by additional triggers, for example, local skin trauma (see below), is dependent upon VP16 transactivation.

Modeling reactivation *in vitro*

The development of an *in vitro* latency model that provides a platform for dissecting host cell signaling pathways involved in viral genome entry into

latency, its maintenance, and its reactivation would be a valuable resource. Ideally such models could yield hypotheses testable in the more complex *in vivo* setting. Defining the limitations to these models and the approaches used to probe outcomes is critical to interpreting results obtained from them.

There is a growing number of *in vitro* latency models for both HSV and VZV that are the topic of several recent reviews (Bloom, 2016; Depledge et al., 2018b; Thellman and Triezenberg, 2017). *In vitro* models have been exploited to study both the establishment of latency and subsequent reactivation of VZV. Major efforts in the use of human embryonic stem cells for the study of VZV have led to several critical improvements, including axon-restricted infection protocols and methods to obtain enriched populations of specific types of neurons (Kurapati et al., 2017; Markus et al., 2015; Pourchet et al., 2017; Sadaoka et al., 2016; Sadaoka et al., 2018). These models are being explored for HSV latency and reactivation as well (Pourchet et al., 2017) and have brought forward the concept of a two-phase paradigm of transcription leading to reactivation. It should be noted that the establishment of latency in most of these models requires the use of antiviral compounds such as acyclovir, and latency is considered to be established after just a few days. It is not yet clear how this "forced" latency parallels latency in vivo. A primary limitation of these models is the disruption of the intricate context of the neuron and the very different mode of entry of the virus into the neuron. Roizman noted that satellite cells which surround neurons and communicate constantly with them may play roles in the establishment and maintenance of latency. Also noted is the fact that the acyclovir employed to suppress virus replication does not prevent the initial stages of reactivation and in effect might serve to eliminate those neurons in which spontaneous reactivation occurs (Roizman and Whitley, 2013). Immune functions also are thought to play important roles in these processes and are absent (for review see (St Leger and Hendricks, 2011)).

Reactivation in the superior cervical ganglion neuron culture model

As discussed above a primary SCG neuronal culture model of HSV quiescence/latency and reactivation was reported to require nerve growth factor (NGF) to maintain HSV latency/quiescence (Wilcox and Johnson, 1987, 1988; Wilcox et al., 1990). One current hypothesis is that the sustained signaling program mediated by NGF via the TrkA receptor, PI3 kinase p110a isoform, PDK1 and Akt represses HSV replication, maintaining the quiescent state (Camarena et al., 2010; Kim et al., 2012). A mutant containing a GFP-US11 fusion gene was employed so that fluorescence would mark lytic gene expression. The PI3 kinase inhibitor LY294002 was found to parallel NGF withdrawal in that the number of GFP positive wells (6 days post treatment) increased 3.5-fold from the baseline of 20% (time 0) to 70% (48-72 hrs post LY294002 treatment). Switching of wells from GFP negative to positive (a surrogate for reactivation) did not occur in 30% of wells and the number of neurons expressing GFP (the marker of lytic cycle employed) averaged 5% (48/1000) (Cliffe et al., 2015). The number of neurons containing latent viral genomes was estimated at 25-50% (stated in the discussion of (Kim et al., 2012)); thus, reactivation occurs in the range of 10-20% of infected neurons. PI3 kinase inhibition does not induce reactivation in most of the neurons in the well that contain the viral genome.

Presumably all of these embryonic SCG express the NGF TrkA receptor, indicating that additional factors are preventing viral genome activation following inhibition of PI3 kinase. Considering reactivation at the level of the individual latently infected neuron treated with the inhibitor is actually rare, sustained signaling through PI3 kinase and Akt would not seem to be universally required to maintain latency in this culture model (Camarena et al., 2010). This model system allowed detailed analysis of the role of individual growth factors; a study not practical in the *in vivo* context. Interestingly, these authors demonstrated that other growth factors expressed in SCG neurons, including epidermal growth factor (EGF) and glial cell-line derived neurotropic factor (GDNF), both of which act through the PI3-kinase pathway, differed in their ability to maintain quiescence/latency in these cultures. This was based

on the abilities of these growth factors to provide sustained signaling through PI3-K and Akt. More recently, the importance of neurotropic factors in adult sympathetic and sensory neurons was examined (Yanez et al., 2017). While NGF and GDNF withdrawal induced HSV1 reactivation in adult sympathetic neurons, in adult sensory neurons NGF deprivation had no effect. Neurturin (NTN) and GDNF withdrawal induced HSV1 and HSV2 reactivation, respectively. Thus, the nature of receptor tyrosine kinase (RTK) signaling appears to be a key host parameter that regulates the HSV1 quiescent to lytic activation in a subset of neurons even in this "homogeneous neuronal" model system. While the importance of neurotrophic factors during development is well established (Indo, 2018), in the adult nervous system a role for continuous NGF or other neurotropic support is less clear as is the relevance of neurotropic factor withdrawal as a trigger of reactivation (Skaper, 2017).

A two phase VP16TF dependent HSV reactivation model

In a second study using the SCG neuron culture model, the expression of 4-5 viral genes from IE, E, Lg_1, Lg_2 kinetic classes were profiled following LY294002 treatment (Kim et al., 2012). Two distinct phases of transcriptional activity were observed. During the first at 20-24hr (Phase I), extremely low levels of UL30 (658 RNA copies/sample*) and UL48 (3,470 RNA copies/ sample*) transcripts were detected when protein synthesis was inhibited (cycloheximide, which blocks all protein synthesis, was added 10 hrs after LY294002). In the absence of the protein synthesis inhibitor, neither infectious virus nor increases in viral DNA were observed during the 25hr Phase I. Phase II encompassed 48hr and onward, during which time modest replication of the viral genome (2.5 rise over baseline) and infectious virus production (250 pfu at 72 hrs) were detected. Thus, Phase II has the hallmarks of a lytic infection. Examination of the SCG neurons for viral protein expression during both phases included three proteins: VP16, ICP0, and ICP27. Curiously, VP16 was detected in 5% of the neurons/well at the time of LY294002 treatment, representing 10-20% of the infected neurons. Following LY294002 treatment and during Phase I, VP16-positive neurons increased 5-fold by 20hrs to 25% of cells which is 50-100% of the infected neurons/well. ICP0 and ICP27

expressing neurons were detected during Phase I but the frequency was not indicated. These proteins appear in clusters of neurons suggestive of cell to cell spread. Characterization of the spectrum of viral proteins expressed during Phase I will be important to determine. The assumption is made that the Phase I transcriptional burst is the source of the viral proteins and, furthermore, that these proteins drive the productive Phase II event. 10%-20% of infected neurons/well express VP16 *before* the transcriptional burst is induced by LY294002 treatment. Whether other proteins are present at this time is not reported but is quite important, as this may represent persistent infection.

This model includes a forced form of latency requiring suppression of viral replication by the anti-viral compound acyclovir (ACV) for a 1-week period, which is quite different than the events and time frame that occur *in vivo*. While it appears that the resulting cultures conform to latency as broadly defined, analysis of latent viral genomes in neurons has revealed that within a given nucleus their association with promyelocytic leukemia protein (PML) containing nuclear bodies and histone variants are features of latency. These are under investigation and likely to be important (Cohen et al., 2018; Lomonte, 2016; Maroui et al., 2016). The potential that this model is characterized by a low level of persistent infection is likely. *In vivo* the presence of viral protein has been examined extensively using approaches at least as sensitive as those employed in the in vitro studies. In vivo viral protein is rarely detected and, in extensive studies using wild type virus, its presence correlates with the detection of infectious virus in what are likely spontaneously reactivating neurons (Sawtell, 1998, 2003; Sawtell et al., 1999; Sawtell et al., 2001). From this perspective, neuronal cultures do not appear to model *in vivo* latency in TG. Even if only 0.1% of latently infected TG neurons expressed cytoplasmic VP16, these ~6 neurons would be detectable assuming similar expression levels of protein *in vivo* as in the cultured neurons. It is possible that host immune functions effectively "remove" these viral protein positive neurons. This is observed in vivo during the final stages of a reactivation event in reactivating neurons and late during the lytic cycle in ganglia (Doll et al., 2020;

Goodpasture, 1929). However, if this were the case and such neurons were removed through time, a reduction in the size of the in vivo latent reservoir would be expected over time, which is not observed (Thompson and Sawtell, 2011). These differences could arise from differences between SCG and sensory neurons, and examination of latent infection and reactivation at the neuronal level in SCG *in vivo* may be informative.

An additional feature of the SCG neuron culture model is the hypothesis is that VP16 remains sequestered in the cytoplasm with HCF-1 until HCF-1 is transported to the nucleus, which is triggered by LY294002 treatment. Whether these HCF-1/ LY294002 studies were done in infected or uninfected cultures is difficult to interpret (Kim et al., 2012). While the HCF-1 correlation is of interest, further studies are needed to test this hypothesis. Simple co-localization studies would provide strong support for the hypothesis that VP16 is sequestered in the cytoplasm by HCF-1 and is then translocated to the nucleus by HCF following LY294002 treatment. These cultures systems are ideal for addressing this type of question. The GFP-expressing neurons would be expected to result from the nuclear translocation of VP16 by HCF-1. Drawing these relationships is especially important since infection is expected to promote cytoplasmic to nuclear translocation of HCF-1 six days prior to LY294002 treatment. During acute infection of TG *in vivo*, HCF-1 relocates to the nucleus in many neurons. Importantly, this nuclear translocation of HCF-1 during acute infection does not return to the pre-infection cytoplasmic localization. Thus, in a latently infected ganglia, there is a persistent high percentage of neurons in which HCF-1 is nuclear.

A histone methyl/phospho switch is required for HSV reactivation

A third study using the SCG neuronal culture model revealed novel concepts regarding the mechanism of HSV reactivation with respect to Phase I and its requirement for Phase II. The conclusions drawn from this study are that (i) a methyl/phospho switch at H3S10 during Phase I is required for HSV reactivation and that (ii) the DLK/JIP-3 JNK pathway is directly linked to this switch. While this study has the potential to be quite important in providing

insight into the molecular signaling underlying HSV reactivation, aspects of the experimental design raise questions and emphasize the challenges faced. First, interpretation of the study relies heavily on the specificity of two inhibitors of JNK, SP 600125 and AS 601245. In an extensive evaluation of the selectivity of protein kinase inhibitors, SP 600125 was tested and 19 of the 69 protein kinases tested were inhibited with similar or greater potency than JNK isoforms (Bain et al., 2003; Bain et al., 2007; Fabian et al., 2005; Tanemura et al., 2010; Uitdehaag et al., 2012). AS 601245 was also determined not to be selective for JNK. Importantly, AS 601245 was found to be an exceptionally potent inhibitor of PIM1, PIM3 and GSK3 with IC_{50} values in the nanomolar range, 50-100 fold lower than the IC_{50} values for JNK1 and JNK2 (Bain et al., 2003). This does not mean that JNK was not inhibited in this study, but it does mean that JNK was not the only kinase inhibited. Thus, it is not possible to attribute the phenotypes observed to JNK inhibition without further studies. It is also possible that the observed effects of the inhibitor over time (Phase I 18hr) and (Phase II 72hr) are related to the inhibition of different kinases and the phenotypes observed in phase I are unrelated to phase II making interpretation of the results difficult.

A second issue arises from additional limitations of the experimental design. The viral genome related phenotypes, i.e., either transcription (ICP27 related RNA) or the histone modifications related to specific viral promoters were measured from the entire population of cells in a given well. There is no information on the distribution of these phenotypes among the many cells in the well. This is critically important because these averaged phenotypes are being connected to the very minor population of reactivating neurons in the well. Thus, while an event occurs in Phase II at 72 hrs post treatment (GFP expression in a small number of cells), whether the RNA detected in Phase I at 18hrs is or is not expressed in these particular cells (at 18 hrs) is not known. Whether the Phase I transcriptional activity has any effect on reactivation remains an open question. VP16 expression in this study was not reported but could have been informative. This becomes even more complex given that the limited number of modified histones detected cannot be ascribed to the

transcriptional activity from any given viral promoter. The fact that GSK-J4 treated neurons during phase I showed no difference in the relative levels of ICP27 RNA in the total cell population does not mean that the distribution of the RNA did not change among individual neurons.

As mentioned, the use of inhibitors or depletion strategies to eliminate or reduce the function of key host proteins without the required supporting studies is problematic. While this type of approach can indicate the importance of that function to the reactivation process (for example GSK-J4 in explant reactivation) whether this is a direct effect (i.e. methylase function on the viral genome) or more likely, a result of effects on host cell functions that then influence reactivation is often difficult to parse out. Additional studies are required before drawing direct links between the host function and the viral genome. For reference, a more detailed comparison between the in vivo reactivation model and the SCG model with respect to latency and reactivation is shown in Figures 2 and 3.

HCF-1: A potential VP16 independent inducer of reactivation

HCF-1 is an essential transcriptional co-regulator that is important for several aspects of cell proliferation based on cell culture genetic studies (reviewed in (Minocha et al., 2016; Wysocka and Herr, 2003; Zargar and Tyagi, 2012)). HCF-1 protein is located at the transcriptional start sites of many genes (over 5400 genes in Hela cells), associating with both sequence-specific DNA-binding proteins, chromatin-modifying enzymes (Dejosez et al., 2010; Michaud et al., 2013), transcription initiation factors, and elongation complexes (Alfonso-Dunn et al., 2017). As such, HCF-1 is a scaffold protein central to normal cell function, bringing together regulatory effector proteins. As discussed above, HCF-1 was first identified as a component of the VP16 induced complex (VIC) and a similar complex Oct-1/ORF10/HCF-1 was identified for IE gene expression regulation in VZV (Narayanan et al., 2005).

The potential ability of HCF-1 to be recruited to the viral genome through binding partners other than VP16 raises the possibility that HCF-1 could play a

role in initiating expression of the viral IE genes apart from its interaction with VP16. This hypothesis was proposed more than a decade ago (Kristie et al., 2010; Whitlow and Kristie, 2009) and is a viable possibility during high multiplicity infection (cultured cells) or explant induced reactivation. These are both *ex vivo* contexts in which viral IE gene expression and viral lytic cycle entry are not dependent upon VP16 transactivation function (Ace et al., 1989; Steiner et al., 1990). However, *in vivo* replication in the absence of VP16 is severely impaired at peripheral sites of infection and in the nervous system (Sawtell and Thompson, 2016; Sawtell et al., 2011; Thompson et al., 2009), indicating that if HCF-1 is facilitating IE gene expression in the absence of VP16 it is quite limited in the *in vivo* context. Consistent with this, hyperthermic stress induced reactivation is VP16 dependent. Although the possibility that distinct triggers could interface with the viral genome *in vivo* in diverse ways has not been fully explored, a VP16-independent role for HCF-1 in driving IE gene expression *in vivo* does not currently have support. Identification of potential HCF-1 binding partner(s) that recruit HCF-1 to the latent viral genome in the absence of VP16 would support the hypothesis. However, VP16 transactivation function mutants do not express any detectable viral proteins in latently infected neurons in vivo following reactivation inducing stresses (Sawtell and Thompson, 2016; Sawtell et al., 2011; Thompson et al., 2009). This strongly suggests any such VP16 independent recruitment of HCF-1 to the latent genome does not initiate viral reactivation and detectable viral protein expression.

JQ1 and reactivation: manipulation of an epigenetic reader perturbs viral homeostasis

Epigenetic features of herpesvirus genomes, including the chromatin associated with latency and changes observed during reactivation of the alpha herpesviruses are the subject of multiple reviews (Balakrishnan and Milavetz, 2017; Lieberman, 2016). Newer findings include the role played by CTCF binding sites on the HSV genome and their cognate binding proteins to the establishment and maintenance of latent infections (Washington et al., 2018a; Washington et al., 2019).

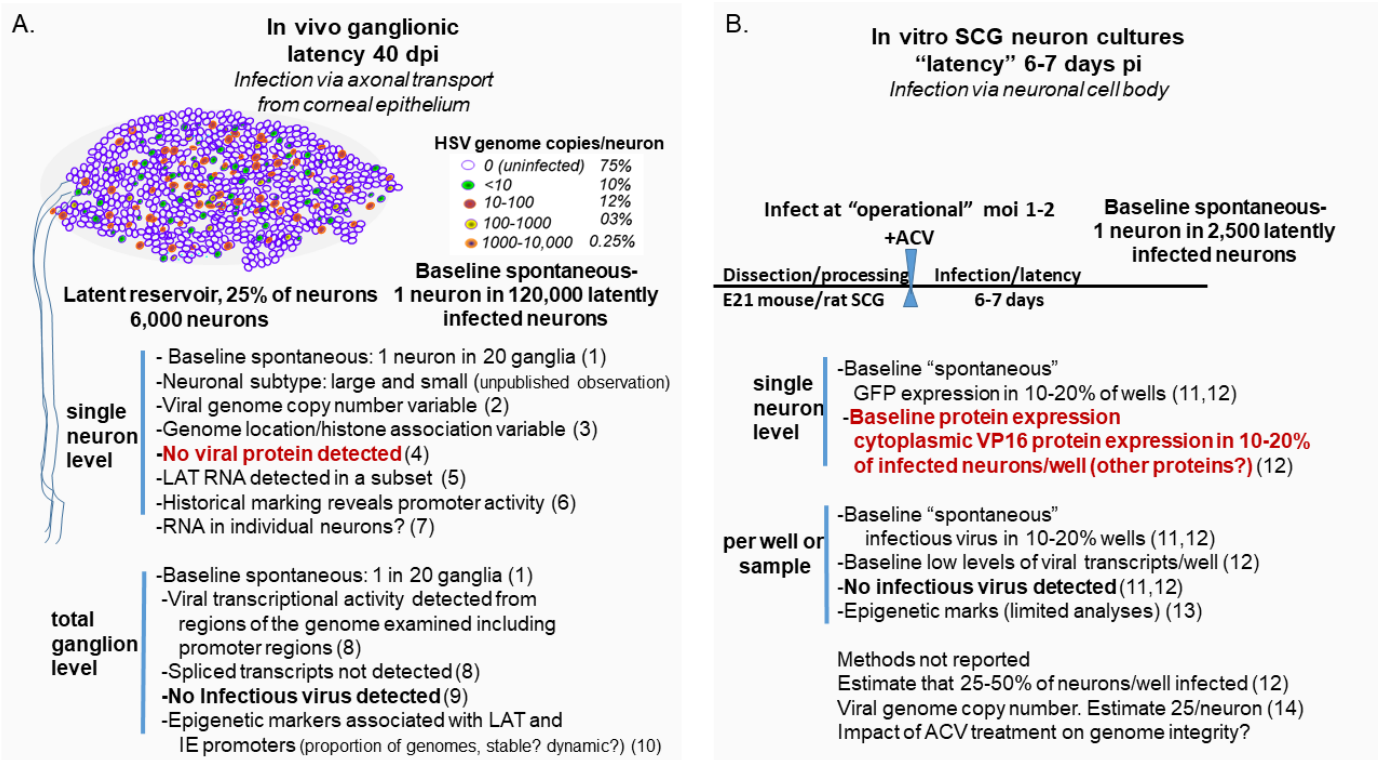

Figure 2. Comparison of latency mouse TG in vivo and in vitro in SCG rodent neuron model. The SCG neuron model appears to have hallmarks of a persistent infection including viral protein expression in 10-20% of latently infected neurons/well and a "spontaneous reactivation rate 50-fold greater than observed in vivo. References (1)= for example (Sawtell, 1998); (2) = for example (Sawtell et al., 1998); (3)= for example (Cohen et al., 2018); (4)= for example (Sawtell and Thompson, 2016); (5)= for example (Stevens et al., 1987); (6)= for example (Proenca et al., 2008; Proenca et al., 2015; Russell and Tscharke, 2016); (7)= (Russell and Tscharke, 2016); (8)= (Thompson and Sawtell, 2006); (10) reviewed in (Knipe, 2015); (11+12)= (Camarena et al., 2010; Kim et al., 2012); (13)= (Cliffe et al., 2015); (14)= (Kobayashi et al., 2012).

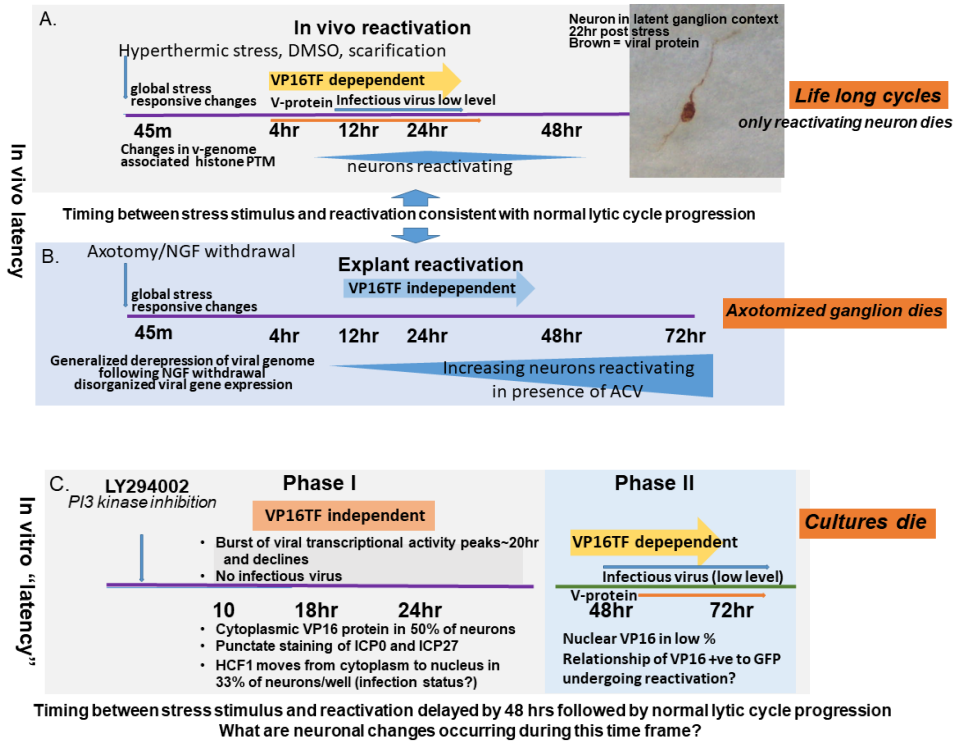

Figure 3. Comparison of the timing of reactivation in latently infected ganglia and SCG cultured neurons. Infectious virus can be detected in latently ganglia following stress in vivo (A) or axotomy (B) within 14 hrs post stimulus. In conrast, infectious virus is not detected until 48 hrs after PI3 kinase inhibition(C).

The availability of small molecule inhibitors of epigenetic readers and writers designed as potential cancer therapeutics have provided probes to test for effects on viral transcription and replication. An epigenetic compound library screen to test for effects on HSV replication in Vero cells revealed that BET (Bromodomain and Extraterminal Domain) protein inhibitor JQ1 increased HSV1 and 2 lytic infection by as much as 100 fold (Ren et al., 2016). This effect was specifically dependent upon the BET protein, Brd4. Paradoxically, knock down of Brd4 inhibited viral replication. The requirement for Brd4 for

transcription, replication and other functions is reported for a number of viruses (McKinney et al., 2016; Wang et al., 2013).

Alfonso-Dunn, et al, tested the ability of JQ1 to modify explant reactivation, finding that consistent with the effect of the cyto-differentiating agent HMBA (that also inhibits Bet bromodomain proteins), JQ1 modestly increased the number of neurons expressing viral protein at 48 hrs post explantation from 2 to 7 (Alfonso-Dunn et al., 2017). The shared function in the release of positive transcription elongation factor, P-TEFb, from the inhibitory 7SK small nuclear ribonucleoprotein (7SK snRNP) complex of the two compounds (HMBA and JQ1) and their well characterized effect on HIV latency, suggest that transient release of P-TEFb facilitated reactivation, although further studies are required to demonstrate this (Abner et al., 2018; Bensussen et al., 2018; Khoury et al., 2018). The effect of JQ1 was also tested *in vivo* with latently infected mice being injected with JQ1 twice at 24-hour intervals. Increased levels of ICP27 and gC transcripts were detected in TG and 2-3-fold enhancement of viral DNA in some eyes were detected at 48hrs. These findings are intriguing but in the absence of infectious virus or viral protein detection, whether these changes represent bone fide reactivation remains an open question. Nonetheless, the ability of JQ1 to perturb viral latency *in vivo* is significant. JQ1 is a broad BET domain inhibitor and not selective for Brd4. Thus, in the absence of additional studies, the mechanism underlying the observed phenotypes is yet to be illuminated.

Frequency of HSV2 genital shedding implies HSV2 is continuously "reactivating" in the ganglion. Corollary: the reactivating neuron must survive the production and release of infectious virus

Multiple studies have now solidified not only the shedding frequency but also the peak amounts of viral DNA and rate of decay with which HSV2 DNA is detected in human genital swabs collected from a specified regional map (Agyemang et al., 2018; Ramchandani et al., 2017; Sacks et al., 2004). These studies have substantially altered what had been established concepts of the frequency of viral presence at the genital mucosal surface and raise important

questions regarding the transmission potential of these frequent PCR detectable shedding events. The results of these studies were best fit by a mathematical model that assumed a nearly constant release of small numbers of virions from ganglionic neurons (Schiffer et al., 2009). While a legitimate hypothesis, it is worth noting that the source of viral DNA detected by PCR in the genital swab is not known but rather assumed to be the innervating sensory ganglia. Thus, the concept is based on a mathematical model which assumes that the only source of viral DNA is (or could be) the sensory ganglion. More recent mathematical modelling from this group is focused on immune control in the mucosa (Gottlieb et al., 2017; Schiffer et al., 2018). Based on the shedding frequency, neurons presumably survive reactivation and are able to undergo repeated reactivation events; otherwise, frequent shedding cannot easily be explained. However, the ability of HSV2 to persist in other cell types is well described in the older literature, including HSV in hair follicles, skin, T cells, B cells, and myeloid cells (Al-Saadi et al., 1988; Claoue et al., 1987; Easty et al., 1987; Nicholls et al., 1996; Scriba, 1977, 1981; Shimeld et al., 1986; Shimeld et al., 1982; Tullo et al., 1985). Additionally, the idea that the reactivating neuron must survive based on this level of shedding does not appear to consider the number of ganglia that could be potentially alternate sources for the viral DNA in genital skin or mucosa. Progress in developing animal models to gain insight into the contribution of other ganglia to vaginal reactivation (Bertke et al., 2007; Lee et al., 2015; Pieknik et al., 2019; Yanez et al., 2017) as well as developing the tools in HSV2 to test hypotheses *in vivo* (Kawamura et al., 2018; Pieknik et al., 2018) will undoubtedly deepen current understanding of HSV1 and 2 reactivation and the distinctions between them.

A recent report detailed the resolution of reactivation of HSV1, characterizing the surrounding cellular context and morphological changes in individual neurons undergoing reactivation in vivo (Doll et al., 2020). Infectious virus was detected in a high percentage (60-80%) of the trigeminal ganglia at 20 hours post-reactivation stimulus but declined by 48 hours post-stimulus (0-13%). Importantly, in addition to intact viral protein positive neurons, fragmented viral

protein positive neurons morphologically consistent with apoptotic bodies and containing cleaved caspase-3 were detected. The frequency of this fragmented phenotype increased through time post-reactivation. These fragmented neurons were surrounded by Iba1+ cells, consistent with phagocytic removal of dead neurons. Treatment with anti-CD4/CD8 depleting antibodies did not alter this outcome. In contrast, blocking viral DNA replication during reactivation prevented neuronal fragmentation within the 48-hour time frame, even though viral protein was expressed. These findings suggest that reactivation in vivo of HSV1 is resolved through the destruction of the neurons which appears to be independent of antigen-mediated T cell cytotoxicity but does require viral replication. Similar studies with HSV2 are needed.

The role of the latency associated transcription unit (LATU) in latency and reactivation: a reexamination of what we think we know

Functions of products from the LATU locus

As more knowledge concerning the rather subtle effects that non-coding RNAs exert on many different processes including transcription, translation, post translational modification, and chromatin structure have been described, the appearance that the non-coding RNAs generated in the LAT locus had no or minor effects on latency/reactivation can be attributed at least in part to the general lack of assays sensitive enough to measure their influences individually and in aggregate (Chen and Aravin, 2015; Dhanoa et al., 2018; He et al., 2014; Zheng et al., 2017). Space restrictions prevent presentation in detail of all of these earlier studies on the LAT locus and its functions here, and readers are directed to a recently published comprehensive review on this subject (Phelan et al., 2017). In brief, the latency associated transcript (LAT) is a ~8.5 kb mRNA that maps largely to the long terminal repeat of the virus and is antisense to the ICP0 and ICP34.5 genes (Wechsler et al., 1988). It is the only mRNA abundantly transcribed during latent infection of neurons and it expression is strongly repressed during acute infection by an ICP4 binding site that includes the transcriptional start site of LAT (Batchelor et al., 1994). The LAT mRNA is spliced, which produces two co-linear introns of 2.0 and 1.5 kb that share the same splice donner and acceptor sites, differing by an internal

splice in the 5' end of smaller intron (Farrell et al., 1991; Phelan et al., 2017). These introns are stable due to an unusual lariat structure that is not resolve efficiently and are partially antisense to the 3' end of the ICP0 mRNA and together are known as the LATs (Mukerjee et al., 2004). The LAT mRNA does not encode any known proteins and has a short half-life. It is processed by cellular mechanisms into several microRNAs and stable small RNAs, the functions of which are the subjects of current research efforts. The LAT locus is more complex, having multiple transcripts originating from both strands, none of which encode known proteins. These mRNAs are also processed into microRNAs and are expressed during lytic and latent infections at low levels. The patterns of microRNA expression and sequences from the LAT transcriptional unit locus (LATU) are not the same for HSV-1 and HSV-2. Curiously, deletion of a small ~200 bp region of the basal LAT promoter including the TATA box and mRNA start site perturbs the expression of most if not all of the transcripts originating in the LATU (Phelan et al., 2017). Thus, many of the phenotypes originally attributed to "LAT", may actually map to other nearby regions or on the strand opposite of the LAT mRNA (Phelan et al., 2017; Roizman et al., 2013).

Leib et al. provided evidence that a LAT null mutant that replicated normally *in vivo* displayed a reduced reactivation phenotype in a dissociated ganglia explant model. One caveat was that the slot blot analysis of latent viral genome DNA may not have been sufficiently sensitive to determine a defect in the establishment of latency by the mutant (Leib et al., 1989a). Sawtell and Thompson first determined that LAT locus mutants establish latent infection less efficiently than wild type. While the mechanism(s) involved still remain controversial, this phenotype is one that has stood the test of time and is shared among the various mouse rabbit and guinea pig models (Phelan et al., 2017; Roizman et al., 2013) (Krause et al., 1995; Perng et al., 2000b; Sawtell and Thompson, 1992a; Thompson and Sawtell, 1997, 2001).

Two important questions remained. Is the LAT locus required solely for the efficient establishment and/or maintenance of latency, or does it also exert a

positive effect on reactivation from latency? What is the mechanism underlying the reduced establishment of latent infections and/or the increase in reactivation from latency? Answering these questions required more sensitive quantitative assays. Using a quantitative single neuron approach, it was found that fewer latent infections were established in mice infected with LAT-null mutants. When methods were employed to reach equivalent latency between wild type and mutant strains, no evidence for a specific defect in viral reactivation from latency was detected (Thompson and Sawtell, 1997). Counting the total number of neurons present in uninfected or latently infected mouse ganglia latently infected with wild type or LAT null mutants in strain 17syn+ revealed twice the number of neurons were killed in mouse TG infected with the LAT-null mutants compared to the rescuants and wild type. Very low levels and similar numbers of neurons undergoing apoptosis were seen in both groups (Thompson and Sawtell, 2001). Whether transcription from the LATU locus exerts a negative effect on virus entry into the lytic cycle in neurons and in its absence viral lytic infection kills more neurons, or something made from the LATU protects neurons from cytopathic effects associated with HSV replication and thereby increases successful establishment and/or reactivation frequencies, is unknown. Whatever the mechanism, LATU expression reduces lytic cycle gene expression during acute infection of mouse TG neurons (Garber et al., 1997). Increased neuro-invasiveness was displayed by mutants lacking specific regions of the LAT locus (Jiang et al., 2016; Jiang et al., 2015; Thompson and Sawtell, 1997). More recent findings demonstrate that the LAT transcription program is expressed early and first in TG neurons during acute *in vivo* infection. Thus, LAT is expressed at a time when it could negatively influence entry into the lytic cycle in neurons. (Sawtell and Thompson, 2016).

HSV LATs and neuronal apoptosis

The hypothesis that the LAT locus protects newly infected sensory neurons from apoptosis immediately after the lytic cycle has ended in the ganglia (hypothesized to be induced as the result of toxic viral virion proteins) is steeped in the lore of HSV1 latency (Perng et al., 2000a). As recently reviewed

this may not be true (Roizman et al., 2013). Significantly, Perng et al. reported extensive apoptosis (>25% of neurons per section) in 66% of TG sections infected with a LAT mutant and in 4% of sections from TG infected with the rescuant or from TG of uninfected rabbits on day 7 postinfection (Perng et al., 2000a). Unfortunately, this experiment has never been repeated. A manuscript that is often cited to support the idea that the LAT locus blocks apoptosis in the mouse model actually reported very low numbers of neurons undergoing apoptosis (<0.01 to <1%) 30 days postinfection with LAT mutants (Branco and Fraser, 2005), and the relationship of this minor amount of TUNEL staining at 30 day postinfection to the "extensive apoptosis" detected by (Henderson et al., 2004; Jin et al., 2005) and colleagues in rabbit TG only at day 7 postinfection, but not at earlier or later times is not obvious. Further studies with mutants in which anti-apoptotic proteins were inserted in place of LAT are considered to support this hypothesis (Jin et al., 2005; Perng et al., 2002). However, only the downstream phenotypes of ocular shedding (rabbit) or explant reactivation (mouse) were examined, and not the apoptosis of neurons per se. That Wechsler and colleagues were unable to demonstrate more extensive apoptosis in mouse TG infected with LAT null mutants but did find suppression of the reactivation deficient phenotype in mice suggests other mechanisms may have been responsible. The pleomorphic functions of the inserted proteins and the absence of assessment of apoptosis precludes attributing the observed effect on the inhibition of apoptosis. Further experimentation is warranted to test this hypothesis, and experimentation might be productively extended to studies in human neuronal cultures as well.

Ectopic expression of portions of the LAT locus interferes with apoptosis induced with drugs like etoposide in cultured cells (Jin et al., 2003; Peng et al., 2003). Two small non-coding RNAs from this locus can inhibit viral replication and can also cooperate to reduce apoptosis in cultured cells (Shen et al., 2009). These functions may be important during productive infection by the virus, which encodes many functions that inhibit apoptosis including ICP0, ICP34.5, gJ, gD, US3, and ribonucleotide reductase (for review see (Yu and He, 2016)).

The LAT locus serves to maintain latent infections

Regardless of the mechanisms involved, it is clear that the latency associated transcript locus of HSV-1 is required for long-term maintenance of reactivation competent latent infections (Thompson and Sawtell, 2011). Mice were infected with a LAT null mutant and a rescued variant maintained for 30 days post inoculation at which time were each divided into two groups. One half of the latently infected mice were maintained an additional 294 days. Additional mice from these same groups were subjected to the hyperthemic stress procedure an average of 2.5 times per week during 280 days after the initial 30-day period. After week 40 the mice receiving the multiple stress treatments were rested for two weeks, and then all of the mice were subjected to a final stress procedure to induce reactivation in vivo. Two measures were employed to quantify the outcome in TG at 22 hours post HS, (i) the quantification of infectious virus and (ii) quantification of the number of neurons exiting latency using whole ganglion immunohistochemistry (WGIHC) for viral proteins.

Does the LAT locus specifically enhance reactivation from latency in the rabbit model?

As discussed above, when the level of latency established by LAT null mutants in mouse TG is equivalent to that of wild type virus, stress induced reactivation from latency is also equivalent, and even more efficient on a per latently infected neuron basis (Thompson and Sawtell, 1997). This locus is also required for the long-term maintenance of latent infections (Thompson and Sawtell, 2011), emphasizing that in the mouse model it serves to reduce lytic infection. However in the rabbit a number of studies support the idea of a direct role for the LAT locus in promoting viral reactivation from latency (Bloom et al., 1996; Hill et al., 1990) as reviewed in (Toma et al., 2008). This may indeed be a difference between the rabbit and murine models as in the rabbit LAT locus transcription is associated with an increase in lytic gene transcripts such as ICP4, TK and gC, (Giordani et al., 2008) whereas the exact opposite, a decrease in these transcripts, was seen in the presence of an intact LAT locus (Garber et al., 1997).

A recent report suggests LAT null mutants may have a specific reactivation deficit. Using an interesting new approach that circumvents this problem, Bloom and colleagues expressed hammerhead ribozymes directed at the 5' exon of the primary LAT (or a control ribozyme) in TG neurons of rabbits latently infected with wild type HSV1. A significant reduction of induced reactivation was found in rabbits receiving the anti-LAT ribozyme (Watson et al., 2018). If this approach and result stand the test of time, a pro reactivation function of the LAT locus will have strong support. Similar approaches in the mouse model could reveal such a pro-reactivation function of LAT as well.

HSV is also well documented to establish latent infections that can be reactivated in a variety of non-neural tissues including denervated mouse footpads or footpads following long-term acyclovir treatment, various non-neural tissues from rabbit, mouse, rat, and guinea pig including cornea, conjunctiva, and hair follicles in humans and LAT expression has been detected in many of these ((Al-Saadi et al., 1988; Claoue et al., 1987; Easty et al., 1987; Nicholls et al., 1996; Scriba, 1977, 1981; Shimeld et al., 1986; Shimeld et al., 1982; Tullo et al., 1985) and see (Kennedy et al., 2011) for a relatively recent review). These extra-neural sites of latency are usually dismissed but may be biologically relevant, especially in recurrent ocular shedding and eye disease in the rabbit and humans. Whether the LAT locus plays a direct role in promoting reactivation from such sites deserves further study.

How does the virus go latent in the first place?

The ubiquity of HSV1 in the human population is the result of the efficient establishment of latent infections that subsequently reactivate throughout the host's lifetime and transmit to new hosts. A long-lived question is how the virus evolved to interact with sensory neurons to promote the efficient establishment of latency, which requires viral replication within the sensory ganglia but also a self-limiting mechanism to prevent neurovirulence and transmission into the CNS. The discovery of the LAT (Stevens et al., 1987) and mapping of its promoter (Dobson et al., 1989) provided a powerful tool to examine latent

phase transcription in TG neurons by *in situ* hybridization for the LATs or by using LAT promoter/beta-galactosidase reporter mutants (Margolis et al., 1992; Sawtell and Thompson, 1992a; Simmons et al., 1992; Speck and Simmons, 1992).

A somewhat unexpected result common to all these studies was that the acute stage of infection in the TG (examined at 48 hours postinfection and later) was characterized by neurons expressing viral proteins and others expressing only the LATs or the LAT promoter. A few neurons were detected that expressed both, but these were rare. These data were interpreted to suggest that latent and lytic pathways in neurons were district from each other (Lachmann and Efstathiou, 1997; Margolis et al., 1992; Sawtell and Thompson, 1992b; Simmons et al., 1992; Speck and Simmons, 1992), and that the lytic and latent viral transcriptional programs in neurons were regulated in ways that were not understood. Hypotheses were proposed to explain this transcriptional programmatic duality including differential lytic/latent expression in neuronal subtypes, (Cabrera et al., 2018; Yang et al., 2000), transition from some acute gene expression into the latent program (Proenca et al., 2008), failure of VP16 to be transported to neuronal nuclei (Sears et al., 1991; Steiner et al., 1990), alternative regulation of gene expression (Kosz-Vnenchak et al., 1993), strict nonnuclear compartmentalization of essential co-activators such as HCF-1 specifically in neurons (Kristie et al., 1999), or that its interaction with alternate neuronally expressed cofactors like HCF-2, Oct-2 or Brn3 might inhibit its function (Liang et al., 2009; Lillycrop et al., 1993; Nogueira et al., 2004). Any or all of these mechanisms may act to constrain virus entry into the lytic phase in neurons, although replication in TG neurons is required to promote the efficient establishment of latency (Field et al., 1982; Field and Wildy, 1978; Katz et al., 1990; Kramer and Coen, 1995; Thompson and Sawtell, 2000), for review see (Hill, 1985; Roizman et al., 2013). To avoid injury or death of the host, this entry into the lytic cycle in neurons must be tightly controlled.

Several lines of investigation support the concept that VP16 and its unusual regulation in neurons plays a central role in the choice between latent and lytic

infection (Sawtell and Thompson, 2016; Sawtell et al., 2011; Thompson et al., 2009). In the absence of the VP16 transactivation function latency is favored over acute viral replication in TG neurons *in vivo* (Sawtell and Thompson, 2016; Sawtell et al., 2011; Steiner et al., 1990; Thompson et al., 2009). During retrograde transport of HSV nucleocapsid/tegument through axons in cultured neurons loosely associated tegument proteins including VP16 are left behind and this presumably favors quiescent/latent infection in cultured neurons infected via axons (Aggarwal et al., 2012; Diefenbach et al., 2008; Hafezi et al., 2012; Koyuncu et al., 2017). Live-cell analysis by fluorescence time-lapse recording of capsid/tegument complexes transporting retrograde in axons of cultured sensory neurons has revealed that while the majority of VP16 is lost from capsids prior to transport to the neural soma, small amounts of the protein co-transport in this experimental setting that could promote a small amount of lytic neuronal infection (Antinone and Smith, 2010; Smith, 2012). This mechanism likely promotes latency in neurons (Hafezi et al., 2012; Koyuncu et al., 2017). Importantly, in all *in vitro* and *in vivo* models, significant viral replication occurs in cultured and sensory ganglion neurons demonstrating that these neurons can support lytic viral replication if the lytic cycle is initiated.

Replacing the VP16 gene promoter with that of another leaky late gene, VP5, resulted in normal viral replication on the mouse eye, but >100-fold reduced virus replication in TG (Thompson et al., 2009). Likewise, mutations in putative factor binding sites near and downstream of the VP16 TATA box also resulted in wild type replication on mouse eyes, and greatly reduced viral replication in TG (Sawtell and Thompson, 2016). In the latter study promoters of various kinetic classes (LAT, immediate early ICP0, and leaky late VP16) were employed to drive expression of E. coli beta-galactosidase (B-gal) in otherwise wild type HSV1 strain 17syn+. Whole ganglion analysis of viral protein expression by immunohistochemistry and B-gal expression from the diverse promoters was employed to examine the earliest events that occur within TG neurons infected via axons from the cornea. The expected asynchrony of TG neuron infection was evident in the analysis of the time postinfection and the

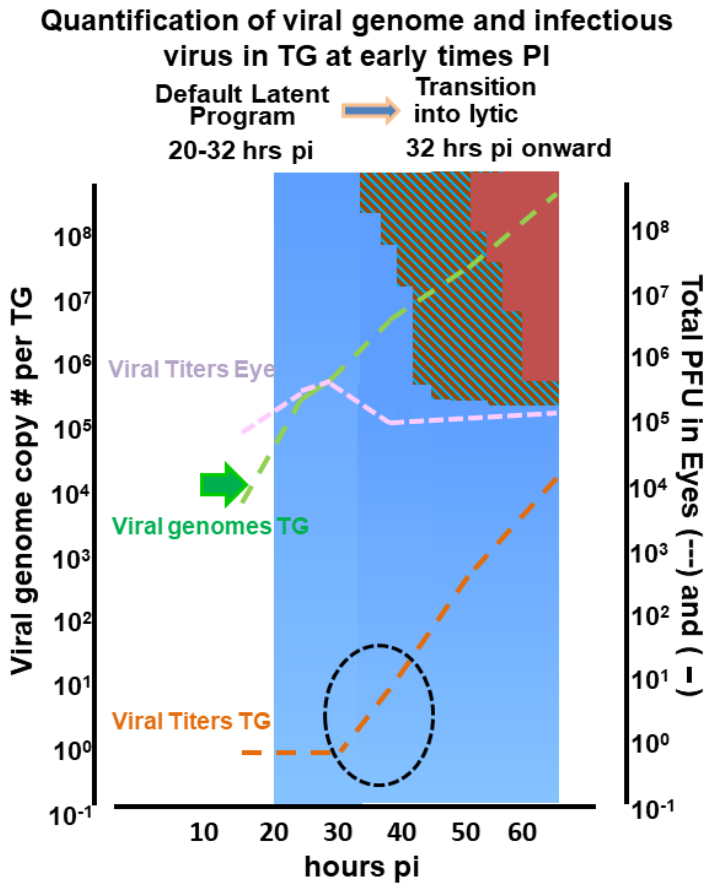

Figure 4. The colored field depicts the percent of positive neurons expressing the LAT promoter (Blue), only viral proteins (brown) or both (striped). Also shown are virus titers in the eye and TG and viral genomes in the TG (dashed lines).

number of neurons expressing viral proteins and promoter-driven B-gal prior to viral spread within the TG (up to 32 hours postinfection, Figure 4). At the earliest times examined (18 to 30 hpi), only LAT promoter-driven B-gal was detected in TG neurons.

Thus, the latent transcriptional program was engaged first in neurons infected via axons, consistent with the idea that VP16 did not arrive with the viral genome to initiate the lytic cascade. Of importance, B-gal activity and cognate

viral protein expression in neurons was detected simultaneously in neurons (e.g. viral proteins including VP16 were detected in all neurons expressing B-gal from the VP16 promoter). The same was true of neurons marked by the ICP0 promoter, indicating simultaneous expression of the native promoters and the transgenes.

Viral proteins were first detected about 14 hours after LAT promoter driven B-gal, and only in neurons previously marked by LAT promoter expression. Since the LAT promoter is strongly repressed by ICP4 in cells including neurons during acute productive infection (Batchelor and O'Hare, 1990; Farrell et al., 1994; Margolis et al., 1992; Sawtell and Thompson, 1992a), we concluded that the viral protein expression observed at these early times postinfection occurred in neurons transitioning out of the acute stage latent program into the lytic cycle.

Mutants expressing a second copy of wild type VP16, the VP16TF deficient mutant, or a third copy of ICP0 driven by the LAT promoter were examined to determine whether the *de novo* expression of either ICP0 or VP16 was sufficient to push neurons out of the default latent transcription program into the lytic program. If so, viral protein expression and virus production in TG would be expected to occur about 14 hours earlier than that seen with wild type HSV1 infection. Early viral protein expression and infectious virus production was seen in TG of mice infected with the mutant expressing VP16 from the LAT promoter, but not in those infected with the LAT driven VP16TF mutant or ICP0 gene. Therefore, *de novo* expression of VP16, but not of ICP0, precipitated entry into lytic infection in TG neurons and the transactivation function of VP16 was required for this.

However, the requirement for the VP16 transactivation function is not absolute in so far as VP16TF viruses replicate to some extent in TG albeit at a ~100 fold reduction (Sawtell et al., 2011; Tal-Singer et al., 1999; Thompson et al., 2009). High genome copy number could potentially override a requirement for VP16TF and this is supported by the finding that strategies increasing surface

replication also increase replication in the TG (Sawtell et al., 2011; Thompson et al., 2009).

A specific region of the VP16 promoter regulated the transition into the lytic transcription program

The region of the VP16 promoter near and downstream of the VP16 TATA box is dispensable for normal leaky late kinetics (Lieu and Wagner, 2000). Thompson and Sawtell found that 3 bp mutations in two putative factor binding sites in this region resulted in wild type virus replication in cultured cells and on the mouse eye, but nearly completely ablated the ability of the virus to transition out of the default latent pathway (2% transition vs. 30% at 44 hours postinfection (Sawtell and Thompson, 2016)). Recently a targeted replacement the HSV1 VP16 promoter sequences with those of HSV2 was generated and tested in mice. This demonstrated that the type 2 VP16 promoter also directs pre-immediate early VP16 expression and does so with greater efficiency during acute infection and following hyperthermic stress induced reactivation from latency (Thompson and Sawtell, 2019).

Combined, the studies offer strong support for the hypotheses that:
(i) VP16 is generally not transported retrograde in axons, favoring latency in neurons.
(ii) The VP16 promoter contains elements required for the *de novo* pre-immediate early production of VP16, which drives exit from a default latent pathway into lytic viral replication in neurons.
(iii) Production of this VP16 is not the result of random "phase 1" type transcription seen in persistently infected cultures of primary neurons treated with AKT or protease inhibitors (Cliffe et al., 2015; Kim et al., 2012).
(iv) Latency is established by default in sensory neurons because VP16 does not arrive with the viral genome. Subsequent reactivation of HSV from the default, or consolidated latent state requires *de novo* VP16 production, and at least for exit from the default latent state this is mediated specifically by the VP16 promoter (Sawtell and Thompson, 2016; Thompson et al., 2009).

Events during the consolidation of HSV latency

Assembly of chromatin structures on latent viral genomes is thought to be an important aspect of silencing the genome. In cultured cells viral DNA is rapidly associated with histones and chromatin is rapidly assembled, occurring in the first thirty minutes post infection (Roizman et al., 2005). This appears to be a multi-step and curiously slow process in TG neurons *in vivo* with initial events detectable at 7 days postinfection and modifications associated with silencing of genes such as heterochromatin formation occurring on lytic promoters after two weeks post infection, whereas the LAT promoter is associated with euchromatin at this time (for review see (Conn and Schang, 2013; Knipe, 2015; Kristie, 2015; Roizman et al., 2013)). Since viral gene expression ceases well before this time, this process may be more important for maintaining latency rather than the initial formation of latent infections. In support of this idea, recent studies suggest that chromatin insulator sequences and their binding proteins may maintain boundaries between regions of the latent HSV genome that are actively transcribed (e.g. the LAT locus) from the lytic phase genes that are silenced (Lee et al., 2018; Washington et al., 2018b; Watson et al., 2018).

Part of the difficulty in understanding the effects of chromatin modifications and boundaries is that latent viral genomes are diverse in these properties. Approaches employed on whole tissues can only detect changes averaged over these millions of latent genomes. Lomonte and co-workers have developed methods to take such analyses to the next level in single neuronal nuclei. As expected, they detected multiple genomes in many neurons. What was not expected is that some of these are intimately associated with PML containing nuclear bodies, whereas others in the same nucleus are not. Also, of interest, some genomes express LAT locus RNAs, whereas others in the same nucleus do not. The silencing properties of these PML-NBs are thought to provide an intrinsic anti-viral defense mechanism that serves to promote latency (Cohen et al., 2018; Lomonte, 2016; Maroui et al., 2016).

Efstathiou and colleagues developed a method to historically interrogate viral promoter expression in individual neurons that survive the acute stage of infection. Employing the Cre reporter mouse strain ROSA26R in combination with viral mutants that express Cre from various promoters they discovered that promoter expression in neurons that survive is far more frequent than previously thought (Proenca et al., 2008; Proenca et al., 2011; Proenca et al., 2015). However, the extent of production of the relevant viral proteins is not yet known. Another group employed this assay to examine the potential expression of viral promoters for genes encoding proteins that are important targets for immune surveillance. Evidence for the expression of these promoters suggest periodic expression of these promoters might be one mechanism whereby long-term immunity is maintained (Russell and Tscharke, 2016).

A role for HSV 1 latency/reactivation in the development of neurodegenerative disorders

The neurotropic nature of HSV and VZV, the extensive data identifying these viruses in the human CNS at autopsy or identification of virus in the cerebrospinal fluid together with the concept of viral latency and reactivation in the trigeminal ganglion led to the hypothesis "that reactivation of the same dormant viral material travelling centripetally instead might be the cause of the "degenerative" lesions typical both of AD and of the normal aged brain." -MJ Ball (Ball, 1982). In 1997 Itzhaki et al. provided evidence that the combination of HSV1 in brain and carriage of an APOE-e4 allele is a strong risk factor for AD (Itzhaki et al., 1997; Lin et al., 1997). Evidence is now emerging that directly supports the concept that HSV1 infection is a major risk factor in the development of Alzheimer's Disease (reviewed in (Itzhaki, 2018; Lathe et al., 2019)). This concept has been met with much skepticism over the years for reasons including the high prevalence of HSV infection relative to AD and the correlative nature of the studies. Clinical trials are now ongoing to test the efficacy of valacyclovir in individuals with mild AD (ClinicalTrials.gov Identifier: NCT03282916). Animal models are under development, and in human-ApoE4-targeted replacement mouse, long-term HSV1 infected but not mock-infected

mice exhibited spatial memory deficits and CNS pathology consistent with AD. HSV protein was detected in the hippocampus but was extremely rarely (work in progress). Such models will allow efficacy of antiviral treatments to be tested and investigation into the mechanisms of disease initiation and progression.

Conclusions

Increasingly sensitive methods to detect viral transcriptional activity from the latent viral genome have modified the notion of a strictly "silent" vs "active" genome (Collins-McMillen and Goodrum, 2017). However, the basic concepts of latency and reactivation based on detection of infectious virus remain solid fundamental concepts. From our vantage point, the field has three major challenges. The first is recognizing that all models have limitations. Recognizing the limitations and advantages of diverse models is necessary for integration of information from all models. Second, harnessing the advancing single-cell approaches for the investigation of HSV latency and reactivation in complex as well as simpler culture contexts. Third, recognizing that *both* complexity and reductionism have a role to play in future progress in understanding the intricacies of latency and reactivation in the nervous system. An excellent example of the combined power of these approaches was recently published (Cabrera et al., 2018). All models have advantages and limitations and the goal remains to relate what is learned to what is known about human/virus interactions, expanding our overall understanding of HSV pathogenesis.

Reactivation is highly restricted at the cellular level both *in vivo* and *in vitro*, consistent with the fact that maintaining latency is essential for the long-term survival of the virus in the host. Importantly, the mere presence of RNA related to viral lytic genes in the context of latency *in vivo* does not necessarily correlate with either viral protein expression (exit from latency) or reactivation (infectious virus). More startling is the revelation that even in homogenous cultures of neurons collectively stimulated by a potent inhibitor, reactivation occurs in only a small percentage of neurons. This feature of reactivation makes it extremely difficult to interpret analyses on the whole population.

Distinguishing viral pro-reactivation from anti-reactivation transcriptional activity and how this activity is distributed on the viral genome as well as among the individual latently infected neurons is required before sufficient understanding of the significance of global transcriptional activation profiles can be interpreted.

We do not yet completely understand the significance of RNA transcription detected in latently infected ganglia, or under phase 1 conditions in cultured neurons. For example, Krause and colleagues demonstrated that viral transcription during latency that occurs in the absence of ICP27 protein results in splicing at sites that are normally suppressed, disrupting viral ORFs. Thus, they hypothesize HSV-1 may take advantage of host splicing machinery to restrict expression of randomly activated antigenic viral genes to achieve immune evasion (Tang et al., 2019). As we search for and query models in which viral reactivation is more robust, it is important to remember that the alpha herpesviruses evolved to remain latent most of the time and that productive reactivation from latency is a rare event at the cellular and viral genome level. Major remaining challenges include the characterization of the composition of the viral transcriptional activity within individual latently infected cells following a reactivation stimulus, the functional relevance of this activity in promoting or suppressing viral protein production, and whether this activity proceeds to reactivation. In the great majority of latently infected neurons the 100s of thousands of latent genomes distributed in them remain latent. From this perspective, the methodologies are rapidly evolving that will support the analysis of transcriptomes at the single neuron level isolated from the relevant tissues (Cao et al., 2017; Hu et al., 2018; Rosenberg et al., 2018).

While the animal models of HSV latency/reactivation are not perfect, they have proven to support the complex viral life cycle with reasonable fidelity and these models approximate the level of complexity encountered in the human host. The simple act of axotomizing and explanting the ganglia results in changes that yield outcomes that do not align with the requirements for productive reactivation in the *in vivo* context such as the requirement of the VP16

transactivation function to initiate detectable viral protein synthesis (compare (Steiner et al., 1990) to (Thompson et al., 2009). It is becoming increasingly apparent that properties of a complex system cannot be reduced to the components of a system because they depend on many interactions. Emergent properties of a complex system are lost when a system is stripped down to eliminate "extraneous" interactions or "complicating" interactions, with surrounding cells including satellite cells, resident glial and microglial cells, and infiltrating immune cells. In the pursuit of understanding HSV reactivation, a revival of reductionist approaches and models has arisen. The development of an *in vitro* latency model that provides a platform for dissecting host cell signaling pathways involved in viral genome entry into latency, its maintenance, and its activation would be an important tool. Ideally such a model could yield hypotheses testable in the more complex *in vivo* setting. However, defining the limitations to all models and approaches used to probe outcomes in these models is a critical component of the scientific process.

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