Review

A cultured affair: HSV latency and reactivation in neurons

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After replicating in surface epithelia, herpes simplex virus type-1 (HSV-1) enters the axonal terminals of peripheral neurons. The viral genome translocates to the nucleus, where it establishes a specialized infection known as latency, re-emerging periodically to seed new infections. Studies using cultured neuron models that faithfully recapitulate the molecular hallmarks of latency and reactivation defined in live animal models have provided fresh insight into the control of latency and connections to neuronal physiology. With this comes a growing appreciation for how the life cycles of HSV-1 and other herpesviruses are governed by key host pathways controlling metabolic homeostasis and cell identity.

Herpesviruses rely on latency for long-term persistence

All herpesviruses use two contrasting infection strategies: productive (or lytic) replication and latency, constituting fundamentally different viral gene expression programs with contrary goals and outcomes. Latency maintains the viral genome for long periods without producing infectious progeny, but allows the virus to re-engage in productive replication, a process known as reactivation. This ensures long-term persistence as well as dissemination to new host cells or organisms. During latency the infected cell is also changed such that its lifespan is extended and the virus is shielded from immune clearance.

Herpesviruses comprise three subfamilies (α, β, and γ) based on cell tropism and genome organization. In humans, there are three α-herpesviruses, herpes simplex virus 1 (HSV-1, HHV1), herpes simplex virus 2 (HSV-2, HHV2), and varicella zoster virus (VSV, HHV3), each responsible for painful, sometimes debilitating, disease [1]. All three replicate in many tissues,but selectively establish latency in the neurons of the peripheral nervous system (PNS). This review highlights recent advances in our understanding of the HSV-1 latency and reactivation cycle, highlighting the positive impact of latently infected primary neuron cultures as experimental models. With this comes an increasing realization that key molecular processes governing the α-herpesvirus life cycle are also used by β- and γ-herpesviruses in non-neuronal cells.

Establishment and maintenance of latency

The sensory neurons of the trigeminal ganglia (TG) innervate the lips, gingiva, and eyes, and are the principal site for HSV-1 latency in humans, although sympathetic and sensory neurons from the vestibular, geniculate, spiral, and sacral ganglia are also documented sites [2,3]. Viruses access the neurons through axon terminals and release the capsid, containing the viral linear double-stranded DNA genome, into the cytoplasm (Figure 1a). From there, the genome is transported over a comparatively long distance to the cell body located in the ganglion, where it is injected into the nucleus and transcribed by host machinery, resulting in either further productive replication or establishment of latency. How the decision to enter latency is made is not fully understood, although it has long been suspected that the distinctive architecture of neurons and the consequent need for active retrograde transport is a determining feature. Indeed, a recent *in vitro* infection study of dissociated chicken ganglia, cultured in chamber devices that partition neuronal cell bodies from the axons, showed that the site of viral entry has a strong influence on infection outcome [4]. Application of virus to the axon-only compartment favors latency, whereas direct infection of the cell bodies and dendrites more often results in productive replication. This supports the idea, first voiced more than two decades ago, that inefficient axonal transport of virion-associated regulatory factors – such as the viral lytic initiator protein VP16 – would promote latency by compromising the onset of productive cycle gene expression [5]. This is borne out by the chamber experiments because axonal infections can be diverted away from latency by secondary infection in the cell body compartment with a helper virus that delivers VP16, or by treatment with a stress-inducing compound that mimics its stimulatory activity. Absence of VP16 is expected to reduce expression of the five immediate-early (IE) genes, including ICP0, an important regulator of viral gene expression and antagonist of host repression factors, that is essential for reactivation in latently infected mice [6,7]. These findings emphasize the spatial challenges presented by the polarized morphology of neurons, and remind us that HSV-1 and other neurotrophic viruses have evolved to harness these features to their own advantage.

During the establishment of latency, the HSV-1 genome is circularized, presumably by the host DNA repair machinery, and is loaded with core histones in the form of regularly spaced nucleosomes [8]. All latent herpesviruses adopt a similar arrangement, and this presumably allows them to persist for long periods without integration [9]. The ~80 HSV-1 genes needed for productive replication are not expressed at this time, and viral transcription is limited to a ~10 kb locus present twice in the genome. This encodes

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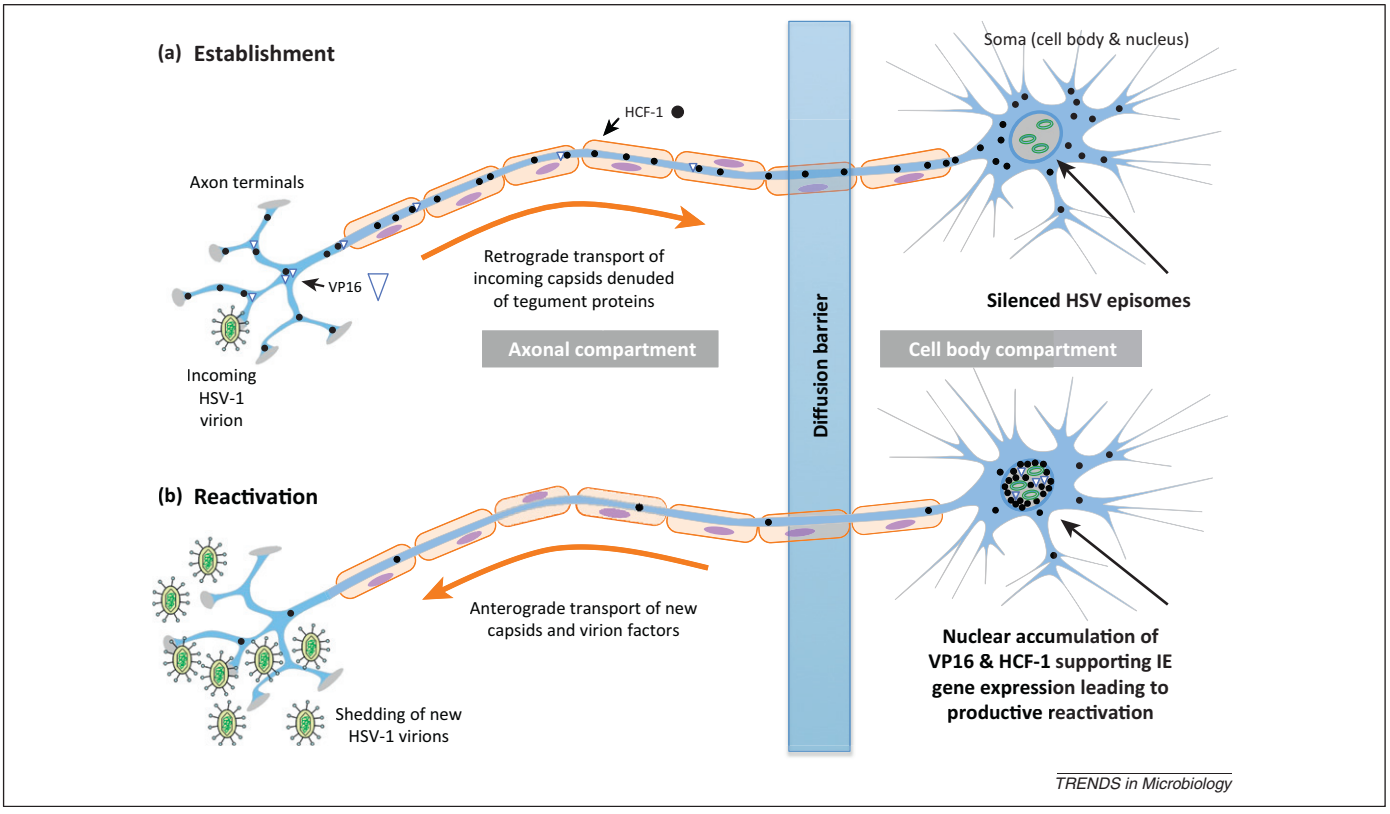

Figure 1. The unique polarized morphology of neurons contributes to the establishment and control of HSV-1 latency. **(a)** During natural infections, HSV-1 enters the nervous system via axon terminals of peripheral neurons innervating the mucosal or corneal epithelial layers, and capsids undergo retrograde axonal transport to the cell body where the genome (green circles) is delivered into the nucleus. Directional infection can be mimicked *in vitro* using chamber devices that allow axons to project through a diffusion barrier, creating physically isolated axonal and cell-body compartments. It is proposed that tegument protein VP16 (blue triangles) dissociates from the capsid almost immediately after release into the cytoplasm, and translocates to the nucleus with very low efficiency, due perhaps to the presence of host transcription factor HCF-1 (black dots) in the cytoplasm of both the axons and cell body. VP16 is required for productive replication in neurons, and thus the absence of tegument-derived VP16 facilitates establishment of latency. **(b)** Reactivation stimuli can elicit many changes in the neuron, including nuclear accumulation of HCF-1 and VP16, which is synthesized *de novo* along with other viral regulatory proteins. Stimulation of viral lytic transcription by VP16 leads to viral DNA amplification and synthesis of virion proteins. Capsids are transported in an anterograde fashion to the axonal termini where they mature and are then released, bringing the HSV-1 life cycle full circle.

the ~8.5 kb latency-associated transcript (LAT) that is rapidly processed into a ~2.0 kb stable intron and set of microRNAs (miRs) [10,11]. The LAT intron corresponds to a circularized intermediate with an extended half-life of hours rather than seconds. As a consequence, it can accumulate to very high levels in the nucleoplasm (≥30 000 copies/neuron). Interestingly, *in situ* hybridization studies reveal considerable neuron-to-neuron heterogeneity in terms of LAT intron abundance. Some neurons accumulate LAT to levels below the detection level of the methodology (LAT^{-}), but are nonetheless positive for the HSV-1 genome [12,13], evidence perhaps for waves of LAT expression that wax and wane over time.

Despite careful studies the function of the LAT locus remains enigmatic, with the weight of evidence pointing to at least a key role in protecting infected neurons from cell death [14]. Differences in the routes of infection and the ganglia analyzed by different teams of investigators have made it difficult to draw firm conclusions. In is clear, however, that in small animal models, mutant viruses lacking the LAT locus are still able to replicate and can also establish latency, although reactivation occurs at reduced levels. *In vivo* marking experiments in mice clearly show that LAT influences the absolute number of latently infected neurons in the TG, but this enhancement is not seen in cervical dorsal root ganglia, suggesting that the route of infection (whisker pad scarification versus ear pinna inoculation) or neuron subtype may be important [15].

Most herpesviruses produce several miRs during latency and influence viral and cellular gene expression [16]. Several of the HSV-1 miRs have been shown to dampen the expression of key lytic regulatory proteins ICP0, ICP4, and ICP34.5 when overexpressed in non-neuronal cells, suggesting that the miRs help to stabilize latency by limiting the cytotoxic effects of spurious viral protein expression [11,17]. By analogy to the γ-herpesviruses, where latency is more easily studied due to the availability of multiple latently-infected cell lines, HSV-1 miRs probably target a battery of host mRNAs to modify the neuronal environment or block antiviral responses. The function of the abundant LAT intron is still a mystery, although it closely resembles a long non-coding RNA (lncRNA), of which there are hundreds in mammalian cells [18,19]. Many cellular lncRNAs function as epigenetic regulators by directing chromatin modifiers to specific genomic locations, or by acting as decoys that draw these factors away from chromatin; the stable LAT intron might perform equivalent functions during HSV-1 latency. Whether LAT specifies any protein products of functional consequence remains controversial. The primary transcript contains at least two candidate open reading frames and can be found associated with ribosomes in infected TGs; however, no genetic

requirement for the protein products or consequence of engaging with the translation machinery have yet been demonstrated [20–22].

Several mechanisms are implicated in lytic gene silencing, including epigenetic controls, recruitment of repressors, competition for activator binding sites, and cytoplasmic sequestration of transcriptional activators [23–26]. Using chromatin immunoprecipitation (ChIP) to probe the composition of latent HSV-1 episomes from murine TG, it has been shown that histones associated with lytic regulatory genes carry marks typical of repressed chromatin, namely the di- or tri-methylation of histone H3 lysine-9 (H3K9me2/3) and lysine-27 (H3K27me3), and reduced levels of acetylated histones [27–29]. In mammalian cells, H3K9me3 is associated with regions of stable heterochromatin that are involved in heritable gene silencing or in the formation of specialized structures such as centromeres and telomeres. These functions requires heterochromatin protein 1 (HP1), which binds to H3K9me3 through its chromodomain and promotes chromatin compaction through oligomeric interactions with HP1 bound to neighboring nucleosomes [30]. H3K9me3 heterochromatic regions have an inbuilt tendency to spread outwards from a nucleation site through direct recruitment of the KMT1 family of H3K9-specific methyltransferases, thereby creating additional H3K9me3 sites.

The presence of H3K27me3 is the signature of epigenetic silencing by the *Polycomb* group (PcG) proteins, comprising the subunits of two functionally distinct PcG repression complexes, PRC1 and PRC2 [31,32]. First characterized in *Drosophila*, the PcG proteins provide a widely used repression system involved in the control of developmental processes in animals and plants. There are several versions of PRC2, but all contain an H3K27me3 methyltransferases subunit (either EZH1/KMT6B or EZH2/KMT6A) that deposits and maintains the signature H3K27me3 mark. This helps to recruit PRC1, and together these complexes antagonize RNA polymerase II elongation through monoubiquitination of histone H2A at lysine-119 or ubiquitination-independent chromatin compaction. PcG-mediated repression is probably used by all herpesviruses during latency. For example, H3K27me3 marks are broadly distributed across the silenced lytic genes of Kaposi's sarcoma-associated herpesvirus (KSHV), a human γ-herpesvirus that latently infects proliferating B cells and endothelial cells [33]. Although the exact subunit composition and function of PRC1 and PRC2 in peripheral neurons is unknown, the PRC1 core subunit BMI1/PCGF4 is detected on latent HSV-1 genomes by ChIP [34], supporting the idea that both complexes are recruited to sustain latency.

How HSV-1 episomes are selected for PcG-mediated silencing is unclear. PRC2 is not recruited to chromatin by default and must be deliberately targeted through *trans*-acting factors. In mammalian cells, this is achieved using lncRNAs that bind to the EZH2 or JARID2 subunits of PRC2 in a phosphorylation-dependent manner [35]. However, JARID2 and EZH2 are considered to be exclusive to dividing cells, being replaced in differentiated non-dividing cells by EZH1, which does not bind to RNA. It will be interesting to see if this generalization holds true for HSV-1-infected neurons, and whether LAT intron or precursor is associated with PRC2. In mice TGs, reduction of LAT transcription leads to increased lytic transcript levels and reduced levels of H3K27me3 on IE promoters, concomitant with increased IE mRNA levels [27]. Paradoxically, when analogous experiments are performed in rabbits, loss of LAT leads the opposite result – with reduced lytic gene expression and reactivation potential [36]. The reasons for this striking discordance are not known. Another pressing question is whether the H3K9me2/3 and H3K27me3 marks are present on the same episome or indicate a mixture of episomes in alternative chromatin states. Based on studies of cellular genes, the latter seems more likely [24], but it will require sequential ChIP or manipulation of the host enzymes responsible for adding or removing each modification to demonstrate this empirically.

Also implicated in lytic gene silencing is the HDAC/LSD1/REST/CoREST (HLRC) complex, which contains both deacetylase (HDAC1/2) and demethylase (LSD1/KDM1) activities [37]. During productive infection HLRC antagonizes early (E) and late (L) gene transcription, but is countered by IE product ICP0, thus allowing transcription to proceed. Recruitment of HLRC to the E promoters is mediated by the DNA-binding REST subunit; in mice, a recombinant virus expressing dominant-negative REST is more virulent than the wild type and replicates better in the PNS, suggestive of reduced entry into latency [38]. Silencing via HLRC may be one consequence of inefficient VP16 transport during axon-mediated infection of neurons due to reduced expression of ICP0.

Reactivation: a race to the finish with multiple hurdles

To reactivate, a latent episome must extensively reorganize its chromatin, ensure that levels of IE gene expression are sufficient to overcome the virus-encoded miRs, and contend with antagonistic host responses. For every HSV-1 genome that produces infectious progeny, it is likely that a greater number will have begun the process but failed at some point. As viral activity increases, the likelihood of a strong counter-response from the host grows, and progression to each successive stage may involve mechanisms that gauge the capability of the host to support the next steps – such as DNA replication, virion synthesis, or dissemination to epithelial cells – to prevent the elimination of infected neurons without producing new virus.

In reviewing the current state of our knowledge, it is important to consider the different experimental approaches used. Most information on latency comes from small animal models, principally mice and rabbits, and we direct the reader to a comprehensive overview by Wagner and Bloom [39]. It must be remembered, however, that HSV-1 co-evolved with humans, and several viral genes are known to function with limited activity in rodent cells [40–42]. Thus the nuances of individual models or methods to elicit reactivation must be kept in perspective. In mice, HSV-1 shows a much lower level of spontaneous shedding at peripheral sites than is seen in humans, and consequently the majority of studies use explant and axotomy to induce reactivation. Ganglia are removed from sacrificed animals (explant), leaving behind the axonal processes (axotomy), and this imposes significant but undefined

stress [43]. The cell bodies and remaining projections are dissociated and maintained in culture media for several days, during which time infectious virus is produced. Lytic mRNAs can be detected within hours of explant and virus is evident in as little as 14 h. Another approach is to apply stress to the live animal before analysis, for example by raising the core body temperature to 43°C for 10 min [44], but here again the exact physiological trigger(s) is undefined. Rabbits and guinea pigs resemble humans in that virus is shed without need for radical stimulation, and this too can be enhanced by local or systemic stress. However, these too are imperfect experimental models because of other issues such as viral strain effects and significantly greater costs [45,46].

Several methods for *in vitro* infection of cultured primary neurons have been developed, yielding quiescent infections that display all the hallmarks of latency defined in animal models and humans [4,47–51]. One advantage is that reactivation can be elicited using a wider range of pharmacological compounds or by defined molecular stimuli such as expression of short hairpin RNA (shRNA) or mutant proteins. Although several primary neuron models are currently being used to good effect, one of the most robust employs sympathetic neurons prepared from prenatal rat superior cervical ganglia (SCG), yielding homogeneous and long-lived cultures that can be latently infected. Treating cultures with a phosphatidylinositol 3 (PI3)-kinase inhibitor results in a protracted reactivation profile, with infectious virus first appearing 24–48 h post-induction (hpi) [41]. Profiling viral mRNA levels reveals two waves of lytic gene transcription. The first (Phase I) occurs at 15–20 hpi, with simultaneous transcription of IE, E, and L genes, and does not require new viral protein synthesis or viral DNA replication, which begins at 25–30 hpi. Phase I is transient, and lytic mRNA levels drop before a second wave of transcription (Phase II) that resembles the cascade observed during acute infections, and which coincides with viral genome amplification and synthesis of infectious particles.

Viral proteins made during Phase I include the lytic initiator VP16, which first accumulates in the neuronal cytoplasm rather than in the nucleus. Not surprisingly, Phase I does not require VP16, as demonstrated by shRNA-mediated depletion or expression of a VP16 transactivation mutant [41]. By contrast, Phase II mRNA levels are reduced fivefold in the absence of VP16-mediated transcription, and infectious virus is not produced. The onset of Phase II coincides with the earliest detection of VP16 protein by immunofluorescence microscopy in the nuclei of a small number of responsive neurons, consistent with its role in boosting transcription of viral IE genes [41]. The strict dependence on VP16 for successful reactivation in response to interruption of PI3-kinase signaling agrees with previous *in vivo* findings using hyperthermia-induced reactivation in mice [52]. Interestingly, explant-induced reactivation is not dependent on VP16, suggesting that productive Phase II can be initiated by other means, possibly by direct stimulation of the IE promoters by stress response pathways [53,54].

The function of VP16 has been studied in considerable detail [55]. All VP16-responsive promoters contain copies of a specific DNA sequence ('TAATGARAT') that binds a core complex of VP16 and two cellular cofactors, Oct-1 and HCF-1. The resulting 'VP16-induced complex' (VIC) recruits additional cellular factors including several chromatin-modifying enzymes, to create a transcription-permissive chromatin environment at each IE promoter. Repressive histone modifications, such as H3K27me3, are removed, and are presumably replaced by activating marks including trimethyl histone H3 lysine-4 (H3K4me3) [26,27,56]. Likewise, the canonical histone H3.1 is replaced by H3.3 and nucleosome density across the genome is reduced [57]. Several chromatin modifications can be attributed directly to known VIC-associated activities, whereas others are mediated by the IE protein ICP0 [58,59]. Mammalian cells contain two enzymes capable of removing H3K27me2/3, UTX/KDM6A and JMJD3/KDM6B [60], and, for KSHV, overexpression of either is sufficient to induce reactivation [33]. It has been shown recently that the non-coding RNA (ncRNA) PAN, which accumulates rapidly after the onset of KSHV reactivation, interacts with UTX and JMJD3, directing them to the gene encoding RTA, the principal lytic activator, creating a strong feed-forward loop [61]. HSV-1 lacks a clear equivalent of PAN, but may have devised another mechanism to mobilize UTX and JMJD3 and displace PRC1 and PRC2, or alternatively this occurs independently of viral products, perhaps coincident with the promiscuous Phase I transcription of viral lytic genes.

Importance of an active signaling program to maintain latency in neurons

It has been known for more than a century that applying trauma to a nerve to treat chronic pain (trigeminal neuralgia) can elicit an outbreak of herpetic lesions in the associated dermatome [62]. This eventually led to the realization that sensory ganglia are the source of reactivating virus, and that reactivation is most likely is due to loss of trophic support. Neurotrophins are growth factors that function in the nervous system to promote survival, proliferation, differentiation, axonal growth, and synaptic plasticity [63]. Many cell types secrete nerve growth factor (NGF), the first identified neurotrophic factor, which uses the membrane-associated receptor tyrosine kinase TrkA expressed by NGF-dependent sensory and sympathetic neurons. NGF–receptor interactions produce signals that travel to the nucleus, causing changes in neuronal gene expression (Figure 1b). Studies on the principal sites of HSV-1 latency have centered upon NGF-dependent neurons. For example, application of anti-NGF antibodies to the eyes of latently infected rabbits resulted in virus shedding, consistent with increased reactivation [64]. This can be recapitulated *in vitro* using neurons from the TG and SCG [50,65,66], allowing systematic dissection of the downstream signaling using a combination of pharmacological inhibitors and RNA interference [50,67]. Although the TrkA receptor activates several discrete signal-transduction pathways, continuous signaling through PI3-kinase, PDK1, and Akt is needed to suppress reactivation [50]. Other growth factor receptors utilize the same downstream kinases, but the signal is transient and they cannot fully substitute for NGF and TrkA [50]. The mTORC1

kinase is a major target of the PI3-K–Akt pathway, and its activity is required to maintain latency [67]. As a central integrator of many nutritional and stress-related inputs, a key function of mTORC1 is to regulate cap-dependent mRNA translation via cellular translation repressors termed eIF4E-binding proteins (4E-BPs). Even transient interruption of protein synthesis, using a pulse of the reversible translational inhibitor puromycin, or by exposure to reduced oxygen environment (hypoxia), a known physiological regulator of cap-dependent mRNA translation, is sufficient to reactivate the virus. This may not be unique to HSV-1 because the inhibition of Akt is also sufficient to reactivate KSHV in primary effusion lymphoma lines [68]. A wealth of data implicates mTORC1 in coupling key cellular functions to the availability of growth factors, oxygen, nutrients, or environmental insults such as genomic damage [69]. Evidently these parameters matter to persistent eukaryotic viruses, and this even extends to bacteriophage lambda, which has tied its lytic–lysogenic switch to nutrient-sensing pathways in its bacterial host [70].

Axons provide a physical connection between the neuronal nucleus protected within the ganglion and the periphery, which connects to the world beyond. This raises a key question: can signals originating in the axon directly influence the episome hidden in the nucleus? One recent study has addressed the issue using compartmentalized neuron cultures where axons are encouraged to cross a diffusion barrier using an NGF gradient, becoming effectively isolated from the cell body and proximal dendrites [67]. Treatment of the axon-only compartment with an mTOR kinase inhibitor for 30 min was sufficient to induce reactivation, indicating that continuous kinase activity away from the cell body is necessary to maintain latency. Although other interpretations are possible, these finding are consistent with the idea that environmental cues can be perceived and interpreted in the periphery, and are then relayed along the axons to the viral genome. Whether this involves long-range transport of neuronal proteins that turn over rapidly, or the propagation of signals that govern factors already in the nucleus, awaits discovery. Axonal signaling is a subject of intense study, and many of the players are known [71].

Peeling back the layers of host control

Environmental triggers that cause HSV-1 reactivation in people include emotional stress, fever, UV exposure,

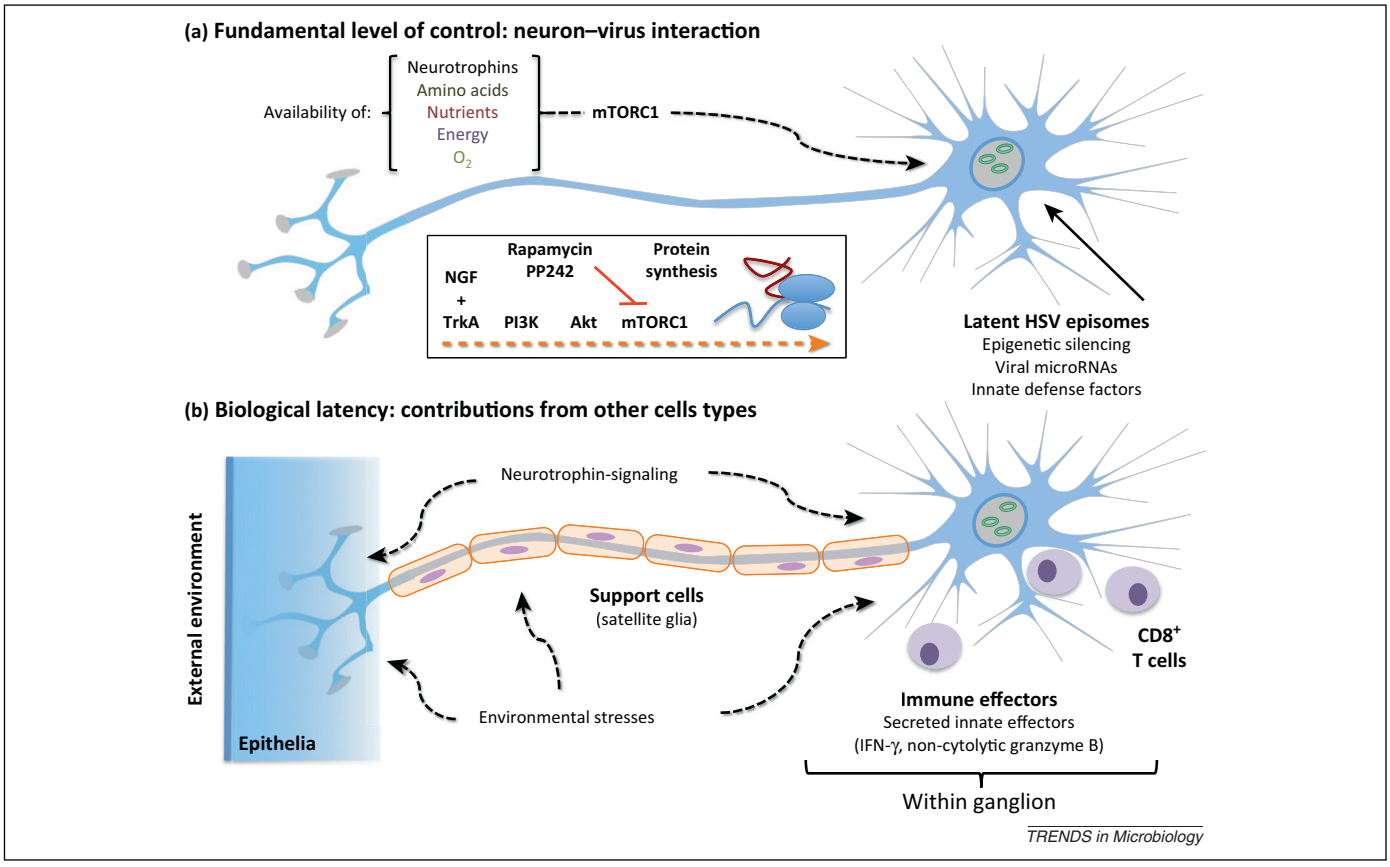

Figure 2. Maintenance of HSV-1 latency by different inputs acting at multiple levels. **(a)** Cell culture experiments have shown that the fundamental unit of HSV-1 latency is the viral genome and its host neuron. Active intracellular signaling is required to maintain latency and suppress lytic gene expression. A key player in this host-mediated control is the cellular kinase mTORC1, whose activity is regulated by neurotrophic factors such as NGF and fundamental indicators of homeostasis, including amino acid sufficiency, oxygen levels, and cellular energy reserves. The inset panel shows how mTORC1 is activated by the neurotrophin NGF, which stimulates PI3K–Akt signaling through its high-affinity receptor TrkA, and is repressed by chemical inhibitors (rapamycin, PP242). A sensor of stress and/or environmental changes, mTORC1 controls the population of mRNAs that are actively translated into proteins, some of which may suppress the lytic cycle by sustaining the repressive chromatin state of the viral genome or by sequestering key host factors in the cytoplasm. Virus-encoded microRNAs help to protect the neuron from apoptosis and dampen the effects of inappropriate lytic mRNA transcription. **(b)** Other cell types found in proximity to the neuron are known or suspected to influence latency, or act as sensors that provide a reactivation stimulus. HSV-specific $CD8^+$ T cells present in the ganglia of latently infected animals and humans suppress reactivation through the secretion of IFN-γ and other non-cytolytic factors. Epithelial cells are well positioned to sample the external environment and act as a source of neurotrophins and other signals. Involvement of satellite glia and other support cells in the regulation of latency has not been demonstrated; however, their physical proximity to neurons makes this a strong possibility. Working together, this complex network of interactions defines the natural phenomenon referred to as biological latency.

hormonal changes, dental surgery, and cranial trauma. Whether these stimuli act directly on the infected neuron, or through some of many other cell types present in the ganglion, is unknown. The emerging consensus is that latency is intrinsic to neurons but that, in the context of a living host, there are additional inputs or layers that involve other cell types (Figure 2). Ganglia are sophisticated tissues containing a variety of non-neuronal cells, including satellite glia and also $CD8^+$ T cells, that are found juxtaposed to latently infected neurons, sometimes connected by immunological synapses. Although some of the T cells may be poised to contain reactivation events once they start, there is evidence of a more active role in maintaining latency through the secretion of effector molecules, such as interferon-γ (IFN-γ) and granzyme B, that suppress the virus without destroying the neuron [72,73]. Psychological and physical stresses are known to influence the behavior of $CD8^+$ T cells through the synthesis and release of neuroendocrine-derived peptides and hormones, potentially linking control of HSV-1 latency to activity in the sympathetic nervous system [74].

Differences in the immune system rather than neurons might contribute to the lack of spontaneous shedding in mice when compared to humans [46]; the discordance between the frequency of shedding at peripheral sites and the appearance of infectious virus in the ganglia is well documented [75]. The viral ICP47 protein, that prevents major histocompatibility complex (MHC) class I antigen presentation on the cell surface, functions 100-fold less efficiently in rodent cells compared to human cells, providing a clear example of a virus–model mismatch that may allow mice to clear spontaneous reactivation events more easily before virus can be shed [42,76]. Restoring this capacity, by incorporating either murine cytomegalovirus (CMV) m152 or human CMV US11 into HSV-1, neutralized the $CD8^+$ T cell response and enhanced reactivation [77,78]. Because immune surveillance and other factors provide a significant layer of host control, there may be value to the field in drawing a clearer distinction between reactivation, in the sense of detectable virus being released in the periphery (full or successful reactivation), and more specific events such as the initial transcriptional response of a latent genome (usefully termed 'animation' [79]).

Concluding remarks

Many aspects of latency and reactivation need to be explored further (Box 1), and this will undoubtedly benefit from the increasing acceptance of cultured neuron models. The lack of simple methods to detect latent virus in live cells has made it particularly difficult to study the temporal or spatial relationships between known events. Do viruses first replicate in the neurons that support latency, explaining the presence of multiple episomes? Do all genomes engage in active lytic gene transcription, or is animation limited to a few? Do LAT^- and LAT^+ neurons differ in their capacity to support reactivation or return to latency if there is abortive reactivation?

The emerging issue of tropism is especially interesting because it may lead us to the molecular determinants of latency. It is now well established *in vivo* and *in vitro* that neurons within a single sensory ganglia differ markedly in their ability to support latency [48]. Using antibody markers to distinguish neuron subtypes, $A5^+$ nociceptive neurons are the principal site of HSV-1 latency in mice following footpad or ocular infection, whereas $KH10^+$ neurons from the same ganglia do not support latency. It may be relevant that A5 neurons are NGF-responsive; however, the underlying mechanism is more complicated because HSV-2 favors KH10 neurons as its site of latency, a difference that maps to a transferable genetic element within LAT [80].

The chief take-home lesson from the studies touched upon here is that the virus and its host neuron represent the fundamental unit of latency. Even in the absence of other cell types, the neuron imposes a significant degree of control over the viral parasite – and this requires the continuous activity of signal transduction machinery used in all cells to convert information about the environment, nutritional status, and cell integrity into changes in gene expression. IFN-γ and other molecules secreted by non-neuronal cells impose a further layer of control, and these may be governed by the same or different environmental cues. Peeling away each layer in the context of a whole animal is very challenging, and there is much to be learned from the judicious use of organotypic cultures. This is especially true for studies of the crucial cellular transcription factors and signaling molecules, many of which are essential for animal viability.

The field needs to address the consequences of using non-human cells to study HSV-1 head-on. We already know that several viral proteins require human molecular targets to function optimally, and more examples may be forthcoming. This prompts a legitimate concern that these various 'mismatches' add up, skewing the relative importance of particular viral proteins or altering the ability of viral regulatory circuits to respond properly to host inputs. Of course, many studies are currently impossible using human tissues, but this may change with advances in induced pluripotent stem cell (iPSC) technology [81,82]. Before long, studying HSV-1 in cultures of iPSC-derived human sensory neurons may be routine, and this will in turn encourage the study of natural viral isolates rather

Box 1. Outstanding questions

- Are all latent genomes competent to reactivate? Most latently infected neurons contain multiple episomes, but it is not known if these share a uniform chromatin composition or if they are equally capable of reactivation. Indeed, elegant studies of productive infection show that only a finite number of incoming genomes can replicate successfully within a single cell but what determines this limit is unknown [84].
- Can viruses that have initiated reactivation return to the latent state? Latently infected neurons are often juxtaposed with virus-specific $CD8^+$ T cells, raising the question of how these neurons are recognized. One possibility is low-level or episodic expression of viral lytic proteins without viral replication, reminiscent of the Phase I transcription observed in cultured neurons [41].
- What determines effective transmission from neurons to epithelial cells and vice versa? An increasing number of non-essential viral genes influence dissemination of virus into and out of the PNS [85]. Identifying the corresponding cellular determinants, possibly through the use of chambered devices and mixed cell cultures, will provide insight into the determinants of cell–cell transmission and the relationship between tissue tropism and pathogenesis.

than laboratory-adapted strains. In closing, we are reminded of a prescient remark by Jack Stevens and colleagues from their 1987 initial description of LAT, noting that 'the metabolic and physiological state of the neuron itself plays a crucial role in viral genetic expression' [83]. This prediction rings all the more true today as the full extent to which the HSV-1 life cycle is directly influenced by host pathways controlling neuronal identity and metabolic homeostasis becomes clear.

Acknowledgments

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