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Strength in Diversity: Understanding the Pathways of Herpes Simplex Virus Reactivation

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Abstract

Herpes simplex virus (HSV) establishes a latent infection in peripheral neurons and can periodically reactivate to cause disease. Reactivation can be triggered by a variety of stimuli that can activate different cellular processes to result in increased HSV lytic gene expression and production of infectious virus. The use of model systems has contributed significantly to our understanding of how reactivation of the virus is triggered by different physiological stimuli that are correlated with recrudescence of human disease. Furthermore, these models have led to the identification of both common and distinct mechanisms of different HSV reactivation pathways. Here, we summarize how the use of these diverse model systems has led to a better understanding of the complexities of HSV reactivation, and we present potential models linking cellular signaling pathways to changes in viral gene expression.

Keywords

Herpes simplex virus; reactivation; epigenetics; NGF-deprivation; axotomy; DLK

Introduction

Herpes simplex virus (HSV) 1 and 2 are ubiquitous pathogens that persist for the life of infected individuals. The ability of these viruses to develop lifelong infections is due to the presence of a latent pool of virus in terminally differentiated neurons, most commonly in the peripheral ganglia. It is estimated that approximately 90% of individuals worldwide are infected with HSV-1, HSV-2 or both of the viruses (Arvin et al., 2007). In the United States, the estimated prevalence rate of HSV-1 and HSV-2 in people aged 14–49 is 47.8% and 11.9% respectively, with higher prevalence in women and Mexican-American and non-Hispanic black persons (McQuillan et al., 2018). HSV infection is often clinically silent. However, HSV periodically re-enters a lytic replication cycle in a process known as reactivation. In immunocompetent persons, reactivation events result in replication at the body surface that can give rise to recurrent blisters or sores, which are typically self-limiting

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and resolve rapidly (Roizman et al., 2013). These lesions most commonly occur at oral, nasal or ocular sites with HSV-1 infection and at the genital skin and mucosa with HSV-2 infection. HSV reactivation can also lead to significant morbidity and mortality in immunocompromised individuals, and, in rare cases, infection of the central nervous system can lead to acute viral encephalitis or a recurrent lymphocytic meningitis.

The ability of HSV to reactivate from latency and re-enter a productive replication cycle significantly contributes to its ability to cause lifelong disease that results in recurrent infection, viral shedding and transmission to new hosts. However, the molecular events that underlie the switch from latent to productive infection are not well understood. This review will examine our current understanding of how different reactivation stimuli may trigger this switch in the HSV viral life cycle and illuminate some of the remaining questions in the field.

Lytic replication and latency: contrasting viral life-cycles

The ability of HSV to establish a lifelong infection can be attributed to its capacity to undergo contrasting infectious events, termed the lytic and latent stages. Primary infection at the body surface results in productive replication in epithelial cells (Roizman et al., 2013). During this initial lytic stage of infection, over 80 viral gene products are expressed in a cascade-dependent manner. Initial viral gene expression is enhanced by delivery of viral tegument proteins, including the viral transactivator, VP16, into the nucleus. This potent transcriptional activator, induces the formation of a transcriptional regulatory complex with multiple cellular co-activators to promote transcription of the viral immediate early (IE) or α genes. Products of the IE genes include proteins required for transcription of the early (E) or β genes, which encode proteins required for viral DNA replication. The final group of genes is the late (L) or γ genes, whose expression is dependent on viral DNA replication. L genes encode structural proteins required for assembly, egress and release of the infectious HSV particle.

During primary infection, HSV is able to enter the terminal axons of neurons that innervate tissue at the initial site of infection. Although viral genomes are most frequently detected in sensory ganglia, particularly the trigeminal ganglia (HSV-1) and lumbar-sacral ganglia (HSV-2), viral DNA and reactivation-competent virus can also be isolated from sympathetic and parasympathetic neurons (Baringer and Pisani, 1994; Baringer and Swoveland, 1973; Richter et al., 2009; Warren et al., 1978). Furthermore, HSV DNA can be detected in the central nervous system, with the frequency of detection increasing with age (Beffert et al., 1998; Fraser et al., 1981; Gordon et al., 1996). Following neuronal infection, the virus can enter latent infection. However, neurons can also support lytic replication, which may be associated with neuronal death (Thompson and Sawtell, 2001). There is also evidence of prior lytic promoter activity in latently infected neurons (Proenca et al., 2008). The mechanisms that regulate entry into lytic replication versus latent infection in neurons remain largely undefined.

HSV latency is defined as the persistence of viral DNA in the absence of detectable infectious virus that retains the ability to reactivate following an appropriate stimulus. While

expression of the viral lytic genes is largely repressed during latent infection, there is active transcription of the latency-associated transcript (LAT), which is composed of a primary 8.3kb unstable transcript that is spliced to give rise to a stable intron of approximately 2kb in length and multiple miRNAs (Kramer et al., 2011; Stevens et al., 1987; Umbach et al., 2008). Latency is usually defined at the level of the ganglia, but within a ganglion only a sub-population of latently infected cells will reactivate at any one time (Sawtell and Thompson, 2004). In addition, there is evidence that different stimuli can result in reactivation from different subtypes of neurons (Yanez et al., 2017). Therefore, the definition of a "latently" infected neuron may depend not only on the neuronal subtype, but also on the nature of the reactivating trigger.

Modeling HSV latency

A strength of the HSV field is the diversity of the model systems used to investigate the pathways to reactivation. Although there may be differences in the interpretation of the data based on the system or stimuli, all these systems will ultimately have relevance to basic science and human health. To elucidate the cellular signaling pathways involved in the reactivation processes, models of latency that allow faithful establishment of latency, robust reactivation and easy manipulation of signal transduction pathways are required. The most commonly used model organism in HSV research is the mouse. Infection of mice with HSV-1 results in initial lytic replication at the body surface and entry of the virus into innervating sensory and autonomic neurons. Following an initial period of acute replication in the ganglia, HSV establishes latency, and reactivation can be triggered by explant of the ganglia (Sawtell and Thompson, 2004), hyperthermic stress (Sawtell and Thompson, 1992), UV irradiation (Shimeld et al., 1996) or hormone treatment (Cook et al., 1991; Miguel et al., 2010). Overall, *in vivo* models of reactivation have the advantage of more accurately recapitulating the natural course of infection and incorporate host antiviral responses that may impact the state and population of latent genomes or modulate viral reactivation. However, the manipulation of cellular pathways *in vivo* can be challenging.

To elucidate the molecular pathways involved in HSV reactivation, *in vitro* models have proven to be invaluable (Thellman and Triezenberg, 2017; Wilson and Mohr, 2012). The optimal model system would utilize mature, human neurons. However, while sensory neurons can be isolated and maintained from human donors (Valtcheva et al., 2016), access to this material is limited, consistency is difficult to achieve, and the tissue may already be latently infected with HSV or varicella zoster virus (VZV). Human sensory neurons differentiated from embryonic stem cells have also been used to investigate latency and reactivation for both HSV and VZV (Markus et al., 2015; Pourchet et al., 2017). A recent study utilizing human differentiated neurons achieved latently infected cultures that could be reactivated with sodium butyrate, a histone deacetylase inhibitor (Pourchet et al., 2017). There is also an emerging interest in human cell lines that can be easily differentiated into neurons, as shown using the HD10.6 cell line, which can be differentiated into sensory neurons with nociceptive properties (Raymon et al., 1999; Thellman et al., 2017). Quiescent infection can be established in the presence of acyclovir, and reactivation can be triggered from a sub-population of neurons following depletion of nerve growth factor (Thellman et al., 2017).

Perhaps one of the best characterized *in vitro* systems to study HSV reactivation utilizes primary sensory or sympathetic neurons isolated from the peripheral ganglia of pre-natal rats and post-natal or adult mice (Camarena et al., 2010; Cliffe et al., 2015; Ives and Bertke, 2017; Wilcox and Johnson, 1987; Wilcox et al., 1990). Infection of these primary neuronal cultures in the presence of acyclovir or phosphonoacteic acid (PAA) results in a quiescent infection that resembles latency. Importantly, to accurately define a quiescent infection in these model systems, it is imperative to show that replicating virus remains undetectable following the removal of viral DNA replication inhibitors. When a quiescent infection is properly established, these systems exhibit all of the known molecular hallmarks of latency, including accumulation of the LAT intron, expression of latency-associated miRNAs, absence of replicating virus and undetectable levels of viral proteins (Camarena et al., 2010; Cliffe et al., 2015; Jurak et al., 2014; Wilcox and Johnson, 1987). Furthermore, these models maintain the capacity to undergo reactivation triggered by a variety of stimuli, including NGF-deprivation, dexamethasone, inhibition of protein synthesis or high intracellular levels of cAMP (Camarena et al., 2010; Cliffe et al., 2015; Linderman et al, 2017; Colgin et al., 2001; Kobayashi et al., 2012; Wilcox and Johnson, 1987). Thus, these systems provide a powerful tool to study the molecular features of latency and reactivation, such as the role of cell stress pathways or chromatin modulation, in primary neuronal populations. Some caveats to these model systems is the absence of support cells that may also impact the nature of the latent infection and/or reactivation. The use of DNA replication inhibitors to promote latency is instead utilized to compensate for missing immune components. Whether DNA replication inhibitors impact the nature of latency or reactivation mechanisms is not known. However, it is worth noting that symptomatic primary HSV-1 is often treated with anti-viral compounds (James and Whitley, 2010). Moving forward, it will be important to determine if the mode infection, age of neurons, presence of immune mediators or addition of DNA replication inhibitors alters the nature of latency or impacts events that occur during reactivation, as this will have relevance to both the model systems used and human disease.

Latent viral chromatin structure: silent but poised?

Following the establishment of latent infection, viral lytic gene expression is silenced, and the lytic gene promoters are associated with repressive heterochromatin (Knipe and Cliffe, 2008). Key experiments performed in the 1980's indicated that latent genomes in the brain stems of infected mice have a nucleosomal structure (Deshmane and Fraser, 1989). Later studies confirmed that the latent viral genome associates with cellular histones in the trigeminal ganglia of mice (Cliffe et al., 2012; Kubat et al., 2004b; Wang et al., 2005). Coinciding with the silencing of lytic transcripts, the viral lytic gene promoters become enriched with characteristic heterochromatic histone modifications, namely histone H3 di- and tri-methylated at lysine 9 (H3K9me2/3) and H3K27me3 (Cliffe et al., 2012; 2009; Kwiatkowski et al., 2009; Nicoll et al., 2016; Wang et al., 2005). While it appears that factors intrinsic to neurons play a key role in the transcriptional silencing of the virus (Cliffe et al., 2012), viral gene products expressed during latent infection can also modulate the chromatin structure (Cliffe et al., 2009; Kwiatkowski et al., 2009; Raja et al., 2016; Wang et al., 2005). This modulation likely promotes long-term latency, while priming the genome for reactivation following the appropriate stimuli (Leib et al., 1989; Trousdale et al., 1991).

In contrast to the lytic gene promoters, the region encompassing the LAT promoter and enhancer elements are enriched with euchromatin-associated modifications, (Cliffe et al., 2009; Kubat et al., 2004a). This apparent demarcation in the nature of the chromatin likely arises due to binding sites for the cellular insulator protein CCCTC-binding factor (CTCF) on the viral genome. Interestingly, CTCF eviction coincides with reactivation (Ertel et al., 2012; Washington et al., 2018b), and depletion of CTCF *in vivo* promotes reactivation (Washington et al., 2018a). Furthermore, one important binding site of CTCF, known as CTRL2, lies downstream of the LAT enhancer and separates it from the nearby lytic ICP0 gene (Amelio et al., 2006b). Deletion of this site from the viral genome results in increased heterochromatin formation on the LAT enhancer region and a paradoxically small increase in LAT gene expression (Lee et al., 2018). The CTRL2 binding site deletion also decreases the mutant virus's ability to reactivate from latency. These studies suggest that the organization of chromatin domains may play a role in both the establishment and maintenance of latency and potentially poise the viral genome for reactivation.

Although regions of the latent viral genome are associated with heterochromatin, there is evidence to suggest that it exists in a state that is primed for reactivation. In mouse models of latency, the viral genome does not contain detectible canonical CpG methylation (Dressler et al., 1987; Kubat et al., 2004b), which is associated with a particularly stable form of gene silencing. In addition, lytic promoters do not appear to be associated with H4K20me3 (Cliffe et al., 2009), which is a modification that is classically associated with transcriptionally silenced regions of stable, constitutive heterochromatin in mammalian cells (Jorgensen et al., 2013). A component of the repressive PRC1 complex, which is a histone reader often found enriched at sites of cellular H3K27me3, is present at very low levels, if at all, on the latent viral genome (Cliffe et al., 2012; Kwiatkowski et al., 2009). Furthermore, a recent study by Alfonso-Dunn et al. (2017) suggests that activation of the super-elongation complex by treatment with BET domain inhibitors enhances reactivation *ex vivo*, implicating RNA polymerase II promoter-proximal pausing as a rate-limiting step in HSV reactivation. Whether this is due to 'poised' RNA polymerase II on the latent viral genome clearly deserves more investigation. Taken together, this data indicates that portions of the viral genome are enriched with a form of heterochromatin that may be readily remodeled to permit rapid gene expression.

Triggers implicated in HSV reactivation

In order to regulate the delicate balance between latent infection and re-entry into the lytic phase, the latent HSV genome must sense neuronal cues that induce the up-regulation of viral lytic gene expression. However, the nature of these stimuli and how they can act on the viral genome to permit gene expression is not well understood. In the context of human disease, exposure to sunlight, psychological stress, fever, menstruation and surgical resection have all been associated with HSV reactivation (Chida and Mao, 2009; Hayderi et al., 2013; Padgett et al., 1998; Roizman and Whitley, 2013). It is likely that reactivation of HSV in a normal human host results from a combination of factors, including signals that act directly on latently infected neurons and suppression of the immune responses that typically prevent reactivation and clear replicating virus. The key role of the host immune response in HSV infection is clearly evident by the significant morbidity and mortality associated with HSV

infection in immunocompromised individuals, and we refer readers to excellent reviews on the immune control of HSV (Divito et al., 2006; Egan et al., 2013; Kinchington et al., 2012). In this review, we will primarily focus on how stimuli can act directly on neurons or the viral genomes to result in reactivation.

When studying the mechanisms involved in latency and reactivation, it is important to make the distinction between the neuronal signaling pathways that trigger reactivation and the downstream factors that may act directly on the genome to modulate viral chromatin or regulate viral transcription. In the first case, stimuli are used to mimic a physiological response in an attempt to recapitulate natural *in vivo* reactivation episodes. In the second case, other triggers, such as down-regulation of a repressive protein or treatment with an HDAC inhibitor, potentially bypass up-stream cellular pathways and act directly on the viral chromatin (Neumann et al., 2007b). These types of stimuli result in efficient re-entry into lytic replication and prove useful for assessing the role of host proteins in maintaining latency. To add another level of complexity, depletion of a cellular protein or HDAC inhibition could also activate a cell stress response within the neuron and result in lytic gene expression, thus indirectly triggering reactivation (Dai et al., 2005). Here, we try to focus on the role of cellular signaling pathways that mimic natural stimulation of viral reactivation in humans.

As reviewed recently, the process of reactivation likely requires multiple steps (Cliffe and Wilson, 2017; Sawtell and Thompson, 2016a). In primary neuronal culture systems, it has been shown that there is an initial relaxing of the chromatin structure, which likely includes multiple changes that may or may not be accompanied by detectable increases in lytic gene transcription. After chromatin relaxation and transcription, viral proteins are synthesized followed by the production of infectious virus and progression to full reactivation (Kim et al., 2012; Sawtell and Thompson, 2016b). At each stage of the pathway, only a subset of neurons will progress and complete the subsequent step (Kim et al., 2012; Thompson et al., 2009), indicating that there are thresholds and multiple controls that regulate progression through reactivation. It is currently not well understood whether these thresholds relate to intrinsic neuronal responses, levels of viral protein synthesis or non-coding RNAs, or changes in the chromatin structure, but it is likely that many, if not all, of these factors contribute. As reviewed in Sawtell et al. (2016), the techniques for measuring HSV “reactivation” vary between different researchers and have contributed to some discrepancies in the literature (Sawtell and Thompson, 2016a). Importantly, while the end point of reactivation, defined as the active production of infectious viral particles, is the same, the intervening steps that regulate this process are likely different depending on the nature of the initiating signal. Below we summarize what we have learned from a variety of model systems on the process of reactivation in response to different stimuli.

Loss of neurotrophin-associated signaling

Using both animal models and *in vitro* systems, progress has been made in recapitulating stimuli that trigger reactivation in humans. However, there remain significant gaps in our understanding of how these stimuli correlate with reactivation of the virus that results in clinical disease. One pathway that has been found to stimulate HSV reactivation in multiple

systems is nerve-growth factor (NGF) deprivation. In humans, this may occur during the developmental period, and particularly through adolescence, as the process of axon pruning helps correctly wire the developing nervous system. In addition, damage to innervated tissues that results in loss of the neurotrophin-producing cells or changes in neurotrophin synthesis may occur following periods of chronic stress (Eckart et al., 2013), changes in hormone levels (Kaur et al., 2007) or UV irradiation (Stefanato et al., 2003). NGF-deprivation was first found to trigger HSV reactivation in primary neuronal models of HSV latency using rat sympathetic neurons (Wilcox and Johnson, 1987). *In vivo* injection of anti-NGF serum into latently infected rabbits has also been shown to enhance reactivation of HSV (Hill et al., 1997). Furthermore, interruption of signals downstream of the NGF receptor triggered reactivation in a variety of *in vitro* models of HSV latency (Camarena et al., 2010; Cliffe et al., 2015; Kobayashi et al., 2012; Linderman et al., 2017), and have been shown to enhance explant mediated reactivation *ex vivo* (Du et al., 2011; Messer et al., 2015).

The changes in cell-signaling in response to NGF-deprivation have been well delineated in both primary sympathetic and sensory neurons. In immature neurons, NGF-deprivation leads to apoptosis due to up-regulation of the pro-apoptotic BH3-only proteins (Biswas et al., 2007). These proteins activate Bax, which results in mitochondrial outer member permeabilization (MOMP) and cytochrome c-mediated caspase activation. However, as neurons mature, they develop mechanisms to restrict both MOMP and downstream caspase activation, permitting their long-term survival (Kole et al., 2013). In murine sympathetic neurons, this occurs at around post-natal day 10. Interestingly, neuronal maturation appears to be intrinsic to neurons and occurs both *in vivo* and *ex vivo*, as seen in primary neurons isolated postnatally (Kole et al., 2013; Annis et al., 2016). Importantly, many studies have shown reactivation of the virus after restriction of the apoptotic pathway, suggesting that apoptosis itself does not play a significant role in HSV reactivation. This is consistent with observations that neither caspase activity nor MOMP are required for reactivation upon NGF-deprivation (Camarena et al., 2010; Cliffe et al., 2015). Therefore, signals up-stream of MOMP appear to be important for triggering HSV reactivation.

In an important study by Camarena et al. (2010), the authors investigated how interruption of cell signaling pathways by NGF-deprivation triggers HSV reactivation in a primary neuronal model of latency. Although NGF can signal through two major receptors present on sympathetic neurons, TrkA and p75, the authors found that NGF binding to the TrkA receptor sustained latency in rat sympathetic neurons. Signaling through TrkA results in the activation of three major signaling pathways: MEK/ERK, PLCγ and PI3K/AKT (Chao, 2003). However, only interruption of the PI3K/AKT pathway triggered reactivation of HSV (Camarena et al., 2010). Since this study, inhibition of PI3K signaling has been found to also trigger reactivation in primary murine sympathetic and sensory neurons (Cliffe et al., 2015; Hukkanen et al., 2015).

Inhibition of PI3K or AKT signaling results in inhibition of the mammalian target of rapamycin (mTOR) signaling pathway. mTOR is a serine/threonine kinase that is a component of two functionally distinct protein complexes, mTORC1 and mTORC2, which regulate a number of cellular processes in response to environmental signals, including cap-

dependent translation and autophagy (Foster and Fingar, 2010). Direct inhibition of mTORC1 activity or transient inhibition of protein synthesis can result in HSV reactivation in rat primary neurons (Kobayashi et al., 2012). This implicates mTOR as another key mediator of the HSV lytic/latent switch in neurons, either directly by sustaining the synthesis of proteins required for the maintenance of latency or indirectly by activating pathways following protein synthesis inhibition. Inhibition of AKT also results in activation of the mixed lineage kinase protein, dual leucine zipper kinase (DLK) (Wu et al., 2015). DLK is a key mediator of apoptosis resulting from global NGF-deprivation, as well as axon pruning following axon-specific deprivation. Key targets of DLK are the cell stress proteins c-Jun N-terminal kinases (JNKs) (Tedeschi and Bradke, 2013). In neurons, JNKs are constitutively active and carry out many physiological roles, including dendritic arborization and synaptic plasticity (Coffey et al., 2000). In response to neurotrophic deprivation, JNKs are redirected to activate a cell stress response through mobilization of DLK and JNK interacting proteins 3 (JIP3) (Sengupta Ghosh et al., 2011). In a study using a mouse primary neuronal system of HSV reactivation, we found that activation of JNK via DLK/JIP3 was an essential step in induction of lytic gene expression (Cliffe et al., 2015). Thus, it is clear that multiple downstream cellular processes are involved in reactivation following inhibition of PI3K.

The ability to study the kinetics of lytic gene expression following interruption of NGF-signaling in tractable *in vitro* systems has highlighted key differences in the mechanism of viral gene expression from silenced latent genomes, as compared to *de novo* lytic infection. In this context, reactivation occurs as a two-stage program that overcomes a more compact viral chromatin structure and the absence of tegument factors, such as VP16 (reviewed in Cliffe and Wilson, 2017). During the first stage, termed phase I, there is a transient burst of lytic gene transcription. This first wave of synchronous lytic gene expression leads to the simultaneous synthesis of many lytic transcripts with the potential to encode IE, E, and L viral proteins (Kim et al., 2012). A similar synchronous wave of gene expression has been observed following *ex vivo* reactivation when explant was combined with NGF-deprivation (Du et al., 2011), indicating that the specific stimuli, and not experimental system, impacts the mechanism of reactivation. However, it is worth noting that NGF-deprivation combined with axotomy does not result in the exact same cellular response as NGF-deprivation performed on intact neurons. Axotomized neurons reach an earlier commitment to death than intact neurons and, unlike intact neurons, are unable to recover from mitochondrial membrane permeabilization (Fletcher et al., 2000). Therefore, further work is required to directly show that differences in the nature of viral gene expression is independent of the nature of the model used. Importantly, phase I is not dependent on the viral transactivator VP16 (Kim et al., 2012) in primary neurons. Furthermore, neither the synthesis of IE proteins nor viral DNA replication are necessary for the expression of viral E or L genes (Kim et al., 2012). Viral gene expression in phase I is dependent on JNK activity, but it is independent of histone demethylases that have been implicated in the removal of repressive H3K9 and H3K27 methylation marks. Instead, histones associated with viral lytic promotors are phosphorylated on S10 in a JNK-dependent manner, resulting in a H3K9me3pS10 histone methyl/phospho switch. This switch could permit increased viral gene expression

without the need to recruit histone demethylases and allow gene silencing to re-occur if the genome does not progress to full reactivation (Cliffe et al., 2015).

The second phase of reactivation closely resembles *de novo* infection and is hypothesized to occur only if threshold amounts of key viral proteins are synthesized during phase I (Kim et al., 2012). Similar to *de novo* infection, viral gene expression during phase II is dependent on histone demethylase enzyme activity and may require the viral transactivator VP16. (Cliffe et al., 2015; Kim et al., 2012). Ultimately, phase II results in the amplification of viral DNA and production of infectious viral particles (Kim et al., 2012).

Axotomy/explant

One of the first clinical observations of a stimulus resulting in HSV reactivation was by Cushing in 1905, when he documented reactivation in a patient following trigeminal root surgery for trigeminal neuralgia (Cushing, 1905). Reactivation of HSV following various forms of surgical treatment for trigeminal neuralgia has now been well documented and is even considered an indication of successful surgery (Tenser, 2015). The most likely reason for surgery-triggered reactivation is damage to the trigeminal nerve and activation of cellular responses induced by axotomy. In animal models of latency, complete axotomy via explant of infected ganglia is a commonly used stimulus for reactivation (Sawtell and Thompson, 2004; Stevens and Cook, 1971) and, therefore, should closely mimic reactivation caused by mechanical injury of neurons in humans.

With respect to the pathways linked to HSV reactivation, the cellular response to axotomy has overlapping features with the NGF-deprivation pathway, but there are also some key differences. In the case of axotomy, calcium influx into the injured axon is a central trigger for changes in gene expression (Cho et al., 2013). The increase in intracellular calcium results in increased electrical activity of the neuron, as well as calcium-dependent modulation of cellular proteins (Rishal and Fainzilber, 2014). Calcium-dependent signaling triggers the rapid cellular changes required for neuronal survival, including membrane resealing, activation of cellular transcription factors and relocalizaiton of specific epigenetic modifiers (Mar et al., 2014; Rishal and Fainzilber, 2014). This likely accounts for the rapid changes in cellular gene expression seen in explant models of HSV reactivation (Sawtell and Thompson, 2004), as compared to other models.

One downstream effect of axotomy-driven calcium influx is activation of adenylate cyclase and increase in intracellular cAMP levels (Mahar and Cavalli, 2018). High intracellular levels of cAMP, which also can be achieved with cAMP mimics and activators of adenylate cyclase such as forskolin, has been shown to trigger HSV reactivation (Colgin et al., 2001; De Regge et al., 2010). This suggests that increased cAMP levels following axotomy may contribute to reactivation, although a dependence of cAMP in axotomy-induced reactivation has not yet been described. Following explant, increased intracellular calcium and cAMP can also trigger activation of DLK (Hao et al., 2016; Yan and Jin, 2012). A key sensor of local axon injury, DLK is required for many downstream signaling pathways that promote axon regeneration following axotomy. This is in contrast to its role in the NGF-deprivation pathway, where it mediates axon apoptosis and pruning (Geden and Deshmukh, 2016;

Tedeschi and Bradke, 2013). Therefore, DLK seems to function as a key regulator of the axon response, promoting different outcomes depending on the nature of the stimulus. DLK may also be a key mediator of HSV reactivation, as one of its downstream targets, JNK, is required for HSV reactivation following explant (Cliffe et al., 2015). Although a direct role of DLK in explant-induced reactivation has not been shown, it will be important to investigate whether this protein acts as a common mediator of HSV reactivation in addition to its role as a central sensor of neuronal stress. If so, it is likely that there will different DLK-mediated reactivation pathways for different stimuli, just as there are different phenotypic outcomes for different mechanisms of DLK activation (Geden and Deshmukh, 2016).

In an NGF-deprivation model of reactivation, DLK/JIP-3-JNK pathway signaling mediates a histone methyl/phospho switch and enrichment of H3K9me3/pS10 on lytic gene promoters (Cliffe et al., 2015). It remains unclear if JNK plays a similar mechanistic role following explant of latently infected ganglia, and, as a whole, very little is known about the mechanisms linking cell signaling pathways and changes in viral chromatin following axotomy. However, a number of important observations about the viral chromatin have been made using the explant model, including changes in histone post-translational modification and changes in CTCF binding (Ertel et al., 2012; Washington et al., 2018b). Most notably, it has been shown that, following axotomy, there is a transient increase in the enrichment of euchromatic histone markers (acetylated H3K9/K14) at IE gene promoters (Amelio et al., 2006a). The mechanism responsible for the increased acetylation has not been determined but may be related to the global increases in histone acetylation that have been observed following axotomy and result from calcium-dependent nuclear export of HDAC5, along with HDAC3 (Cho et al., 2013; Fischle et al., 2002). Notably, reactivation can be triggered by inhibition of HDAC activity, which also results in similar changes in histone acetylation on viral promoters (Neumann et al., 2007a).

Following explant of sensory ganglia, there is also a rapid re-localization of host cell factor-1 (HCF-1) from the cytoplasm to the nucleus (Kristie et al., 1999). Once in the nucleus, HCF-1 quickly associates with gene enhancer domains of IE proteins, which was found to correlate with RNA polymerase II occupancy and gene expression (Whitlow and Kristie, 2009). Interestingly, HCF-1 occupancy at IE gene promoters was detected as early as 1hr post-explant, indicating a potential role of HCF-1 in the initiation of reaction transcription (Whitlow and Kristie, 2009). Furthermore, the activities of LSD-1 and JMJD2 proteins, which are histone K9 demethylases that interact with HCF-1, are required for the early induction of lytic gene expression (Liang et al., 2013; 2009). The HCF-interacting histone H3K4 methyltransferases may also be recruited to promote active euchromatin on IE promoters following explant (Kristie et al., 2010). VP16, which induces a transcriptional complex with HCF-1 and Oct-1 during lytic infection, is dispensable for explant-induced reactivation (Sears et al., 1991; Steiner et al., 1990). This is in contrast to reactivation in intact ganglia, in which VP16 is required (Thompson et al., 2009), again indicating that the mechanisms of reactivation likely differ depending on the nature of the trigger. Lastly, it has been shown that CTCF domains positioned around IE (ICP0 and ICP4) and LAT gene regions lose CTCF occupancy following explant-induced reactivation (Ertel et al., 2012;

Washington et al., 2018b), suggesting that disruption of CTCF-mediated chromatin domains may contribute to reactivation.

Psychological stress

Psychological stress has been extensively linked to HSV-associated disease in humans (Chida and Mao, 2009; Cohen et al., 1999; Glaser and Kiecolt-Glaser, 1997). During times of high emotional stress, there is activation of the sympathetic nervous system and release of catecholamines, as well as activation of the hypothalamic pituitary adrenal axis and release of glucocorticoids (Carrasco and Van de Kar, 2003). The downstream actions of these two stress pathways have been shown to play significant roles in HSV latency and reactivation, and stress hormones have been well-utilized as tools to trigger efficient reactivation. Iontophoresis of epinephrine has been widely used to reactivate HSV-1 in different animal models (Creech and Neumann, 2010; Rootman et al., 1990; Willey et al., 1984). In addition, dexamethasone, a synthetic corticosteroid, stimulates reactivation of HSV-1 both *ex vivo* and in primary neuronal cultures (Cliffe et al., 2015; Du et al., 2012), and the closely related bovine herpesvirus 1 (BHV-1) can also be reactivated in latently infected calves by intravenous injection of dexamethasone (Workman et al., 2012).

Although stress hormone-mediated immunosuppression likely plays a role in recurrence of disease in humans, there is evidence that stress hormones can have a direct effect on neurons, as well as on HSV gene expression (Du et al., 2012; Ives and Bertke, 2017; Workman et al., 2012). During productive replication in non-neuronal cells, BHV-1 lytic gene expression is stimulated by dexamethasone (Zhu et al., 2017). Furthermore, differential expression of adrenergic and glucocorticoid receptors on sensory and autonomic neurons has been well-characterized, and differences in expression correlate with the effect of stress hormones on HSV-1 and HSV-2 replication (Ives and Bertke, 2017). The signaling pathways that ultimately result in viral gene expression in response to stress hormones are not fully understood. However, in a rabbit model of HSV latency, reactivation triggered by transcorneal iontophoresis of epinephrine results in an increase in enrichment of the euchromatin marker H3K4me2 at the ICP4 promoter region (Creech and Neumann, 2010). How this treatment results in the observed changes in the chromatin structure is not clear.

Binding of epinephrine to the β_2-adrenergic receptor, which is expressed on sensory and sympathetic neurons, activates adenylate cyclase and results in increased levels of the second messenger cAMP (Gold et al., 1997; Vásquez and Lewis, 2003). Interestingly, forskolin, which also activates adenylate cyclase, is commonly used in cultured neurons to trigger HSV reactivation (Colgin et al., 2001; De Regge et al., 2010; Halford et al., 1996). While the cAMP-dependent signaling pathways that ultimately promote HSV reactivation have not been elucidated, a recent study using pseudorabies virus (PRV) found that the repression of an axon-specific infection could be overcome by addition of forskolin in a JNK-dependent manner (Koyuncu et al., 2017). Dexamethasone can also trigger JNK activity (Lee et al., 2014; Zhang et al., 2000), and dexamethasone-induced HSV reactivation in primary sympathetic neurons has been found to be JNK dependent (Cliffe et al., 2015). Although the contribution of adenylate cyclase and JNK activity to stress-hormone induced reactivation have not been fully elucidated, these observations suggest that cAMP and JNK may be key

regulators of the HSV quiescence/lytic switch in response to stress. However, the activation mechanisms may be divergent and dependent on the stimulus.

Heat shock/Fever

Historically, fever has been one of the most strongly associated environmental triggers of HSV reactivation in humans (Roizman et al., 2013). However, like many stimuli, the underlying mechanisms and cellular pathways that induce this reactivation remain correlative, at best. Reactivation in response to fever could be due to a variety of different factors including direct heat stress to latently infected neurons and/or the resultant release of pyrogenic cytokines. In animal models, whole-body hyperthermia can mimic the effects of fever and stimulate the release of pyrogenic cytokine, IL-6. Transient hyperthermic stress can also trigger HSV-1 reactivation *in vivo* (Sawtell and Thompson, 1992), and there is evidence for a role of IL-6 secretion in response to heat stress in mice, which likely occurs due to glucocorticoid-mediated activation of the hypothalamic pituitary adrenal axis (Noisakran et al., 1998). Additional cytokines and prostaglandins produced during fever also have the capability of acting directly on neurons (Poon et al., 2015). IL-6 and prostaglandins can both trigger sensory neuron hyperstimulation (Black et al., 2018), which, at least for prostaglandins, appears to occur as a result of increased levels of cAMP (Emery et al., 2011). As mentioned previously, elevated cAMP levels can trigger HSV reactivation. Therefore, it will be interesting to explore the direct effects of pyrogenic cytokines on the latent viral genome and potential cell signaling pathways involved.

Heat shock of cultured neurons can trigger reactivation (Halford and Schaffer, 2001), and in non-neuronal systems viral lytic promoters can be activated in response to heat shock (Kushnir et al., 2009), suggesting indicating a direct, intrinsic role. The cellular heat shock response involves increased synthesis of the heat shock proteins (HSPs), along with activation of the mitogen-activated protein kinase family, including JNK, which as previously discussed has been linked to HSV reactivation. The signaling proteins important in triggering HSV reactivation resulting from heat-stress are not known, although in non-neuronal cells heat-stress activation of lytic-promoters has been linked to NF-Y (Kushnir et al., 2009).

An advantage of the hyperthermia model of HSV reactivation is the ability to induce *in vivo* reactivation. Using this model, Sawtell and Thompson (1992) provided evidence that reactivation was neuron-specific, only occurred in 1–3 neurons per trigeminal ganglia and led to degenerative changes that indicated cell death. Whether similar numbers of reactivating neurons occur in the sympathetic ganglia is not known. Subsequent *in vivo* studies using this model have indicated that *de novo* expression of VP16, but not ICP0 or ICP4, may be a requirement for the initiation of reactivation (Thompson et al., 2009; Thompson and Sawtell, 2006), which is in contrast to axotomy induced reactivation in which VP16 is dispensable, highlighting that the mechanism of reactivation may differ depending on the trigger. Interestingly, this study suggested that the VP16 promoter could be activated in neurons during acute infection in the absence of other viral proteins (Thompson et al., 2009), suggesting a neuronal specific pre-IE VP16 promoter that may be activated during reactivation. However, the mechanisms responsible for this non-canonical VP16 expression

remain unknown. The identification of the precise region of the VP16 promoter important for pre-IE expression and the corresponding interacting cellular proteins will be key to narrowing down the cellular pathways that are responsible.

UV radiation

To model sunlight-induced reactivation, exposure of the cornea or skin to UV radiation can trigger reactivation in mice infected with the most pathogenic strains of HSV (Laycock et al., 1991; Shimeld et al., 1999). The molecular events that trigger reactivation in this model are not well understood, as UV radiation at the body surface results in multiple neuronal effects that could be relevant to reactivation. For example, UV radiation-induced danger signals at the body surface can lead to the changes in the levels of regulatory neuropeptides, neurotrophins, neurotransmitters and oxygen products that could signal to the innervating sensory and autonomic nerves (Roosterman et al., 2006). In addition, UV treatment in mice results in increased serum levels of cortisol (Han et al., 2017) and may act through a pathway that is similar to psychological stress-induced reactivation. Clearly the molecular pathways that trigger UV-induced reactivation deserve a more thorough investigation, especially when considering that this common insult can result in HSV-related disease in humans.

Conclusions and future directions

Significant progress has been made in understanding the mechanisms that underlie HSV reactivation, but considerable gaps remain in our knowledge of how different signaling pathways ultimately act on the latent genome. Although the activation status of cellular proteins, including mTORC1, adenylate cyclase, JNK and DLK, have been linked to reactivation under certain conditions, it is not clear whether these proteins are common regulators of the latent to lytic switch in response to different stimuli. However, these proteins represent potential targets for therapeutics. A particularly intriguing cellular protein is DLK because it appears to serve as a master regulator of axonal processes, including degeneration and regeneration (Tedeschi and Bradke, 2013). Highly expressed in neurons (Hirai et al., 2005; Kelkar et al., 2000), DLK is a key candidate for therapies that counteract neurodegeneration and may also be an ideal target to prevent HSV reactivation.

Interestingly, the outcome of DLK activation depends not only on the type of stimuli, but also on the location of the stimulus. This is especially highlighted by the differential effects of localized NGF deprivation. Global deprivation of NGF results in apoptosis of immature neurons, whereas axon-specific deprivation results in the degeneration of the deprived axon – a process known as axon pruning (Cusack et al., 2013; Geden and Deshmukh, 2016). Following NGF deprivation, mature neurons will restrict apoptosis but fail to restrict axon pruning (Cusack et al., 2013; Easton et al., 1997; Kole et al., 2013). Because axons of infected neurons are usually exposed to a different milieu from the soma, it will be important to investigate whether stimuli exhibit localization specific outcomes in terms of HSV reactivation, as well. It has been shown that axon-specific mTOR inhibition can trigger reactivation, indicating that inhibition of local protein synthesis in the axon is a sufficient stimulus for reactivation (Kobayashi et al., 2012). Determining whether axon-specific

deprivation of neurotrophins can reactivate HSV, and, if so, whether HSV traffics down the same axon from which deprivation occurs would also be of significant physiological importance.

It is likely that there are many uncharacterized modulations of the viral chromatin structure that occur either prior to or concurrent with lytic gene expression, as well as other changes that are involved in the transition to full reactivation. In addition, little is known about the cellular chromatin remodelers, histone modifying factors and transcription factors that contribute to different triggers of reactivation. The removal of H3K27me3 from the viral genome is required for full reactivation (Cliffe et al., 2015; Messer et al., 2015), but which K27 histone demethylases are required and how these proteins are recruited to the viral genome are not known. Similarly, a JNK-dependent histone methyl/phospho switch has been shown to occur on viral lytic promoters following interruption of NGF-signaling (Cliffe et al., 2015), but the mechanisms regulating gene expression following histone methyl/phospho switches are still poorly defined (Fischle et al., 2003; Noh et al., 2014; Winter et al., 2007). Further investigation of how histone phosphorylation influences transcription of the viral genome will uncover mechanisms regulating HSV gene expression and provide insights on the control of cellular gene expression. Furthermore, there is clear evidence that the insulator protein CTCF and components of the polycomb repressive complex (Washington et al., 2018b) are evicted from sites on the viral genome following different reactivation stimuli, but the cause-and-effect relationships and mechanisms of these processes are less clear.

Likewise, the regulation of RNA polymerase recruitment and elongation during the different phases of reactivation has not been fully explored. Recent results suggest that inhibition of BET domain proteins enhances HSV reactivation following explant, indicating that formation of the RNA-polymerase super-elongation complex may promote reactivation (Alfonso-Dunn et al., 2017). In unpublished studies, we have also found that BET-domain inhibitors are sufficient to trigger robust reactivation in human differentiated and primary neurons. One interpretation of this data is that RNA polymerase II is present on HSV lytic promoters in a poised form and switches to an elongating form following a reactivation stimulus. However, the presence of RNA polymerase on lytic promoters during latency has not been shown. It is worth noting that BET domain proteins do exist on the Kaposi's sarcoma associated herpesvirus genome during latency, where they have been found to regulate the three-dimensional genome structure (Mo et al., 2015; Stroud et al., 2017). Thus, BET domain proteins could potentially play a similar role in HSV latency.

There is now a growing body of evidence demonstrating heterogeneity in latently infected ganglia in terms of localization of viral genomes, copy numbers of viral DNA and expression of lytic and latent transcripts (Catez et al., 2012; Ma et al., 2014; Proenca et al., 2008; Sawtell, 1997). This likely results in different viral chromatin states that are more or less primed to reactivate depending on the stimulus. How this heterogeneity arises is unclear, but it could occur due to a variety of factors, including the exposure of the neuron to different cytokines and signaling molecules, the amount of infecting virus, or heterogeneity in neuronal populations themselves. There is great diversity in sensory and sympathetic neurons, which can differ in their physiological, anatomical, structural and molecular properties (DeLeón et al., 1994; Gold et al., 1997; Lallemend and Ernfors, 2012; Liu and

Ma, 2011), and this may have significant implications for HSV infection. Certain sensory neuron subtypes, characterized by the presence of neurofilaments (NefH) and calcitonin gene related peptide α (CGRP), as well as positive staining with the A5 antibody, have the highest levels of LAT promoter activity, are less permissive for HSV-1 productive infection, and preferentially undergo early-phase reactivation (Bertke et al., 2009; 2011; Cabrera et al., 2018). Sympathetic and sensory neurons also vary in regards to their expression of different stress hormone receptors, neurotrophin receptors, and ion channels, which also influence how these neurons respond to cues during both acute infection and reactivation from latency (DeLeón et al., 1994; Gold et al., 1997; Lallemend and Ernfors, 2012; Liu and Q. Ma, 2011). Neurons acquire differences in their epigenetic signatures resulting from experience dependent activity (Mo et al., 2015; Stroud et al., 2017), and there is even evidence of DNA copy number variations, at least in the central nervous system (McConnell et al., 2013). In addition, there is evidence of heterogeneity in how individual neurons respond to axotomy (Hu et al., 2016), and this is likely the case for other triggers of reactivation. Following a given stimulus, only a subpopulation of neurons fully reactivates, perhaps suggesting that certain viral genomes or populations of neurons are not capable of progressing through to full reactivation. Understanding how heterogeneity contributes to reactivation potential of latently infected neurons will have significant therapeutic and basic science value.

In summary, a growing body of literature in the field has shed light on the mechanisms of HSV reactivation, but there is still an incredible amount that is not known about these processes. By using a variety of model systems, strains of virus and reactivation stimuli, studies have uncovered both commonalities and differences in the mechanisms of reactivation. Diversity in the neuronal populations, methods of infection and modes of detecting reactivation should be capitalized upon to understand a) how different stimuli can act on different viral genomes in distinct neuronal populations and b) why some genomes may be refractory to reactivation. As an extra-chromosomal, heterochromatin-associated piece of DNA, the HSV genome serves as a sensor of alterations in the neuronal environment and provides a trackable system to understand the resulting changes in gene expression. Ultimately, the goal is to better understand how the viral genome goes from a silent form to fully expressing lytic genes and develop therapies that target latent infection. One potential strategy for developing therapeutics could be to manipulate latency into a form that is completely refractory to reactivation stimuli.

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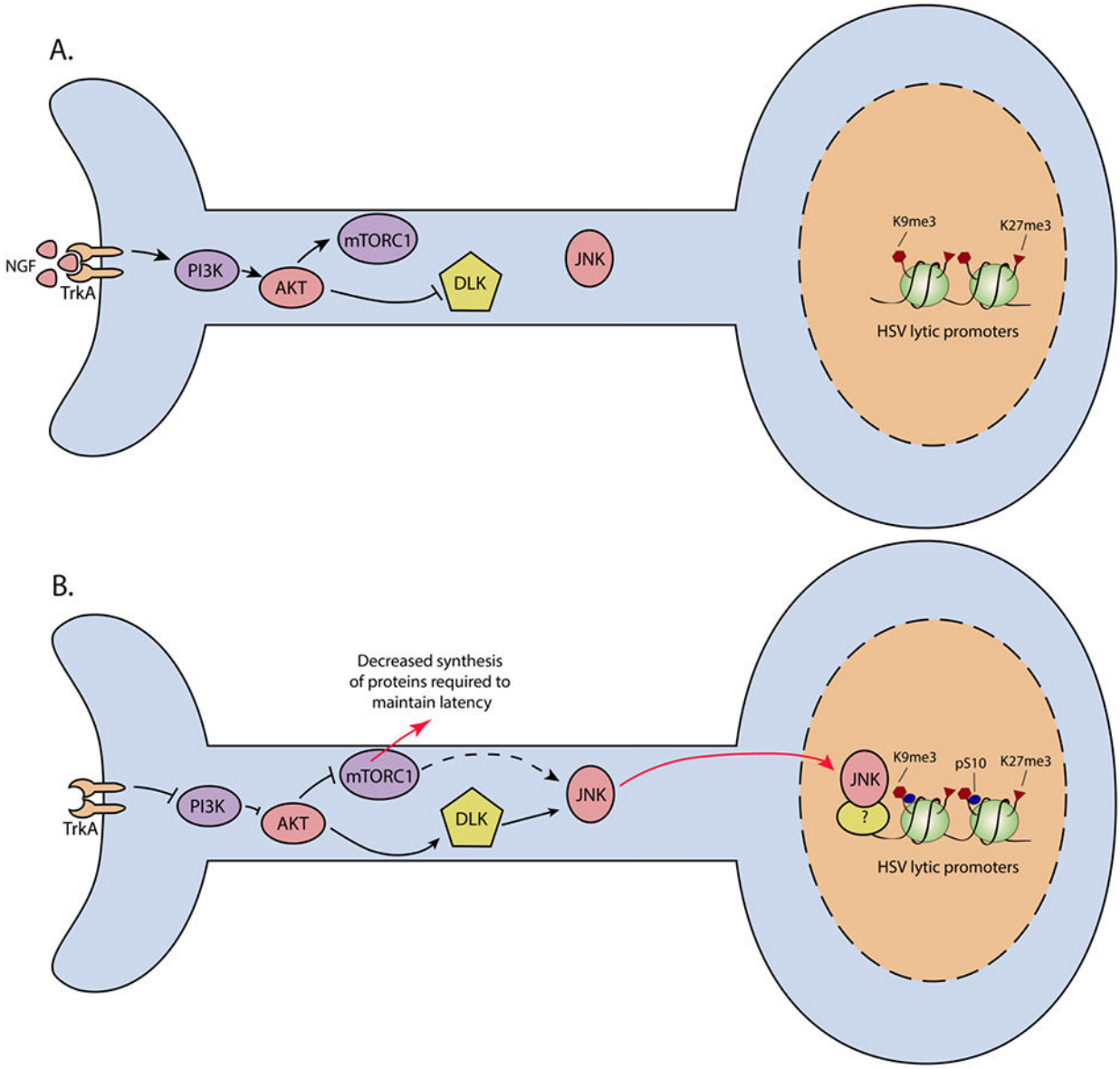

Figure 1. Model of HSV reactivation in response to NGF-deprivation.

(A) In primary neurons, sustained NGF-signaling through the TrkA receptor maintains latency via activation of PI3K/AKT. AKT activation maintains mTOR activity, while inhibiting the activation of DLK. JNK is constitutively active but does not mediate a stress response. Viral lytic promoters associate with repressive histones. (B) Following loss of NGF binding to TrkA, or inhibition of PI3K activity, HSV reactivates. Inhibition of AKT results in mTOR inhibition and redirection of JNK to a cell stress role via DLK. Decreased mTOR activity could result in decreased synthesis of proteins required to maintain latency and possibly activation of JNK. JNK is found enrichened on lytic gene promoters and there is an accompanying H3K9me3pS10 histone methyl/phospho switch.

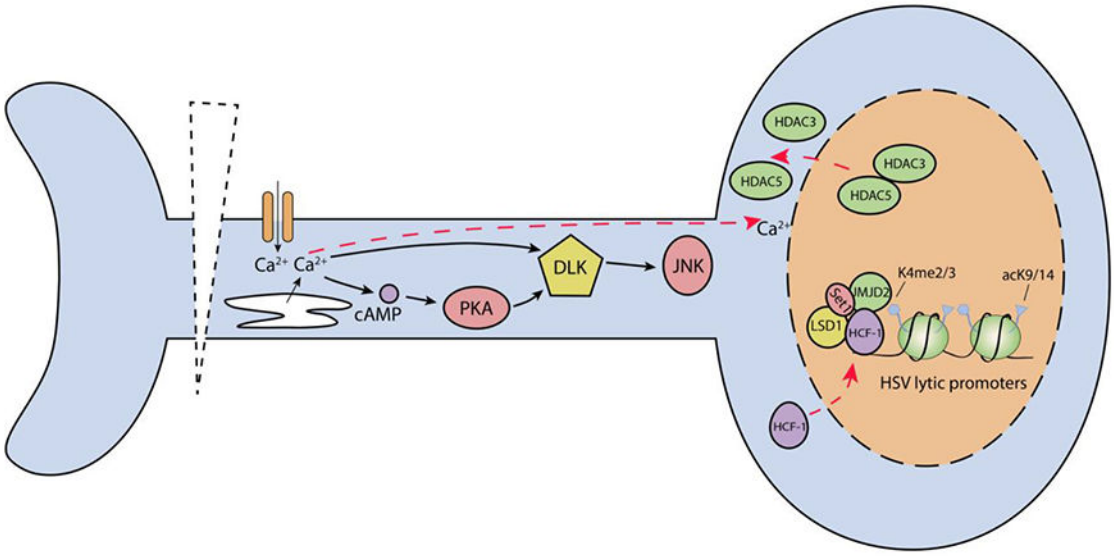

Figure 2. Model of HSV reactivation in response to axotomy/explant.

Following axotomy, there is a rapid increase in intracellular calcium levels triggering a calcium wave that is propagated to the soma. This permits rapid cellular responses, including a global increase in histone acetylation due to the export of HDAC5, along with HDAC3, from the nucleus. This may explain the increased histone acetylation observed on HSV lytic promoters following explant. Increased intracellular calcium also activates adenylate cyclase and DLK, which activates JNK. JNK activity is required for explant induced reactivation. HCF-1 has been found to rapidly translocate to the nucleus and bind to HSV IE promoters. Viral gene expression is dependent on LSD-1 and JMJD2 histone K9 demethylase activity, which are presumably recruited along with Set1, by HCF-1.