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Neurons versus herpes simplex virus: the innate immune interactions that contribute to a host–pathogen standoff

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Abstract

Herpes simplex virus (HSV) is a prevalent neurotropic virus, which establishes lifelong latent infections in the neurons of sensory ganglia. Despite our long-standing knowledge that HSV predominately infects sensory neurons during its life cycle, little is known about the neuronal antiviral response to HSV infection. Recent studies show that while sensory neurons have impaired intrinsic immunity to HSV infection, paracrine IFN signaling can potentiate a potent antiviral response. Additionally, antiviral autophagy plays an important role in neuronal control of HSV infection. Here we review the literature of antiviral signaling and autophagy in neurons, the mechanisms by which HSV can counteract these responses, and postulate how these two pathways may synergize to mediate neuronal control of HSV infection and yet result in lifelong persistence of the virus.

Keywords

autophagy; herpes simplex virus; interferon; latency; neurons

Viral infections can be broadly placed into two categories; acute and persistent. Acute infection usually results in clearance of the pathogen by the immune system, and failure to do so may lead to severe pathologies and possibly death of the host. Lack of viral clearance may also result in relatively benign long-term persistence, resulting from a balance between host defense and viral countermeasures. Disruption of this balance, especially through host immune compromise or viral immune escape, can result in a switch that favors the virus, resulting in more frequent or severe disease pathologies. The herpesviruses are highly successful and efficient at establishing such persistent infections that humans and most other animals carry for life. Such infections, while very frequent and clinically significant, rarely result in death of the host. This success is facilitated by the establishment of latency during which the virus remains largely dormant. The family *Herpesviridae* is comprised of three subfamilies; α -, β - and γ -herpesvirinae. While subfamily classification is largely based on

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genomics, it can also be based on the cell type in which latency is established *in vivo*. The alphaherpesviruses include the closely related herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), and the more distantly related varicella-zoster virus, all of which establish lifelong latent infections in neurons. Among the betaherpesviruses, cytomegalovirus, a prevalent human pathogen, establishes latency in monocytes. The human gammaherpesviruses which comprise Epstein–Barr virus, and Kaposi’s sarcoma-associated herpesvirus, generally establish latency in lymphocytes and have the additional capability of transforming latently infected cells [1].

The simplex viruses, HSV-1 and HSV-2, establish latency in neurons of sensory ganglia innervating the site of primary infection, most commonly the cells that comprise the susceptible mucosal epithelium of the eyes, nose, mouth or genitalia. While this latent infection is largely asymptomatic, a disruption of latency and derepression of viral replication can lead to orolabial lesions, allowing spread to naive susceptible individuals. Such reactivating virus can result in more severe disease pathologies such as herpes simplex encephalitis (HSE) [2] and herpes stromal keratitis (HSK) [3], although these syndromes can also result from primary infection. While HSV predominantly resides in sensory neurons, the response of neurons to HSV or other neurotropic viral infections is poorly understood. As neurons are a largely irreplaceable cell type critical to host survival, they were previously thought of as immune-privileged bystanders that were thereby protected from potentially damaging immune pathology. More recent studies suggest that neurons do indeed mount innate and intrinsic immune responses to virus infection, although the nature of these responses likely depends upon the type of virus, and the neuronal subtype infected [4–9].

Through use of *in vitro* culture models, there have been significant advances in our understanding of the interactions between HSV and neurons. While many studies have used neuronal cell lines or embryo-derived neurons, it is becoming increasingly clear that the differentiation state and subtype of neuron can greatly affect antiviral responses, making it difficult to meaningfully compare data [4,9–10]. Additionally, it is now evident that the route of neuronal entry (infection of the axon terminal versus the neuronal cell body) can impact the outcome of viral infection [11,12]. It is also important to recognize that *in vitro* systems do not fully account for the anatomic and physiologic complexities of sensory neurons *in vivo*, with their multiple connections to other neurons, exposure to a variety of soluble factors, and interactions with non-neuronal support cells. It is thus important to interpret such systems with caution and validate *in vitro* studies with *in vivo* infection models. Continued use and development of *in vitro* culture models that most closely mimic *in vivo* infection will also be crucial for an accurate understanding of the interactions of HSV and other viruses with neurons. Herein, we review studies examining the innate antiviral response of neurons to HSV and other neurotropic viruses. We also describe HSV countermeasures to these responses and how the biological properties of the neuron and HSV often result in a unique lifelong standoff between host and pathogen.

Herpes simplex virus

HSV belongs to the family alphaherpesviruses and consists of two serotypes; HSV-1 which generally causes orofacial lesions, and HSV-2 which is commonly the causative agent of

genital herpes [13]. Herpesviruses are structurally characterized by their large genome (125–240 kbp) and shared virion architecture composed of an icosahedral capsid that encapsulates a linear double-stranded DNA genome. This is further encased in a cellular derived lipid bilayer envelope decorated with viral glycoproteins. Between the capsid and envelope is the viral tegument, where viral proteins and selective RNAs are packaged [14].

HSV entry, lytic replication & egress

HSV initially infects the cells of the mucosal epithelium. While the predominant mode of viral entry into the cell is through membrane fusion, entry can also occur through endocytosis. Upon entry, viral capsid and tegument proteins are released into the cell and are trafficked via the microtubule transport system to the nucleus, a process that is facilitated by the ATP-driven microtubule motor, dynein. Microtubule transport is particularly critical for neuronal infections where, upon entering axon terminals, viral tegument proteins and capsid must be transported a relatively long distance before arriving at the nucleus [15].

After docking to nuclear pore complexes, viral DNA is released from the capsid into the nucleus where viral gene expression proceeds in ordered temporal waves comprised of immediate-early (IE), early (E) and late (L) genes [16]. The viral tegument protein, VP16, can mediate initiation of IE gene transcription through association with the cellular proteins HCF-1 and Oct-1 and binding to the regulatory element present in the promoter of each of the five IE genes [17]. The IE protein, ICP4, through direct binding of upstream DNA promoter regions, then stimulates transcription of E genes [18]. The expression of IE and E genes is still not sufficient for the majority of L gene transcription, as a subset of L genes require DNA replication in order to be expressed [16]. The linear HSV genome circularizes and DNA replication is mediated by the HSV DNA polymerase complex [19].

The viral capsid is then formed in the nucleus and exits through the double membrane of the nuclear envelope in a process called nuclear egress. It is then thought that the virion envelope is acquired at the trans-golgi network and is subsequently encased in a secondary vesicle which then fuses with the plasma membrane of the cell thereby releasing the virus [20]. Virions resulting from this lytic infection then enter the axonal terminals of innervating sensory neurons. The viral capsid and tegument proteins then travel in a retrograde direction to the neuronal cell body, which resides in sensory ganglia, namely the dorsal root ganglia (DRG) or trigeminal ganglia (TG). It is within the sensory ganglia that HSV establishes a lifelong latent infection.

Latency

During latency, the HSV genome exists as a circular episome in the nucleus of the neuron, is largely transcriptionally silent and produces little to no detectable infectious virus. The mechanisms governing the selective nature of latency establishment in neurons, as well as the factors determining the lytic-latent balance of infection remain largely unknown. An interesting and prevailing model stems from the unique anatomy of the neuron [12,21]. Upon viral entry into axon terminals, capsid and tegument proteins must travel relatively far to reach the cell body via retrograde transport, and do so independently of each other. The tegument protein VP16 is less efficiently transported, thereby resulting in little VP16 in the

neuronal nucleus [12]. Additionally, HCF-1, an important component of the initiation complex to promote IE gene expression, is uniquely sequestered in the cytoplasm of neurons and thus cannot form the necessary complex with VP16, which is already at limiting amounts [22]. Together, these factors result in minimal transcription of IE genes, and in turn, reduced expression of subsequent viral genes [17]. Consequently, the herpes genome remains wrapped in heterochromatin thereby promoting silencing of the genome [23].

While the latent genome is largely transcriptionally silent, there is one region that is highly active encoding the latency-associated transcripts (LATs). Despite their abundance during latency, LATs are dispensable for the establishment, maintenance and reactivation from latency. It is likely that LATs play a regulatory role, however, as LAT null mutants establish latency at a lower frequency. LATs also mediate chromatin remodeling thereby regulating viral gene expression and can promote neuronal survival [24,25].

While LAT is unquestionably the most abundant transcript expressed during latency, lytic transcripts can also be found in latently infected TGs [26]. These findings suggest that latency may not be as stable as previously thought and limited stochastic reactivation events may occur, only to be suppressed by host immunity. This low-level gene expression may provide the neuron with peptides to present on MHC class I to CD8+ T cells. Indeed CD8+ T cells can directly interact with infected neurons to prevent reactivation through noncytolytic mechanisms and are thus important for the maintenance of latency [27].

Reactivation

Reactivation from latency can be triggered by various stimuli including immunosuppression and UV light [28,29]. Upon reactivation, the virus replicates to create new virions which, through anterograde transport, access the peripheral mucosal epithelium and undergo subsequent rounds of lytic replication, enabling viral shedding and host-to-host transmission [15]. While the molecular mechanisms governing reactivation remain unclear, it is hypothesized that the nature of gene expression during reactivation may differ from lytic infection. One study demonstrated *in vitro* that within hours of a reactivation stimulus, an early burst of unordered gene expression occurs, comprised of all viral gene classes (IE, E and L) [30]. This scenario would allow for the actions of VP16 and the IE transactivator ICP0 to subsequently initiate the conventional sequence of ordered gene expression. Indeed, while the kinetics of VP16 expression likely differs, the role of VP16 during reactivation mirrors that of lytic replication as there is evidence that *de novo* synthesis of VP16 can trigger effective viral reactivation [31].

Clinical presentation & epidemiology

The ability of HSV to establish latency enables persistence in the host, resulting in high (65–90%) seroprevalence [32]. HSV-1 infection can be asymptomatic or result in self-limiting orolabial lesions, ocular disease or encephalitis [13]. The annual incidence of new ocular HSV disease is estimated at 11–12 in 100,000 persons in the USA [3], with HSK as the most common cause of recurrent ocular disease and infectious blindness in developed countries [33]. Symptoms of HSK include corneal opacity, edema and neovascularization. Nucleoside analogs such as acyclovir (ACV) are the preferred treatment and in some cases

corticosteroids may be used in conjunction with ACV. While ACV does help to control outbreaks, it does not eliminate latent viral reservoirs, and thereby cannot eliminate recurrent disease [3].

Despite the high seroprevalence of HSV-1, the incidence of HSE is relatively rare with estimates ranging from 1 in 250,000 to 500,000 cases per year [2]. Symptoms of HSE include those typically associated with viral infection (fever, headache and fatigue) along with neurological symptoms such as seizures, confusion, aphasia and focal deficits. These symptoms reflect the involvement of the orbitofrontal and temporal lobes, characteristic of HSE [2]. Like with HSK, ACV is used to treat HSE and its use has reduced mortality from 70 to 28% [2]. Survivors, however, are often left with long-term sequelae including cognitive defects and seizures. HSE can result from primary infection, which commonly affects newborns, and in adults may be a result of reactivation of latent HSV, although there is evidence that HSE can also result from secondary HSV infection [2].

Studies by Casanova and his colleagues demonstrate a role for IFN signaling in protection against HSE as patients with genetic defects in antiviral signaling frequently suffer from HSE [34–36]. Furthermore, neurons derived from induced pluripotent stem cells isolated from patients with such genetic deficits are more permissive to HSV-1, suggesting the neuronal antiviral response is important in preventing HSE [37]. While clinical trial outcomes utilizing recombinant IFN for HSE pathology are varied [38], this therapy proved promising in treatment of HSV lesions [39]. Preclinical demonstration of effective neuronal IFN signaling in combating HSV infection, discussed below, warrants further clinical investigation into the use of recombinant IFN in conjunction with antivirals.

Overview of IFN-driven antiviral signaling

As a result of a significant body of research, it is well established that the IFN-driven antiviral response is critical for controlling HSV infections [34–36,40–41]. This response is initiated when infected cells detect the presence of viral pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs) (Figure 1). PRRs can be found on the cell membrane, endosomal compartments or in the cytoplasm. A major group of PRRs are the toll-like receptors (TLRs) including TLR2, which is found on the cell surface and can detect viral glycoproteins, and TLRs 3, 7, 8 and 9, which are found in endosomal compartments and can detect viral nucleic acids. Cytoplasmic sensors that can detect viral nucleic acids include RIG-I and MDA5, which can sense different forms of RNA in the cytosol. Other cytoplasmic sensors can detect the presence of DNA or RNA:DNA hybrids, which include DAI, cGAS and IFI16 [42–44].

PRRs signal through adaptor molecules to mediate activation of key transcription factors. MyD88 is the predominant adaptor molecule used by all TLRs except for TLR3, which signals through the adaptor molecule TRIF. The cytoplasmic sensors RIG-I and MDA5 signal through MAVS, while IFI16, cGAS and DAI act mainly through STING. These adaptors activate other downstream molecules ultimately leading to the phosphorylation of three key transcription factors; IRF3, IRF7 and NF- κ B [42,44].

Once activated, IRF3, IRF7 and NF- κ B localize to the nucleus and induce the production and secretion of Type I IFN (herein referred to as IFN). IFN acts in an autocrine or paracrine manner to engage the IFN receptor and activate the JAK and STAT pathway, mainly through the transcription factor STAT1. Once phosphorylated, STAT1 forms a homodimer and localizes to the nucleus, inducing the production of interferon-stimulated genes (ISGs). This pathway self-amplifies via successive secretion of IFN α/β and stimulation of IFN receptors. Importantly, uninfected cells can also respond to secreted IFN, establishing an antiviral state to further halt viral spread [43].

There are hundreds of known ISGs with various different antiviral functions including inhibition of transcription and translation, stimulation of cytokine production and promotion of apoptosis. Many ISGs include PRRs and transcription factors that are important in upstream antiviral signaling, thus amplifying this pathway. There are several main ISG effector molecules that have been well characterized and are important for exerting antiviral effects. A few key examples include Mx1, PKR, OAS and RNaseL, which all work to impair viral replication at different stages. Mx1 can degrade incoming viral nucleocapsids and is thought to act by surrounding and directing capsids for degradation through GTPase activity. OAS works to activate RNaseL, which subsequently degrades RNA, including incoming nucleic acid or virally transcribed RNA. Finally, PKR can inhibit mRNA translation through phosphorylation and inactivation of eIF2 α , a key regulatory factor in protein synthesis [45].

A series of important studies have now shown that humans with genetic impairments in TLR3- and STAT1-dependent pathways suffer from recurrent HSE [34–36,46], consistent with a wide body of work in mice [40–41,47–48]. IFN signaling not only controls HSV replication, as infection of knockout mice results in increased viral titers, but it is also important for determining tissue tropism [41], also seen with other neurovirulent viruses [6,49]. Together these studies underscore the importance of IFN-driven antiviral signaling in controlling HSV infection in the nervous system, and help establish the validity of the murine studies.

HSV genes involved in evasion of the IFN response

HSV has a long history of coevolution with its human host and has multiple ways to combat the immune response. Many of these viral proteins target the IFN response, further underscoring the importance of antiviral signaling to both host and pathogen [50]. Outlined below are HSV proteins that modulate the IFN response, categorized by kinetic gene class (Table 1 & Figure 1). Most studies examining the IFN-modulating capacity of these proteins have been done *in vitro* and have not looked specifically at their function in neurons. The importance and function of HSV IFN-modulating proteins may therefore differ in neurons versus non-neuronal cells due to differential expression and cellular localization.

Immediate-early genes

IE genes are expressed prior to any other *de novo* viral gene expression and are thus able to exert IFN-modulating effects early in the viral replication cycle. One such protein is ICP0, an E3 ubiquitin ligase, which can promote viral gene expression and regulate lytic and latent

infections. ICP0 can also disrupt PML (i.e., ND10) bodies, which are important for maintaining heterochromatin, thus further promoting viral gene expression. As such, viruses lacking ICP0 are restricted in replication *in vitro* and *in vivo* [76]. ICP0 can mediate resistance to IFN through counteraction of the antiviral response at multiple stages [77–80]. This is in part through disruption of IRF3 signaling [51,52], which can lead to reduced expression of ISGs [81]. Additionally, ICP0 can regulate NF- κ B signaling through direct degradation of NF- κ B or degradation of an inhibitor of NF- κ B (I κ B α) thereby stimulating transcription of NF- κ B-targeted genes [53,54]. ICP0 can also interfere with PRR signaling by blocking TLR2-mediated signaling [55] and promoting the degradation of IFI16 [56] although this may be cell type specific [82].

Another IE protein is ICP27, which is a multifunctional protein that is necessary for viral replication. ICP27 functions to facilitate export of unspliced, largely viral, mRNAs from the nucleus and recruit RNA polymerase II to stimulate viral gene transcription [83]. Due to the requirement of ICP27 for viral replication, it is difficult to study the IFN-modulating properties of this protein, particularly *in vivo*. Johnson and colleagues, however, demonstrated *in vitro* that ICP27 can block STAT1 phosphorylation and partially block its translocation to the nucleus [57]. Interestingly, they further show that ICP27 is sufficient to induce the release of a soluble factor that can partially inhibit IFN receptor signaling, leading to reduced STAT1 nuclear translocation [58].

Late genes (tegument/structural)

As part of the virion, tegument proteins are poised to exert immediate effects upon entering host cells, including disruption of antiviral signaling. As outlined above, VP16 is a component of the tegument that is important for initiating IE gene transcription [17]. A recent study demonstrated that ectopic expression of VP16 could block upregulation of IFN β upon RNA viral infection. This study found that while VP16 was not able to inhibit IRF3 phosphorylation or nuclear translocation, it can competitively bind to CBP, a coactivator of IRF3 [59]. Additionally, VP16 can inhibit NF- κ B activation, a key transcription factor that can mediate upregulation of IFN β [59].

Another important tegument protein is vhs, an RNase produced by the late gene *UL41* that mediates host and viral RNA degradation leading to shut-off of protein synthesis [84]. Vhs-mutants are hypersensitive to the effects of IFN and infection with a vhs-deficient virus leads to increased IFN production and ISG expression, supporting the role of vhs in counteracting IFN signaling [60,61]. Vhs has been proposed to mediate this IFN resistance by degrading viral RNAs that activate PKR and antiviral signaling through PRRs, but this has yet to be proven [60,85–86]. Vhs mutants are profoundly attenuated *in vivo* and this is partially due to lack of immune modulation, as replication is mostly restored in IFN signaling-deficient mice [40,61,87]. That being said, there is also an inherent growth defect of vhs-deficient mutants, as IFN-independent mechanisms lead to attenuation via corneal infection [88].

US11 is an abundant tegument protein that binds RNA in a sequence-specific manner [89,90]. US11 can counteract antiviral responses by preventing PKR activation thereby

enabling viral transcription [62,63]. While HSV is a DNA virus, dsRNA is produced during HSV transcription, likely a product of complementary transcripts from both strands of the viral genome, thus stimulating RNA PRRs [91]. Through its dsRNA-binding domain, US11 is believed to sequester RNA thereby preventing activation of the OAS pathway [64]. Additionally, US11 interacts with the RNA sensors, MDA5 and RIG-I and through binding to the dsRNA binding protein, PACT, it can disrupt activation of RIG-I. These functions lead to reduced IRF3 activation and IFN β production [65,66]. US11 can also counteract autophagy and xenophagy, cellular processes that may mediate viral degradation, discussed below [67].

VP1/2 (*UL36*) is a large tegument protein that associates with the viral capsid. Among other functions, through binding to the microtubule motor protein dynein, VP1/2 facilitates retrograde capsid transport [92]. VP1/2 also contains a unique deubiquitinase domain [93] and through this domain, can deubiquitinate TRAF3, an adaptor molecule that mediates downstream signaling through recruitment of tank-binding kinase (TBK) which is responsible for phosphorylation of IRF3. This activity, thereby leads to reduced downstream IFN β production [68].

Late genes (nonstructural)

Nonstructural genes that are expressed later during viral replication can be important for disruption of an already established antiviral state and prevention of further antiviral signaling. One such protein is US3, a Ser/Thr kinase that exerts multiple effects including, but not limited to, prevention of apoptosis, inhibition of histone de-acetylases and cytoskeletal rearrangement [94]. US3 also plays a role in modulating the IFN response, as US3-deficient mutants are more sensitive to IFN and infection with these mutants results in increased IRF3 activation and downstream signaling [69,70]. This disruption of antiviral signaling may be mediated through hyper-phosphorylation of IRF3 by US3 and subsequent dysregulation of downstream signaling [71].

A key viral protein responsible for counteracting antiviral signaling is ICP34.5. Through binding with PP1 α , ICP34.5 can reverse PKR-mediated phosphorylation of eIF2 α thereby relieving translational arrest [72,73]. ICP34.5 can also prevent TBK-mediated phosphorylation of IRF3 [74]. ICP34.5 is a neurovirulence factor, as strains lacking ICP34.5 are significantly attenuated in mouse models of intracerebral and corneal infection. This is largely due to the ability of ICP34.5 to counteract the IFN response, as the virulence of ICP34.5 mutant viruses is fully and specifically restored in IFN $\alpha\beta\gamma$ R $^{-/-}$ and PKR $^{-/-}$ mice [95]. Additionally, the demonstrated safety of ICP34.5-deficient oncolytic HSV-1 vectors upon intracerebral infection in clinical trials highlights the importance of ICP34.5 as a neurovirulence factor in humans [96]. Work from our lab has shown an important role for ICP34.5 specifically in countering paracrine IFN signaling in sensory neurons, supporting its role as a neurovirulence factor [97]. ICP34.5 can also inhibit autophagy, as discussed below [75].

IFN-driven antiviral signaling in neurons

Antiviral signaling is critical for controlling HSV infection on a global level, however, the scope and impact of IFN responses in neurons remains unclear. Historically, neurons were not thought to mount a robust immune response as this could potentially lead to death of a largely irreplaceable cell type. Recently, however, studies have emerged demonstrating that neurons are capable of detecting the presence of PAMPs and viral infection, and subsequently mounting an effective immune response to several neurotropic viruses [4–8].

While there is evidence demonstrating neuronal antiviral responses, it is crucial to appreciate the heterogeneity of neurons, which differ in developmental state, subtype and anatomic location. This heterogeneity is reflective in the antiviral responses [4,9–10], thus it is important to recognize the limitations of *in vitro* neuron culture models that rely on cell lines or primary neurons which the virus does not infect *in vivo*. This also underscores one of the many difficulties of comparing the behaviors of relatively simple and homogeneous cultured neuron systems with the high complexity heterogeneous networks of cells within the nervous systems of living mammals.

Detection of viral PAMPs

There is accumulating evidence that neurons express PRRs that enable detection of viral infection. Of particular note are studies of the dsRNA sensor, TLR3. West Nile virus (WNV), a neurotropic flavivirus, replicates to higher titers in primary cortical neurons isolated from TLR3-deficient mice compared with wild-type [5]. This is consistent with neuronal cell lines silenced for TLR3, which support increased Japanese encephalitis virus (JEV) replication [98]. Using a similar neuronal cell line, other studies have demonstrated upregulation of IFN β transcripts upon stimulation with polyI:C, a TLR3 agonist, or infection with the RNA viruses, Rabies or Sendai [99,100]. With the caveat of neuronal cell lines being used in place of primary neurons, this supports a role for neuronal TLR3 in control of neurotropic RNA viral infection.

TLR3 may also function as an important PRR in the neuronal sensing of HSV. A compelling study from Casanova and colleagues demonstrates that neurons derived from induced pluripotent stem cells obtained from patients with a genetic defect in TLR3 are more permissive to HSV infection [37]. These patients suffer from recurrent HSE and thus disrupted neuronal TLR3 signaling may contribute to this disease state [36]. Additionally, HSV-2-infected TLR3-deficient mice support increased viral titers in the nervous tissue, suggesting a role for neural TLR3 signaling in controlling HSV-2 replication [101]. While these studies support a role for PRR signaling in neurons, data from our lab have shown that neurons lacking transcription factors and adaptor molecules downstream of PRR signaling (namely STING, IRF3 and IRF7) support equivalent levels of HSV replication compared with wild-type neurons [97]. These data suggest that this pathway is inefficiently upregulated upon HSV infection, at least as studied in adult sensory trigeminal neuron cultures. That being said, in an *in vivo* infection, PRRs including TLR3 may be upregulated in neurons through paracrine IFN β signaling, enabling detection of PAMPs and further amplification of antiviral signaling, although this has not been directly tested. As such,

further studies are required to elucidate the capacity of sensory neurons to detect HSV infection.

IFN production

Experiments examining neuronal production of IFN upon viral infection have produced conclusions that likely reflect neuronal heterogeneity and distinct responses to different virus families. For example, infection of mice with the RNA viruses, La Crosse encephalitis virus (LCEV) or Theiler's murine encephalomyelitis virus (TMEV), results in IFN production by CNS neurons as measured by *in situ* hybridization and immunofluorescence [102]. Another study demonstrated that while glial cells are likely the largest contributors of IFN in the brain, neurons are capable of producing IFN *in vivo* upon LCEV infection [103]. Furthermore, the number of IFN-producing neurons increased upon infection with an LCEV strain lacking the NSs protein, a major IFN antagonist [103]. This suggests that while neurons may be capable of producing IFN, this response is likely tightly regulated and easily shut off by viral immune modulating proteins.

Unlike RNA viruses, there is little evidence to suggest that HSV infection can promote neuronal IFN production. Indeed, HSV infection of DRG neuron cultures results in little to no IFN production [104] and infected neuronal cell lines supported restricted transcriptional upregulation of IFN β and ISGs [100]. Supporting these findings, data from our lab demonstrated limited IFN production and STAT1 nuclear localization in TG neurons upon HSV infection [97]. Additionally, this pattern was observed in neurons infected with vesicular stomatitis virus (VSV), a prototypic IFN-sensitive RNA virus [97], suggesting that the muted response of sensory neurons to HSV-1 is reflective of an intrinsic impairment and not viral counteraction of this pathway.

ISG expression

In line with IFN production, many studies demonstrate neuronal upregulation of key antiviral effector molecules and other ISGs in response to neurotropic RNA viral infection. For example, neurons deficient in PKR and RNaseL support increased WNV titers, demonstrating the importance of these ISGs in controlling neuronal WNV replication [7]. Additionally, the ISG IRF1 can mediate inhibition of VSV replication in neurons of the CNS [105]. Upregulation of ISG expression was also found in neurons infected with WNV or lymphocytic choriomeningitis virus (LCMV) although this response varied with neuronal subtype [106]. As with IFN production, there is scant evidence to suggest any significant upregulation of ISGs upon neuronal HSV infection. One study demonstrated that HSV infection of a neuronal cell line resulted in limited transcriptional upregulation of ISGs [100]. Supporting this, infection of DRG neurons with HSV-1 led to only modest upregulation of the ISG, Mx1 [104]. While it may be that HSV-1 potently inhibits ISG upregulation in neurons, taken together with data described above, these results suggest that sensory neurons have a muted intrinsic response to HSV infection.

IFN receptor signaling

There have been many studies examining the antiviral response of neurons to exogenous IFN, demonstrating a probable role in restricting neurotropic viral infection. Neuronal IFN signaling can restrict replication of WNV, VSV and other RNA viruses [6,8] although the magnitude of this response varies with neuronal subtype [4]. Additionally, studies demonstrate upregulation of ISGs in neurons in response to IFN [4,107]. Similarly, there is *in vitro* evidence suggesting that exogenous IFN can restrict HSV replication in primary neurons under certain conditions such as high IFN concentrations [108,109], or upon axonal infection [11,110]. Contrasting studies, however, have found that IFN signaling in primary DRG and TG neurons results in little to no restriction of HSV replication *in vitro* [97,104]. In TG neurons this resistance to paracrine IFN signaling is mostly due to counteraction by ICP34.5 [97]. One unifying hypothesis to explain these disparate *in vitro* findings is that axonal transport can impact the susceptibility of HSV to IFN signaling. One can envision a scenario whereby limited amounts of VP16 due to inefficient trafficking leads to reduced expression of downstream genes, particularly those that counteract IFN signaling (Figure 2). Indeed, recent studies from our lab have found that upon axonal infection, HSV replication can be restricted by neuronal IFN signaling both *in vivo* and *in vitro* [ROSATO P, LEIB D, UNPUBLISHED DATA].

IFN receptor signaling & latency

As with acute replication, there is also evidence that neuronal IFN signaling may be important in regulating HSV latency. Early experiments by Wigdahl and Rapp utilizing *in vitro* neuron cultures found that a combination of IFN and the antiviral drug Brivudine (BVDU) can promote the establishment of HSV latency [111,112]. This has been further explored in the context of axonal infection where De Regge and colleagues demonstrate that IFN α can promote HSV latency in porcine TG neuron cultures [11]. While the *in vivo* contribution of neuronal type I IFN signaling on latency remains to be explored, HSV infection of IFN γ -deficient mice demonstrates that IFN γ signaling is dispensable for the establishment of latency [113], but may regulate the number of latently infected neurons [114]. Additionally, IFN γ may play a role in the control of HSV-1 reactivation [115], though this may be indirect through actions of CD8 $^{+}$ T cells [27,116]. Additional evidence comes from clinical studies of patients that suffer from recurrent HSE with genetic defects in antiviral signaling. As HSE is postulated to arise from reactivation of latent virus [2], these observations suggest a function for antiviral signaling in the maintenance of latency and/or control of reactivated virus in humans [34–36,46]. While it is apparent that neuronal IFN signaling can regulate HSV-1 latency, it remains unclear at what stage of latency it is important, if direct neuronal IFN signaling is required, and how this relates to *in vivo* infection.

Autophagy & xenophagy as an antiviral mechanism

Autophagy is a catabolic process of cytoplasmic protein and organelle degradation through engulfment of cytoplasmic contents by autophagosomes and subsequent fusion with the lysosome. This pathway can also be triggered by invading pathogens in a process known as

xenophagy. The role of xenophagy in combating viral infection is multifaceted; xenophagy can directly engulf and degrade invading virus, deliver viral nucleic acids to endosomes containing TLRs thus enabling activation, and deliver endogenous viral peptides to MHC class I and II, thus activating adaptive immunity [117].

The importance of autophagy as an antiviral mechanism is underscored by the fact that many viruses encode proteins that can counteract this pathway, including HSV [117]. HSV encodes two autophagy modulating proteins, US11 and ICP34.5. ICP34.5 can inhibit autophagy through binding Beclin-1, thus suppressing xenophagy and antigen presentation [75,118]. It is clear that xenophagy plays an important role in HSV pathogenesis as mutants lacking the Beclin-binding domain of ICP34.5 show decreased virulence upon intracranial [75], corneal [119] and intravaginal infection [104]. US11 can also inhibit autophagy through direct interaction with PKR, a protein that can upregulate autophagy [67].

Autophagy is an especially important process for neurons, as postmitotic cells cannot dilute protein aggregates and cellular debris by cell division. As such, conditional knockout mice lacking the capacity for neuronal autophagy succumb rapidly to neurodegeneration [120]. Given the importance of autophagy for the homeostasis of neurons, and the potentially damaging effects of conventional innate immune defenses, it has been hypothesized that autophagy represents a nondestructive antiviral defense pathway for neurons [104,121]. Indeed autophagy protects against CNS infection with Sindbis virus [122], and recent work has shown that autophagy, rather than IFN signaling, may be the dominant pathway for the neuronal antiviral response to HSV-1 [104].

While IFN-induced autophagy has only been directly studied in other cell types [123], there is some evidence to suggest a potential role for IFN in the upregulation of antiviral autophagy in neurons [97]. Levine and colleagues have shown that HSV-induced autophagy is dependent on PKR and the eIF2 α pathway [124,125]. Additionally, PKR is upregulated upon IFN signaling in HSV-infected neurons [108], and it is required to restrict HSV infection in TG cultures treated with IFN [126]. Given the muted intrinsic responses to viral infection in sensory neurons [97], these studies taken together suggest that paracrine IFN signaling may be important in upregulating PKR thereby increasing xenophagy in addition to transcriptional arrest. Additionally, the demonstrated marked attenuation of HSV lacking ICP34.5, which counteracts both autophagy and IFN signaling, highlights the importance of these two pathways in countering neuronal HSV infection. Indeed it is likely that autophagy and IFN signaling synergize to mediate neuronal control of HSV (Figure 2).

Conclusion & future perspective

Neurons are postmitotic, largely irreplaceable cells and during viral infection, these properties pose a unique challenge of balancing viral clearance with cell survival. There is mounting evidence demonstrating that neurons are immune capable cells, and their participation in antiviral clearance is likely conditional on the type of viral infection and neuronal subtype. It has additionally been demonstrated that neuronal autophagy can aid in mediating viral clearance, particularly in the context of HSV infection [104]. In addition to autophagy, there is strong evidence from our group and others that paracrine IFN signaling

is important in mediating neuronal control of HSV infection *in vitro* and *in vivo* [11,108–109, ROSATO P, LEIB D, UNPUBLISHED DATA]. While autophagy and IFN signaling are often studied independently of each other, there is evidence suggesting that neuronal IFN signaling may upregulate autophagy through PKR signaling, and thus these two antiviral strategies may synergize to control neuronal HSV infection [97,108,125].

While much has been elucidated about the importance of autophagy and IFN signaling in the context of acute HSV infection, the role of these pathways during latency remains very poorly understood and will be an area of active research in the future. Newly emerging data highlighting the impact of neuronal subtype and differentiation state on antiviral signaling will also encourage new *in vitro* models studying HSV infection of neurons which the virus encounters *in vivo*, namely fully differentiated adult sensory neurons [4,9]. In conjunction with *in vivo* transgenic mouse models, future research will be aimed at utilizing these *in vitro* neuron cultures to discover the role of autophagy and paracrine IFN signaling in the establishment, maintenance and reactivation from latency.

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EXECUTIVE SUMMARY

Herpes simplex virus

- Herpes simplex virus (HSV) establishes latency in sensory neurons, however the intrinsic and innate responses of neurons to HSV remain unclear.

Clinical presentation & epidemiology of HSV infection

- HSV infection is generally asymptomatic but disruption of latency can result in recrudescence and disease.
- HSV usually causes self-limiting mucocutaneous lesions, but can also cause ocular diseases, generalized infections and encephalitis.

Overview of IFN-driven antiviral signaling

- Human and murine studies demonstrate an essential role for IFN signaling in mediating control of HSV pathogenesis.
- Patients with genetic defects in IFN-signaling succumb to recurrent herpes simplex encephalitis.

HSV genes involved in evasion of the IFN response

- HSV has many proteins that can modulate and interfere with the IFN response.
- In particular, the HSV protein ICP34.5 is a neurovirulence factor that can disrupt IFN signaling and establishment of the antiviral state.

IFN-driven antiviral signaling in neurons

- The antiviral response to RNA and DNA viruses differs in neurons.
- Sensory neurons have a muted response to HSV but can mount an effective antiviral response through paracrine IFN signaling.
- HSV can counteract IFN-driven antiviral responses, largely through γ 34.5.ICP34.5.
- Antiviral responses are heterogeneous and reflect differences in neuronal subtype, differentiation state and anatomic location.

Autophagy & xenophagy as antiviral mechanisms

- Neuronal autophagy/xenophagy is important for controlling HSV infection.
- Autophagy may be upregulated in neurons through paracrine IFN signaling.
- The HSV proteins ICP34.5 and US11 counteract the autophagic pathway.

Future perspective

- *In vitro* models that more closely represent the subtype and differentiation state of neurons that HSV infects *in vivo* will add to our understanding of the interactions between the virus and neuron.

- Use of neuron-specific knockout mouse models will further our understanding of neuronal responses to HSV *in vivo*.
- The role of neuronal IFN signaling in controlling HSV latency and reactivation is unclear and will be an area of active research to possibly facilitate design of novel antiviral strategies.

Figure 1. IFN-driven antiviral signaling and HSV-1 subversion thereof

Multiple PRRs can detect the presence viral PAMPs including nucleic acids and glycoproteins. TLR2, located on the cell surface, can detect viral glycoproteins, while cytosolic RNA detection is mediated by RIG-I and MDA5, and endosomal RNA detection is mediated by TLR3 and TLR7. TLR9, also located in endosomes, and cGAS, IFI16 and DAI, located in the cytoplasm, can mediate DNA detection.

These PRRs then signal through distinct adaptor molecules. Toll-like receptors signal through MyD88 except TLR3 which signals through TRIF. Cytosolic DNA sensors

predominately signal through STING while cytosolic RNA sensors signal through MAVS. These adaptor proteins then mediate phosphorylation of the transcription factors IRF3, IRF7 and NFκB, leading to Type I IFN production, which is then secreted. IFN can act in an autocrine manner or through paracrine signaling to uninfected cells. Engagement of the IFN receptor leads to JAK phosphorylation of the transcription factor STAT1 and upon nuclear localization, results in the production of IFN stimulated genes (ISGs) which establish the antiviral state of the cell. Among these ISGs include PKR which can halt translation, Mx1 which can degrade incoming capsids, and Oas/RNaseL which can mediate RNA degradation. HSV-1 can counteract this pathway at multiple stages and details of the major genes involved can be found in Table 1.

Figure 2. Innate immune factors contributing to neuronal control of herpes simplex virus-1 infection

(A) Lytic replication in the mucosal epithelium leads to production and secretion of cytokines, including type I IFN. (B) IFN can signal through IFN receptors on the neuronal cell body (or possibly axon terminals) resulting in dimerization and nuclear localization of STAT1. (C) STAT1 promotes transcription and translation of ISGs including PKR. (D) PKR can promote autophagy and inhibit translation of both cellular and viral transcripts. (E) Autophagy can degrade viral proteins through engulfment and subsequent fusion with the lysosome. (F) Upon infection at axon terminals, the viral capsid is transported along microtubules to the neuronal nucleus while the tegument protein VP16 is inefficiently transported, leading to reduced VP16 in the nucleus. (G) HCF-1 is sequestered in the cytoplasm of neurons and coupled with low levels of VP16 promotes limited transcription of IE genes. Translation is inhibited by PKR and translated gene products may be degraded through autophagy.

Table 1
Herpes simplex virus control of antiviral responses

Protein	Gene/Alt. names	Major function	Kinetic class	Antiviral function	Ref.
ICP0	<i>$\alpha 0$, Vmw110</i>	E3 ubiquitin ligase	IE	Disruption of IRF3	[51,52]
				Regulates NF- κ B signaling	[53,54]
				Blocks TLR2-mediated signaling	[55]
				Degrades IFI16	[56]
ICP27	<i>$\alpha 27$, Vmw63, UL54, IE63</i>	Translational regulator	IE	Blocks STAT1 phosphorylation	[57]
				Inhibits IFN receptor signaling	[58]
VP16 [†]	<i>α-TIF, Vmw65, UL48, ICP25</i>	Transcriptional activator	L	Inhibits IFN β upregulation by competitively binding to IRF3 coactivator	[59]
				Inhibits NF- κ B activation	[59]
vhs [†]	Virion-host shutoff, UL41	RNase	L	Associated with decreases in ISG expression/IFN production	[60,61]
US11 [†]		RNA binding protein	L	Prevents activation of PKR	[62,63]
				Sequesters RNA to prevent OAS pathway activation	[64]
				Interacts with and disrupts MDA5, RIG-I signaling	[65,66]
				Counters autophagy through PKR binding	[67]
VP1/2 [†]	UL36	Deubiquitinase	L	Deubiquitinates TRAF3, preventing recruitment of TBK	[68]
US3		Ser/Thr kinase	L	Hyperphosphorylates IRF3 resulting in dysregulation of downstream signaling	[69–71]
ICP34.5	<i>$\gamma 34.5$, RLI</i>	Protein binding	L	Reverses PKR-mediated phosphorylation of eIF2 α	[72,73]
				Prevents TBK phosphorylation of IRF3	[74]
				Inhibits autophagy through binding to Beclin-1	[75]

[†]Protein is part of the tegument.