Differential regulation of herpes simplex virus-1 and herpes simplex virus-2 during latency and post reactivation in response to stress hormones and nerve trauma in primary adult sensory and sympathetic neurons

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ABSTRACT

The contrasting infection strategy of herpes simplex virus (HSV) consists of an initial primary lytic infection in epithelial cells, followed by establishment of lifelong latency in sensory and autonomic neurons of the peripheral nervous system that innervate the site of infection. Any cellular stress trigger, ranging from external stimuli such as UV radiation or nerve injury to psychological and physiological stress, can reactivate HSV from latency in the neurons, resulting in recurrent disease episodes. Stress hormones and deprivation of neurotrophic factor (NTF) both have a strong correlation with HSV reactivation from neurons. However, neuronal signaling pathways cardinal to HSV latency and reactivation are still not clear. This dissertation provides new understanding of HSV latency and reactivation in response to two orthogonal stress stimuli, viz. stress hormones epinephrine (EPI) and corticosterone (CORT), as well as NTF deprivation that simulates a nerve injury in primary neuronal cultures.

In this dissertation, we demonstrate that physiological stress hormones EPI and CORT differentially regulate HSV-1 and HSV-2 reactivation in adult neurons. Both EPI and CORT treatment reactivated only HSV-1 in sympathetic superior cervical ganglia (SCG) neurons, while HSV-2 was reactivated only by CORT in both sensory trigeminal ganglia (TG) neurons and sympathetic superior cervical (SCG) neurons. EPI utilized the combination of α and β adrenergic receptor complex, while CORT signaled through

glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) to reactivate HSV in the neurons. NTFs are tissue-target derived growth factors required for neuronal protection and survival. Neurotrophins are also required for maintaining HSV latency, as NTF deprivation reactivates both HSV-1 and HSV-2 in adult sensory TG and sympathetic SCG neurons. In addition, assessing the temporal kinetics of HSV gene expression showed differential expression profiles of viral immediate-early (IE) genes ICP0, ICP4, ICP27 and trans-activator VP16 following treatment with stress hormones and NTF deprivation in HSV-1 and HSV-2 infected neurons.

We also show that different molecular mechanisms are involved in HSV latency and reactivation, which are dependent on the stimuli and the type of neurons. Tyrosine kinase receptor-mediated PI3K-Akt-mTORC signaling cascades have been studied for their role in maintaining HSV latency. Activation of β-catenin signalosome expression has also been implicated during HSV latency and following reactivation. GSK3β is a key effector molecule that inter-connects Akt and β-catenin mediated pathways, forming an Akt-GSK3β-β-catenin signaling axis. Analyzing the Akt-GSK3β-β-catenin signaling in response to stress hormone and NTF deprivation revealed significant differences in protein expression levels between HSV-1 and HSV-2 infected sensory and sympathetic neurons. In HSV-1 infected neurons, the Akt-GSK3β-β-catenin maintains the signal transmission in order to keep the neurons alive, but HSV-2 infections obliterated the entire axis in the adult neurons, particularly in sympathetic neurons. In summary, we demonstrate that HSV-1 and HSV-2 do not have a ‘one for all’ infection mechanism. Establishment of latency and reactivation by HSV is *virus specific, stimulus specific and neuron specific.*

Differential regulation of herpes simplex virus-1 and herpes simplex virus-2 during latency and post reactivation in response to stress hormones and nerve trauma in primary adult sensory and sympathetic neurons

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GENERAL AUDIENCE ABSTRACT

Herpes simplex viruses (HSVs) are common global viral pathogens that are responsible for causing lifelong painful infections and debilitating disease. The two serotypes of HSV include HSV-1, which is associated with oral or ocular disease but can also cause genital disease, and HSV-2, which is predominantly associated with genital herpes. Once infected, both HSV-1 and HSV-2 are present as lifelong reservoirs in our peripheral neurons.

Stress stimuli mediated by our stress hormones or external triggers, such as nerve trauma or an axonal injury, can periodically reactivate the latent virus to cause recurrent disease. Clinical manifestation of HSV recurrences range from asymptomatic viral shedding to painful blisters, cold sores, or herpetic keratitis. In some cases, the virus can spread to the central nervous system, causing encephalitis or recurrent meningitis. No vaccines have been approved yet, and the current treatment utilizes nucleoside analogs, such as acyclovir and its prodrug valacyclovir, to ameliorate the symptoms of HSV infection by halting viral replication and if taken as a daily prophylaxis, reduces the chances of clinical recurrence. Given the route and transmission efficiency of HSV, it is practically impossible to prevent herpes infection. To develop strategic therapeutic interventions to lock the virus in its latent phase in the neurons and prevent it from reactivation, a better understanding of neuronal signaling pathways cardinal to HSV latency and reactivation is necessary. However, neuronal signaling pathways cardinal to HSV latency and reactivation are still not clear.

In this dissertation, we make contributions to better understand HSV latency and reactivation in response to stress stimuli. We show that different stress stimuli exert preferential reactivation between HSV-1 and HSV-2, and are further dependent upon the neurons where they establish latency. Our study specifically focuses on three neuronal stressors that have been associated with HSV recurrences: two stress hormones, epinephrine (EPI) and corticosterone (CORT), as well as deprivation of neurotrophic factors (NTF) that simulates nerve injury. We also focused on a neuronal signaling cascade involved in the response to all of these stimuli, Akt-GSK3β-βcatenin, and viral gene transcripts that respond to these stimuli during reactivation. Comprehensive understanding of the neuronal processes and viral gene transcripts involved during HSV-1 and HSV-2 reactivation in neurons will help the herpes virology field towards development of targeted therapies and vaccines to prevent reactivation and recurrent disease.

For you, Ma.

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Chapter 1 Introduction

Herpes simplex viruses 1 and 2 (HSV-1 and HSV-2), belonging to the alpha-herpesviruses family, are causal organisms of ‘herpetic lesions’ across the globe. Tropism of the two HSV serotypes is different. HSV-1 is predominantly transmitted through oral contact and is associated with orofacial infections, while HSV-2 is sexually transmitted and results in genital herpes. However, these anatomical sites are not exclusive, as HSV-1 can cause genital herpes and HSV-2 can cause orofacial disease. Once infected herpes simplex virus infection is present as lifelong reservoirs in the ganglia of our peripheral nervous system, and can occasionally reactivate from them to cause recurrent disease. Clinical presentation of the disease varies from asymptomatic shedding to painful blisters or ulcers around oral and genital mucosal sites, depending upon the serotype. Severe cases of herpes infection can lead to life-threatening herpes simplex encephalitis (HSE) (1-4). According to the website of the World Health Organization, currently 67% of the population under the age of 50 have HSV-1 infection, while around 13% of people aged 15-49 are infected with HSV-2. Cross reactivity between the two serotypes for the same anatomical site is also common, with HSV-1 being associated with ~30% of primary genital infections, and around 10-20% of them experiencing HSV-1 genital recurrence. However, overall HSV-2 shows higher probability of recurrences throughout a person’s life, with 60-90% of HSV-2-infected people experiencing recurrences (5).

In comparison to other alpha-herpes viruses such as varicella zoster virus (VZV), currently there is no approved prophylactic or therapeutic vaccine available in the market for HSV. A major hindrance to the development of a vaccine is that HSV can persist lifelong in a latent state in non-replicating neurons of the peripheral ganglia, which are considered to be immune-privileged sites. Recently, some promising results have been demonstrated from therapeutic vaccine trials targeting clinical lesions and reducing viral shedding in genital herpes (4, 6). Use of guanosine analog anti-viral drugs, such as acyclovir and its pro-drug valacyclovir, is the only approved regimen for treatment of HSV infection. However, the drugs are directed more towards symptomatic treatment of HSV infection, and are unable to eliminate the virus (4). The pharmaco-kinetic action of acyclovir includes phosphorylation by the virus-encoded thymidine kinase to an active tri-phosphate form, which then gets incorporated into the growing HSV DNA chain by viral DNA polymerase and stops HSV DNA replication. Therefore, HSV must already be reactivating from latency in the neurons for the drug to function. Apart from acyclovir, helicase primase inhibitors, such as amenamevir and pritelivir, are also used as HSV anti-viral drugs that inhibit helicase-primase enzyme complex during viral replication (Figure 1.1).

The limitation of these anti-viral drugs is that they are focused on suppressive treatment with short-lived effectiveness, hence requiring life-long dependency, making HSV a debilitating disease (4). Because of the ubiquitous nature and high rate of prevalence in the population, it is not practically feasible to prevent HSV infection, but understanding the mechanism of latency and reactivation following a trigger will help us to plan for an '*ultimate*' therapeutic intervention that will essentially lock the virus in latency, and prevent future recurrences.

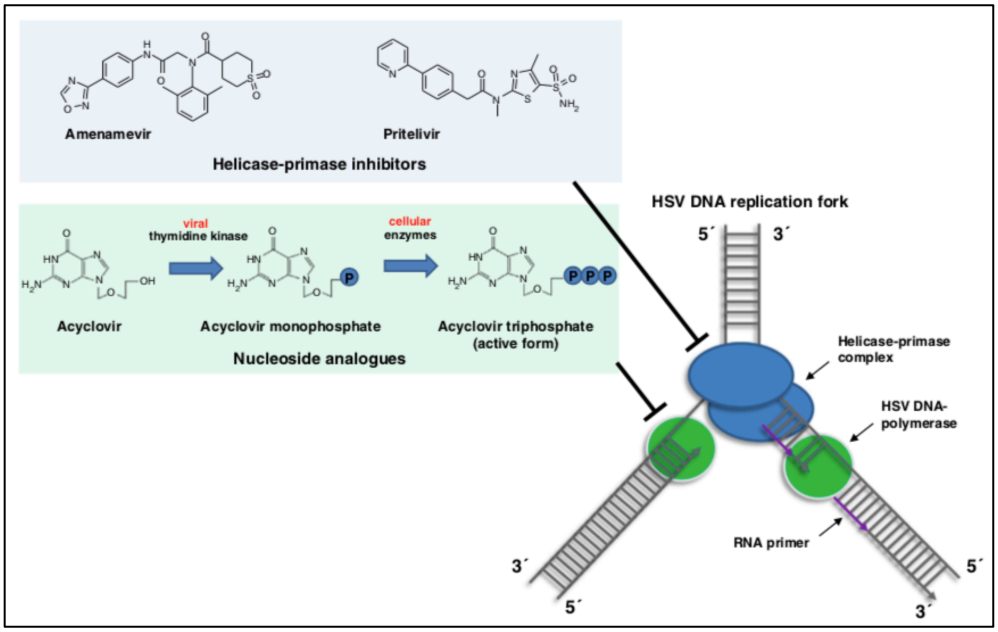

Figure 1.1. Pharmaco-kinetics mechanism of nucleoside analog and helicase-primase inhibitors.
Acyclovir (nucleoside analog) is phosphorylated by HSV-encoded thymidine kinase from an inactive mono-phosphate to the active tri-phosphate drug. It is then incorporated into the growing HSV DNA chain by viral polymerase and stops replication. Helicase-primase inhibitors targets HSV helicase-primase enzyme complex.
Source: *Birkmann and Zimmermann, Current Opinion in Virology, 2016.*

Mechanism of HSV latency and reactivation cycle: from *in-vivo* animal models to *in-vitro* adult primary neurons

HSV-1 and HSV-2 are large double stranded DNA viruses having two contrasting infection strategies. Primary productive or de-novo infection occurs in the epithelial cells at the site of infection. Following primary infection HSV ascends retrograde through the nerve axon and establishes latency by forming episomes in the neurons that innervate the site of infection (7). During latency, the general understanding is that active transcription of the viral genome is repressed and no infectious progeny is produced. The primary gene transcript expressed by HSV in the latent period is an ~8.3 kb long non-coding RNA called the latency-associated-transcript (LAT). Several microRNA (miRNA) are also produced that aid in inhibiting lytic gene expression and protecting the HSV genome from recognition by the immune system of the host cell. Expression of LAT also contributes to maintenance of latency and survival of neurons, as targeted mutations in LAT lead to HSV reactivation and neuronal cell death through caspase-8 and caspase-9 pathways (3, 8). External triggers, such as UV radiation, nerve trauma and injury, or internal signals, such as emotional or metabolic stress, can reactivate the latent HSV episome (Figure 1.2).

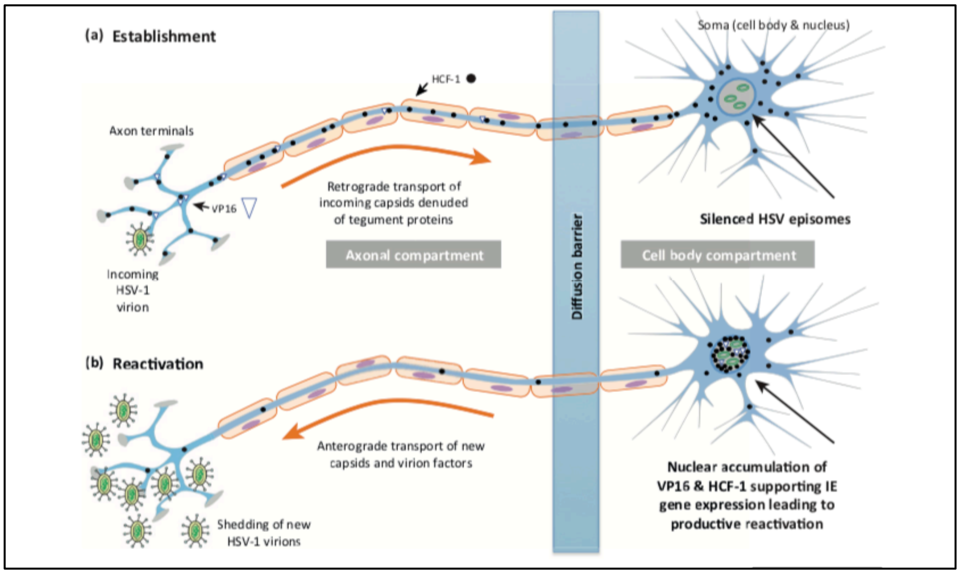

Figure 1.2. Establishment of latency and reactivation of HSV in neurons.
HSV transports retrograde through the axon to the nucleus and establishes latency as an episome. Following a reactivation stimulus, the virus begins expressing viral genes, replicating, and producing new viral progeny, which move anterograde down along the axon to cause recurrent episodes of disease.
Source: Wilson and Mohr, Trends in Microbiology, Cell Press, 2012.

HSV replication occurs through three classes of transcriptional genes— α or immediate-early (IE) genes that initiate lytic infection by activating other viral gene transcription and interfering with the cellular immune response, β or early (E) genes that help in DNA replication and packaging, and finally γ or late (L) genes that are mostly structural genes involved in virion production. These three classes of genes often act as a feed forward loop (1). However, this temporal cascade of HSV productive infection was identified in epithelial cells and it is not clear if the same process is followed in neurons, where the virus establishes latency, or if the same process is followed during reactivation. HSV tegument protein, VP16, also known as viral lytic initiator or trans activator, has been reported to express *de-novo* in latently infected trigeminal ganglia *in-vivo* and helps HSV to enter into a reactivation cycle from the latent phase (9). VP16 is also believed to be a facilitator of immediate-early (IE) gene expression along with host cellular factor 1 (HCF-1). Infected cell proteins 0, 4, 27, and 47 (ICP0, ICP4, ICP27, ICP47) are expressed as IE gene transcripts that alter the neuronal environment to help efficient expression and regulation of HSV genes, particularly the early genes. Further, ICP0 is especially involved in the ubiquitin-proteasomal pathway and ubiquitinates virus and host proteins for HSV's advantage (1, 7).

To study HSV pathogenesis and immune response, several animal models have been utilized, including mice, guinea pigs and rabbits. The mouse model of infection has been extensively used to study the HSV-1 immune response during acute infection. Conversely, guinea pig and rabbit models are significant in understanding the clinical presentation of HSV-1 and HSV-2 during latency and spontaneous reactivation (which does not happen in mice) that is comparable to human infection (10). However, *in-vivo* studies and clinical presentation of HSV infection are unable to comprehensively explain the mechanisms of latency and reactivation. Single cell neuronal cultures of primary sensory and autonomic neurons can provide critical knowledge about the mechanisms and various stress stimuli that can potentially reactivate HSV in neurons. Combined *in-vivo* animal studies and *in-vitro* neuronal cultures have provided new insights on HSV serotypes. Even though HSV-1 and HSV-2 share 83% homology, the two viruses show differential preferences during establishment of latency and reactivation following induction of a stimulus in different types of neurons (11-13).

Stress stimuli mediated HSV-1 and HSV-2 reactivation

Epinephrine (EPI) or adrenaline is a catecholaminergic hormone that mediates our body's flight or fight response through the adrenergic pathway and is crucial to maintaining homeostasis. Studies have shown that iontophoresis with EPI reactivates latent HSV-1 and increases viral shedding in rabbit and mouse models (14-16). HSV-2 latent infection shows reduced reactivation in the rabbit eye model following adrenergic reactivation (17). We show (this work is described in Chapter 2) that EPI treatment reactivates only HSV-1 from sympathetic superior cervical ganglia (SCG) neurons, but not HSV-2 (18).

Cortisol, a glucocorticoid, is a homeostatic anticipatory hormone released by the adrenal cortex through the hypothalamus-pituitary axis in response to stress. The kinetics of glucocorticoid are effectively mediated by glucocorticoid receptors (GRs) and mineralocorticoid receptors (MR), with GRs being extensively present in brain and peripheral tissues (19). We have demonstrated that GRs are selectively expressed in sympathetic and sensory neurons, and CORT plays a differential role during HSV productive infections in sympathetic neurons through GR and MR (20). *In-vivo* studies in calves and explants from mice trigeminal ganglia have shown that dexamethasone, a GR agonist, can reactivate bovine herpes virus (BoHV1) and HSV-1 from latency (21, 22).

Neurotrophic factors (NTFs) are endogenous soluble target-derived ligands, produced in skin and other axonal targets tissues and traveling retrograde along the axon, required by the neurons not only for their survival, but also for neuronal healing post axotomy or injury (23). Maintenance of HSV latency and subsequent reactivation from latency in neurons depend on the presence of trophic support. Studies show that nerve growth factor (NGF) maintains HSV latency in neonatal or embryonic sympathetic and sensory neurons (7, 11, 24-27). Our lab has shown that apart from NGF, glial cell-line derived neurotrophic factor (GDNF) family of ligands, GDNF and neurturin (NTN), are also crucial for maintaining both HSV-1 and HSV-2 latency in different mature neuronal types. NTN deprivation causes HSV-1 to reactivate in sensory neurons, whereas loss of GDNF reactivates HSV-2 from the same subset of neurons (28).

The neuronal signaling pathway downstream of these stress stimuli that govern HSV mediated latency and reactivation is still elusive. The canonical β-catenin signaling cascade has recently gained significant prominence for maintaining HSV latency and helping neurons to survive. Reactivation of HSV has coincided with a decrease in β-catenin expression in the dying neuronal population (29-32). Studies have shown that the PI3K-Akt pathway is another crucial signal transduction pathway that is affected during HSV reactivation post treatment with either CORT or NTF deprivation (26, 27, 33). GSK3β is an integrator of signals from different pathways. It is rendered inactive through phosphorylation by Akt, and becomes part of the β-catenin destruction complex. Inactive GSK3β subsequently allows β-catenin to translocate to the nucleus and bind to transcription factors that in turn switches on the transcription of neuronal survival genes (34). Thus, understanding the exact neuronal mechanisms through which stress hormones and NTF deprivation regulate HSV-1 and HSV-2 reactivation in neurons will provide us with more insight on HSV reactivation. Importantly, understanding these mechanisms will allow for the intelligent design of approaches to effectively prevent reactivation from latency.

Synopsis of Dissertation

Stress has a strong correlation with the exacerbation of acute HSV symptoms and subsequent recurrence from latency. Different stress stimuli, ranging from nerve injury to intrinsic stress hormones epinephrine and cortisol, exert preferential levels of reactivation between HSV-1 and HSV-2 in different neurons.

This dissertation aims to understand the complex cellular and viral consortium that comes into play between HSVs, different types of neurons and stress stimuli that perturbs the latent phase and subsequently reactivates the virus.

Chapter *2* of the thesis is focused on the stress hormones epinephrine (EPI) and cortisol (corticosterone in rodents, CORT) , and how these hormones selectively reactivate HSV-1 and HSV-2 from adult neurons. EPI reactivates HSV-1 through a combination of α and β adrenergic receptors, while CORT uses its cognate cytosolic glucocorticoid (GR) and mineralocorticoid (MR) receptors to reactivate HSV-1 in sympathetic neurons. Only CORT reactivates HSV-2 in both sensory and sympathetic neurons. Quantification of temporal kinetics of HSV immediate early (IE) gene expression of ICP0, ICP27, ICP4 and transactivator VP16 revealed different transcript profiles of these viral genes in response to EPI and CORT.

Chapter *3* compares the signaling mechanisms between two distinct stress stimuli, neurotrophic factor (NTF) deprivation and the stress hormone CORT, immediately post reactivation in HSV-1 and HSV-2 infected neurons. The Akt-GSK3β-βcatenin axis signaling pathway under NTF deprivation and CORT treatment shows significant alteration in protein expression between HSV-1 and HSV-2 infected sensory and sympathetic neurons. The signal transduction attempts to remain active following reactivation in HSV-1 infected neurons, but HSV-2 completely depletes the signaling axis. Expression of the latency-associated transcript (LAT) demonstrates that latency is more dynamic than previously thought and HSV-2 latent infection in neurons is leaky when compared to HSV-1.

Chapter *4* concludes with an overall general discussion and summary of the findings in this dissertation and future research avenues.

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Chapter 2 Stress Hormones Epinephrine and Corticosterone Selectively Reactivate HSV-1 and HSV-2 in Sympathetic and Sensory Neurons

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Angela Ives and Poorna Goswami contributed equally to this work. Andrea Bertke is the principal investigator in this project. Angela Ives and Poorna Goswami both contributed to Figure.1. Angela Ives contributed to the work presented in Figures 2, 3, 6 and 7. Poorna Goswami contributed to the work for Figures 4, 5, 6 and 7. Angela Ives, Poorna Goswami and Andrea Bertke contributed to the writing, review and editing of the manuscript.

Abstract

Herpes simplex viruses 1 and 2 (HSV-1 and HSV-2) establish latency in sensory and autonomic neurons, from which they can reactivate to cause recurrent disease throughout the life of the host. Stress is strongly associated with HSV recurrences in humans and animal models. However, the mechanisms through which stress hormones act on the latent virus to cause reactivation are unknown. We show that the stress hormones epinephrine (EPI) and corticosterone (CORT) induce HSV-1 reactivation selectively in sympathetic neurons, but not sensory or parasympathetic neurons. Activation of multiple adrenergic receptors is necessary for EPI-induced HSV-1 reactivation, while CORT requires the glucocorticoid receptor. In contrast, CORT, but not EPI, induces HSV-2 reactivation in both sensory and sympathetic neurons through either glucocorticoid or mineralocorticoid receptors. Reactivation is dependent on different transcription factors for EPI and CORT, and coincides with rapid changes in viral gene expression, although genes differ for HSV-1 and HSV-2, and temporal kinetics differ for EPI and CORT. Thus, stress-induced reactivation mechanisms are neuron-specific, stimulus-specific and virus-specific. These findings have implications for differences in HSV-1 and HSV-2 recurrent disease patterns and frequencies, as well as development of targeted, more effective antivirals that may act on different responses in different types of neurons.

Introduction

Herpes simplex viruses 1 and 2 (HSV-1 and HSV-2) infect mucosal cells, then establish lifelong latent infection in sensory and autonomic ganglia innervating the site of infection. The viruses can reactivate to cause recurrent disease; HSV-1 is more commonly associated with recurrent orolabial lesions and herpes keratitis, while HSV-2 causes recurrent genital lesions. Although HSV-1 is diagnosed in approximately 30% of new primary genital herpes infections, only 10–25% of individuals infected genitally with HSV-1 experience recurrences, while 60–90% of those infected with HSV-2 experience recurrent genital disease [1–3]. Symptomatic and asymptomatic genital herpes recurrences can also be associated with autonomic dysfunction, including urinary retention, constipation, erectile dysfunction, or impotence, although differences between HSV-1 and HSV-2 have not been assessed in human patients [4]. More rarely, HSV-1 causes necrotizing encephalitis with a high mortality rate and HSV-2 causes recurrent sacral meningitis, often unrecognized as an HSV-2-related disease [5,6]. The mechanisms that cause these differences in recurrent disease frequency and anatomical preferences are not understood.

Stress has been defined as a force that elicits a somatic response beyond the regular bodily functions. Any natural stimulus, ranging from physiological and pathological to social and psychological, can cause a stress response under intense pressure [7]. Social, psychological, physical, and persistent stress is strongly correlated with recurrent HSV disease in humans, and the stress hormone epinephrine induces reactivation in animal models [8–13]. Receptors for the two major stress hormones, epinephrine and cortisol, are expressed selectively in different types of neurons, including sensory neurons in the trigeminal ganglia (TG) and autonomic neurons in the superior cervical (SCG) and ciliary ganglia (CG) [14–16].

Epinephrine (EPI), also known as adrenaline, is a catecholaminergic hormone secreted by the adrenal medulla and regulated by the sympathetic nervous system to induce a "short-term" stress response referred to as the "fight or flight response". EPI induces HSV-1 reactivation in mouse and rabbit in vivo models of infection [11,12], although adrenergic reactivation is significantly reduced if the latency-associated transcript (LAT) is deleted or mutated [17]. Iontophoresis of EPI during HSV-2 latent infection induces reactivation significantly less frequently compared to HSV-1 in the rabbit eye model [18,19], suggesting that HSV-1 and HSV-2 may be differentially responsive to EPI-induced reactivation. Furthermore, we recently showed that EPI enhances DNA replication and the production of viral progeny during productive infection of HSV-1 in sympathetic but not sensory neurons, demonstrating that EPI acts on HSV preferentially in specific types of neurons [16]. Thus, EPI may selectively induce HSV reactivation in both a neuron- and virus-specific manner.

Cortisol, a glucocorticoid secreted by the adrenal cortex, induces a “long-term” stress response. Cortisol binds to glucocorticoid (GR) and mineralocorticoid receptors (MR), and its major effects on humans and mammals include regulation of metabolism and suppression of the immune system. Cortisol (corticosterone in rodents) induces HSV-1 and HSV-2 reactivation in vivo, which is presumed to occur through immune system suppression [20,21]. However, stress-induced transcription factors expressed in sensory TG neurons stimulate the HSV-1 ICP4, ICP27, and ICP0 promoters [22–26], and ICP0 alone can initiate reactivation of HSV-1 from latency [27–29]. In addition, we previously demonstrated that corticosterone (CORT) modulates acute HSV-1 and HSV-2 replication during productive infection in sympathetic but not sensory neurons [16]. Thus, CORT may potentially induce HSV reactivation by acting directly on neurons that harbor latent virus, rather than acting through immune system suppression.

To determine if stress hormones differentially reactivate HSV-1 and HSV-2 at a cellular level in different types of mature neurons, we administered either EPI or CORT to latently infected primary murine adult sensory and autonomic neurons in culture. Quantification of viral replication, production of viral progeny, and gene expression show that stress hormones differentially induce reactivation depending on the type of neuron and the infecting virus. Furthermore, administration of stress hormone receptor agonists and antagonists demonstrate that EPI and CORT exert their effects through specific adrenergic and corticosteroid receptors to selectively induce HSV-1 and HSV-2 reactivation from latency in primary adult sensory and autonomic neurons.

Materials and Methods

Cell Lines and Virus Strains

HSV-1 strain 17+ was originally isolated and transferred from John Hay (SUNY, Buffalo, NY, USA) to the Krause lab (FDA, Bethesda, MD, USA). HSV-2 strain 333 was originally isolated and transferred from Gary Hayward (Johns Hopkins, Baltimore, MD, USA) to the Krause lab (FDA, Bethesda, MD, USA). Virus was propagated in Vero 76 cells (CRL-1586, ATCC) in the Krause lab and Passage 1 aliquots were stored at −80 °C; most new virus stocks propagated in the Krause lab were propagated from the first passage aliquots, resulting in Passage 2. A Passage 2 aliquot was transferred to the Margolis lab (UCSF, San Francisco, CA, USA); new virus stocks were propagated and stored at −80 °C (Passage 3). A Passage 3 aliquot was transferred to the Bertke lab (Virginia Tech, Blacksburg, VA, USA); new virus stocks were propagated and stored at −80 °C (Passage 4). All virus stocks used in the Bertke lab were propagated from the Passage 4 stocks in Vero 76 cells (Passage 5). Stocks were titrated on Vero 76 cells in quadruplicate to determine concentration. Stock viruses were diluted in Neurobasal A medium (Thermo Fisher, Waltham, MA, USA) for inoculation of primary adult murine neuronal cultures.

Primary Adult Murine Neuronal Cultures

Female six-week-old Swiss Webster mice (Hilltop Laboratories, Scottsdale, PA, USA) were euthanized with CO_2 and transcardially perfused with cold, calcium- and magnesium-free phosphate-buffered saline (PBS). Sensory trigeminal ganglia (TG), sympathetic superior cervical ganglia (SCG), and parasympathetic ciliary ganglia (CG) were collected into Neurobasal A medium (Gibco) supplemented with penicillin–streptomycin and B27 Supplement (ThermoFisher) on ice. Ganglia were enzymatically dissociated sequentially in papain and collagenase/dispase (Worthington Biochemical, Lakewood, NJ, USA), followed by mechanical trituration by pipette. TG neurons were enriched on Optiprep gradients. SCG and CG did not go through a gradient step because they contain less ganglionic debris after dissociation. Neurons were plated on culture plates coated with Matrigel Matrix Basement Membrane (Corning) and maintained in Complete Neuro Media, consisting of Neurobasal A medium supplemented with B27 Supplement (Thermo Fisher), penicillin–streptomycin (Thermo Fisher), Glutamax (Thermo Fisher), and neurotrophic factors (NTFs from PeproTech, Cranbury, NJ, USA; nerve growth factor (NGF), glial cell-derived neurotrophic factor (GDNF), neurturin (NTN), ciliary neurotrophic factor (CNTF; for CGs only)), and 5-fluorodeoxyuridine (Sigma) to deplete nonneuronal cells [30]. Primary adult neuronal cultures have previously been assessed for maintenance of in vivo characteristics, neuronal markers, and specific receptors relevant to these studies [16,30–32]. All animal care and handling were in accordance with the Virginia Tech Institutional Care and Use Committee (IACUC# 15-237 approved 5 February 2016, 18-237 approved 2/8/2019, and 21–244 approved 11 January 2022).

Establishment of Latent Infection

Four days after plating, neurons were inoculated with 30 multiplicity of infection (moi) of HSV-1 (strain 17+) or HSV-2 (strain 333). We previously showed that at 30 moi, 90% of the total neurons become infected and establish latency [30]. After a one-hour adsorption period at 37 °C/5% CO_2, viral inoculum was removed and replaced with fresh Complete Neuro media with no 5-fluorodeoxyuridine and with 300 μM acyclovir (Sigma, St. Louis, MO, USA) for seven days to establish and maintain a latent infection in vitro.

Reactivation with Stress Hormones

Seven days after establishment of a latent infection, Complete Neuro media with 300 μM acyclovir was removed and replaced with fresh Complete Neuro media with no acyclovir and with either 10 μM epinephrine (Sigma) for adrenergic reactivation or 10 μM corticosterone-HBC (Sigma) for corticosterone-induced reactivation. Four different concentrations of each hormone (0.01, 0.1, 1, 10 μM) were tested for effects during productive infection [16]. To ensure maximum effects, 10 μM epinephrine or corticosterone were used for all reactivation and inhibitor studies. Concentrations for inhibitors were based on previously published characterizations and studies for each agent. Corticosterone-HBC is a water-soluble corticosterone conjugated to (2-Hydroxypropyl)-β-cyclodextrin (HBC), a carrier molecular that enables solubility in media without harming the cultured neurons. HBC was used as a control to ensure that corticosterone and not the vehicle was inducing reactivation. Untreated controls had acyclovir removed but were otherwise untreated to ensure that removal of acyclovir did not promote replication. Approximately 30% of the total neurons reactivate in response to a reactivation stimulus [30,32].

Reactivation with Receptor Agonists & Antagonists

For testing adrenergic receptor specificity, seven days after establishment of a latent infection, media containing acyclovir was removed and Complete Neuro media with no acyclovir and with one, two, or three adrenergic agonists were added. Adrenergic receptor (AR) agonists included phenylephrine (α-1 AR agonist), clonidine (α-2 AR agonist), dobutamine (β-1 AR agonist), or terbutaline (β-2 AR agonist) (Sigma). Adrenergic receptor antagonists phentolamine (nonspecific α-AR antagonist) or timolol (nonspecific β-AR antagonist) were also added with 10 µM epinephrine to fresh Complete Neuro media. For testing corticosterone receptor specificity, Complete Neuro media with no acyclovir and with either the glucocorticoid receptor agonist dexamethasone (GR agonist) or mineralocorticoid receptor agonist aldosterone (MR agonist) (Sigma) were added to Complete Neuro media (10 µM). The corticosterone antagonists mifepristone (GR antagonist), eplerenone (MR antagonist) (Sigma), or both at 10 µM were added to fresh Complete Neuro media with 10 µM corticosterone-HBC.

Reactivation with Protein Inhibitors

For testing molecular inhibition of intermediates in signaling cascades, seven days after establishment of latent infection, media containing acyclovir was removed. Complete Neuro media with no acyclovir and 10 µM β-catenin inhibitor iCRT14 (Sigma), 10 µM CREB inhibitor 666-15 (EMD Millipore), 20 µM JNK inhibitor SP600125 (Sigma), 20 µM PI3-K inhibitor LY294002 (Sigma), 30 µM p300/CBP inhibitor C646 (Sigma), or 1 µM HDAC1 inhibitor pyroxamide (Sigma) with 10 µM epinephrine or corticosterone-HBC were added to primary adult neuronal cultures latently infected with HSV-1 or HSV-2.

Quantitation of HSV Viral Load

Viral DNA was extracted from neuronal cultures 24 h post-reactivation (hpr) with TRI reagent (Thermo Fisher), according to the manufacturer's instructions. Viral DNA load was determined by qPCR using HSV-1 or HSV-2 thymidine kinase (TK) gene-specific primers and probes. All assays were normalized to 18s rRNA (Applied Biosystems, Waltham, MA, USA) and reported as viral copy number in 200 ng of total DNA, which represents approximately 120–240 neurons.

Plaque Assay

Twenty-four hours post-reactivation, neurons and media were collected and viral titer was determined by standard plaque assay on Vero E6 cells.

Quantitation of HSV Gene Expression

Viral RNA was extracted from neuronal cultures during latency (after 7 days latency, acyclovir was not removed) and between 1 h and 25 h post-reactivation with TRI reagent (Thermo Fisher), according to the manufacturer's instructions. RNA was reverse-transcribed to cDNA with iScript (Biorad, Hercules, CA, USA). Gene expression was determined by quantifying the HSV-1 or HSV-2 immediate early genes ICP0, ICP4, ICP27, and transactivator VP16 by digital droplet PCR (ddPCR) using HSV-1 or HSV-2 gene-specific primers specific for each gene and EvaGreen QX200 Master mix (Biorad) on a QX200 ddPCR system (BioRad). All assays were normalized to 18s rRNA and reported as transcripts/neuron (calculated from number of neurons per well) and fold-change of epinephrine or corticosterone treated samples compared to untreated neurons (latent for 7 days, acyclovir not removed).

Quantification and Statistical Analysis

All experiments were performed using a minimum of three neuronal cultures performed on different days to ensure reproducibility, with 2–3 biological replicates for each treatment in each culture. All assays were performed in duplicate or triplicate for technical replicates of each sample. Data are presented as means ± standard error of the mean. Statistics were performed using parametric analyses with MS Excel and JMP Pro version 12, including analysis of variance with contrast tests. Where indicated, asterisks denote statistical significance as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Results

Stress Hormones Differentially Induce HSV Reactivation In Vitro

To determine if stress hormones induce HSV-1 and HSV-2 reactivation from latent infection in different types of mature neurons, primary adult murine sensory trigeminal (TG), sympathetic superior cervical (SCG), and parasympathetic ciliary ganglia (CG) neuronal cultures latently infected with HSV-1 (17 syn+) or HSV-2 (333) were treated with EPI or CORT. We previously determined active concentrations of EPI and CORT that produce biologically and statistically significant effects on productive HSV-1 and HSV-2 infection, without toxic effects in these neurons [16]. Viral DNA was quantified by qPCR with primers and probes specific for the HSV-1 or HSV-2 thymidine kinase (TK) gene.

Neither EPI nor CORT were able to induce HSV-1 reactivation in sensory TG or para-sympathetic CG neurons. However, both EPI and CORT induced HSV-1 reactivation in sympathetic SCG neurons, as shown by significantly increased viral DNA compared to untreated latently infected neurons (UNT; ACV removed but no other treatment) (Figure

2.1A). Trichostatin A (TSA), a nonspecific histone deacetylase (HDAC) inhibitor that has previously been shown to reactivate HSV-1 reliably from latency [30,33,34], was used as a positive reactivation control. Although EPI and CORT both induced HSV-1 reactivation in sympathetic neurons, viral DNA did not reach the same quantity as TSA-induced reactivation, suggesting that stress hormone-induced reactivation may not be as strong of a stimulus as some other reactivation stimuli. In contrast, CORT induced reactivation of HSV-2 in both sensory TG and sympathetic SCG neurons (Figure 2.1B), but EPI had no effect on HSV-2, regardless of neuron type. Although we also observed a trend of increased HSV-2 DNA in parasympathetic CG neurons following CORT treatment, neither EPI nor CORT had a statistically significant effect on HSV-1 or HSV-2 in parasympathetic CG neurons. TSA produced a significant increase in HSV-1 and HSV-2 DNA in sensory TG and sympathetic SCG, but not in parasympathetic CG neurons (Figure 2.1A,B), suggesting that parasympathetic CG neurons may regulate HSV differently than sensory or sympathetic neurons.

Taken together, our results show that EPI and CORT have differential effects on HSV-1 and HSV-2 reactivation in a neuron-specific manner. EPI induces HSV-1 reactivation only in sympathetic neurons, but has no effect on HSV-2. CORT induces HSV-1 only in sympathetic neurons, but induces HSV-2 reactivation in both sensory and sympathetic neurons.

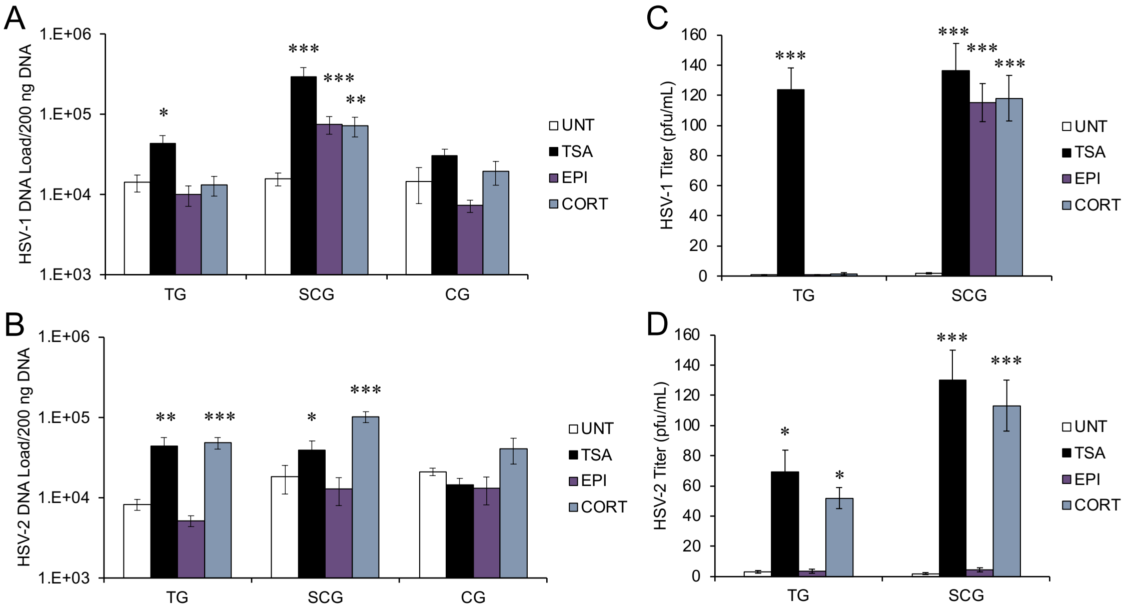

Figure 2.1. EPI and CORT induce HSV-1 and HSV-2 reactivation selectively in sensory, sympathetic, and parasympathetic neurons.
Primary adult neuronal cultures from sensory trigeminal (TG), sympathetic superior cervical (SCG), and parasympathetic ciliary ganglia (CG) were infected with HSV-1 or HSV-2 in the presence of acyclovir for 7 days, followed by treatment with epinephrine (EPI; 10 μM), corticosterone (CORT; 10 μM), or trichostatin A (TSA; 1.2 μM), compared to untreated (UNT) neurons. HSV-1 (**A**) and HSV-2 (**B**) viral DNA was quantified by qPCR 24 h post-reactivation. In parallel, HSV-1 (**C**) and HSV-2 (**D**) infectious viral titers were quantified by plaque assay on Vero cells 24 h posttreatment. Data are shown as means ± SEM, n > 6; results were compared to untreated cultures by ANOVA and post hoc Tukey's HSD; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Adrenergic Receptor Specificity of EPI-Induced Reactivation of HSV-1

EPI can bind to and activate several different adrenergic receptors that are expressed by peripheral neurons, including alpha 1 and 2 (α1-AR, α2-AR) and beta 1 and 2 (β1-AR, β2-AR). Therefore, we next sought to determine which adrenergic receptors are important for the EPI-induced reactivation of HSV-1. We previously demonstrated that adrenergic receptor expression is maintained in cultured primary adult murine neurons similarly to expression in vivo [16]. Furthermore, we showed that HSV-1 antigens generated during productive infection are detected in neurons expressing adrenergic receptors [16].

Since EPI only induced HSV-1 reactivation in sympathetic SCG neurons, we treated latently infected SCG neurons with EPI and nonspecific adrenergic receptor antagonists (blockers) to block activation of either α- or β-ARs. The nonspecific β-AR antagonist (blocker) timolol, which blocks both β1- and β2-AR activation, inhibited EPI-induced HSV-1 reactivation (Figure 2.2A), suggesting that β-AR activation is necessary to induce HSV-1 reactivation following EPI treatment. In contrast, latently infected neurons treated with the nonspecific α-AR antagonist phentolamine produced quantities of HSV-1 DNA that were still significantly higher than untreated sympathetic neurons (Figure 2.2A), showing that general blockade of α-ARs cannot effectively block EPI-induced HSV-1 reactivation in sympathetic SCG neurons.

To further assess receptor involvement, we reasoned that if EPI induces reactivation through a specific adrenergic receptor, an agonist that activates that specific receptor would also induce reactivation without EPI treatment. Therefore, SCG neurons latently infected with HSV-1 were treated with agonists for one of four different adrenergic receptors: α1, α2, β1, or β2. In comparison to untreated (UNT) neurons and those treated with EPI, which induced reactivation in those neurons, none of the four adrenergic receptor agonists alone significantly induced reactivation of HSV-1 in sympathetic SCG neurons (Figure 2.2B).

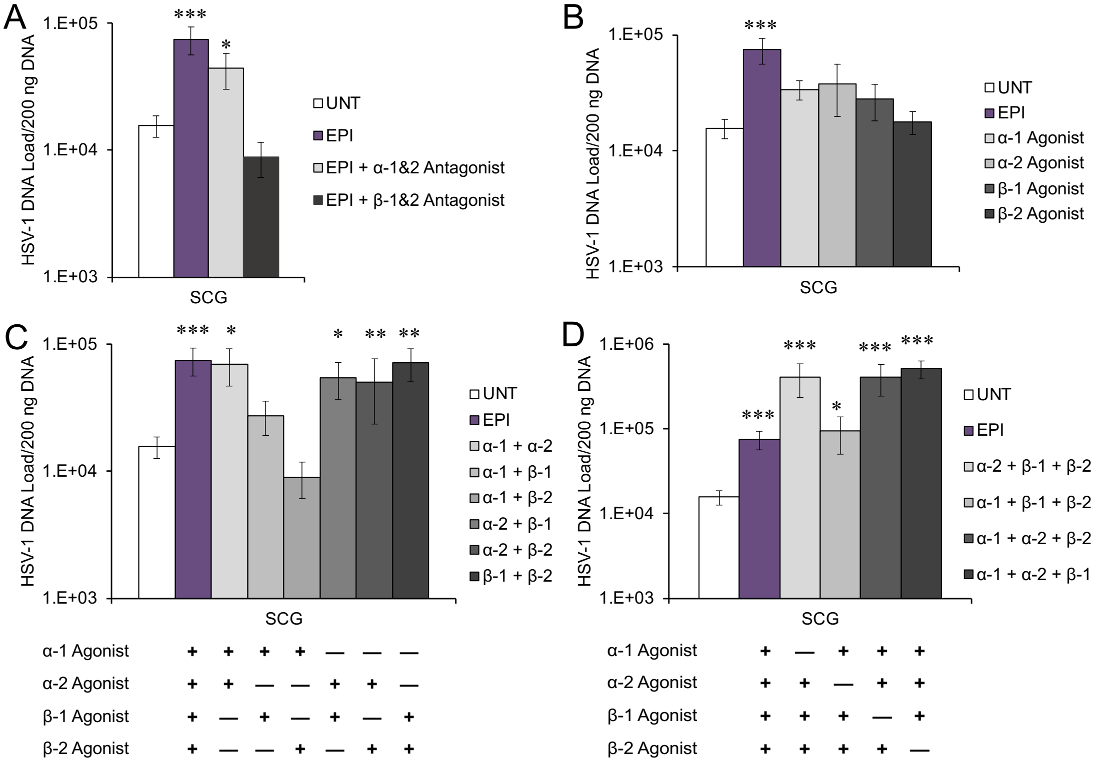

Figure 2.2. Multiple adrenergic receptors are required for EPI-induced HSV-1 reactivation in sympathetic neurons.

(**A**) Reactivation of HSV-1 with EPI in primary adult murine sympathetic SCG cultures either alone, with nonspecific α-AR antagonist (phentolamine), or nonspecific β-AR antagonist (timolol). (**B**) Reactivation of HSV-1 in primary adult murine SCG cultures with either EPI, α-1 agonist (phenylephrine), α-2 agonist (clonidine), β-1 agonist (dobutamine), or β-2 agonist (terbutaline). (**C**) Reactivation of HSV-1 in primary adult murine SCG cultures with either EPI or a combination of two adrenergic agonists utilized in Figure B. (**D**) Reactivation of HSV-1 in primary adult murine SCG cultures with either EPI or a combination of three adrenergic agonists utilized in Figure B. Reactivation was quantified in A–D by viral DNA collected 24 *h* posttreatment measured by qPCR. (**E**) Data summary: activation of α2 and any other AR induces HSV-1 reactivation in adult sympathetic SCG neurons. Alternatively, activation of β1 and β2 simultaneously can also induce reactivation. Data are means ± SEM, n > 6; results were compared to untreated cultures by ANOVA and post hoc Tukey's HSD; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Since a nonspecific beta blocker was able to prevent EPI-induced HSV-1 reactivation but individual receptor agonists were unable to induce reactivation, we reasoned that activation of a single adrenergic receptor is insufficient for HSV-1 reactivation from latency in sympathetic SCG neurons. However, our results suggested that at least one of the β-adrenergic receptors is necessary for EPI-induced reactivation. To determine whether EPI induces HSV-1 reactivation by activating multiple adrenergic receptors, latently infected SCG neurons were treated with agonists for two of the four different adrenergic receptors simultaneously (Figure 2.2C). Combined treatments containing the α2-AR agonist clonidine induced reactivation similarly to EPI (Figure 2.2C). However, a combination of β1- and β2-AR agonists also induced reactivation without clonidine. Therefore, combined activation of the α2-AR and any other adrenergic receptor can induce HSV-1 reactivation in sympathetic SCG neurons to the same extent as EPI, but α2-AR activation alone is not sufficient. Alternatively, combined activation of β1- and β2-AR can also induce HSV-1 reactivation to the same extent as EPI. Although treatment with the combination of α1 and α2 agonists induced reactivation (Figure 2.2C), the nonspecific α1/α2 antagonist was unable to block EPI-induced reactivation (Figure 2.2A). Thus, reactivation can proceed through activation of β1- and β2-ARs if the α-ARs are blocked.

To verify these results, latently infected neurons were treated with agonists for three of the four different adrenergic receptors, omitting one. We reasoned that if the omitted one was required for reactivation, reactivation would not occur. All treatments induced HSV-1 reactivation, as demonstrated by significantly increased viral DNA following treatment compared to untreated controls (Figure 2.2D), but combined treatments that included clonidine (α2-AR agonist) induced HSV-1 reactivation above EPI-induced levels. Thus, activation of α2-AR is necessary for the full reactivation potential of HSV-1 in sympathetic neurons, although activation of β1- and β2-AR simultaneously can compensate.

Our results also suggest that multiple signaling pathways are involved in the process of reactivation, since the adrenergic receptors are coupled to trimeric G-protein complexes that contain different alpha subunits, which activate or inhibit different signaling pathways. The α2-AR couples with the inhibitory G-protein alpha subunit ($G\alpha_{i/o}$), which inhibits adenylate cyclase and downstream cyclic AMP (cAMP) production, while also activating the Src pathway leading to cAMP response element binding protein (CREB) activation [35]. The α2-AR also couples with the G-protein alpha subunit q ($G\alpha_q$), activating phospholipase C (PLC) and downstream mitogen-activated protein kinase (MAPK) cascade, resulting in activation of CREB and c-Jun terminal kinase (JNK) [36,37]. While β1-AR couples with $G\alpha_s$, which stimulates adenylate cyclase and downstream cAMP production, the β2-AR can couple with either the $G\alpha_s$ or the $G\alpha_{i/o}$ proteins [38]. Additionally, different Gα proteins modulate β-catenin signaling, with $G\alpha_s$ potentiating and $G\alpha_q$ suppressing the β-catenin-signaling pathway [39]. Thus, EPI activation of several different signaling pathways simultaneously is necessary for the full reactivation of HSV-1.

Corticosteroid Receptor Specificity of CORT-Induced Reactivation of HSV-1 and HSV-2

CORT can bind to and activate glucocorticoid (GR) and mineralocorticoid (MR) receptors [40]. We previously showed that receptor expression is maintained in cultured primary adult murine sensory TG and sympathetic SCG neurons, and that HSV antigens generated during productive infection are present in the same neurons as receptor expression [16]. As shown in Figure 2.1, CORT induced reactivation of HSV-1, but only in sympathetic SCG neurons, and reactivation of HSV-2 in both sensory TG and sympathetic SCG neurons.

To determine whether CORT-induced reactivation occurs through binding of CORT to GR or MR, latently infected neurons (SCG for HSV-1, SCG and TG for HSV-2) were treated with the GR agonist dexamethasone (DEX) or the MR agonist aldosterone (ALDO).

The GR agonist (DEX), but not the MR agonist (ALDO), induced HSV-1 reactivation similarly to CORT in sympathetic SCG neurons (Figure 2.3A), demonstrating that HSV-1 reactivates following activation of the GR, but not the MR. HBC (2-hydroxypropyl-β-cyclodextrin), the vehicle for CORT, and DMSO (dimethyl sulfoxide), the vehicle for the CORT agonists and antagonists, had no significant effects on the latent virus (Figure 2.3A–C). To determine if we could block CORT-induced reactivation of HSV-1 in sympathetic neurons, SCG neurons latently infected with HSV-1 were treated with CORT and the GR antagonist mifepristone, which effectively inhibited CORT-induced reactivation of HSV-1 (Figure 2.3D). Thus, CORT-induced HSV-1 reactivation occurs through the GR.

In both sympathetic SCG (Figure 2.3B) and sensory TG neurons (Figure 2.3C), both GR and MR agonists (DEX and ALDO) individually induced HSV-2 reactivation similar to CORT treatment. Thus, HSV-2 reactivates following activation of either GR or MR in sympathetic and sensory neurons. Neither the GR antagonist mifepristone nor the MR antagonist eplerenone alone was able to block CORT-induced HSV-2 reactivation in SCG neurons (Figure 2.3E) or TG neurons (Figure 2.3F). However, administration of mifepristone and eplerenone together, along with CORT, effectively blocked CORT-induced reactivation in both sympathetic SCG and sensory TG neurons (Figure 2.3E, F). Therefore, CORT is able to induce HSV-2 reactivation in sympathetic SCG and sensory TG neurons through activation of either the GR or MR.

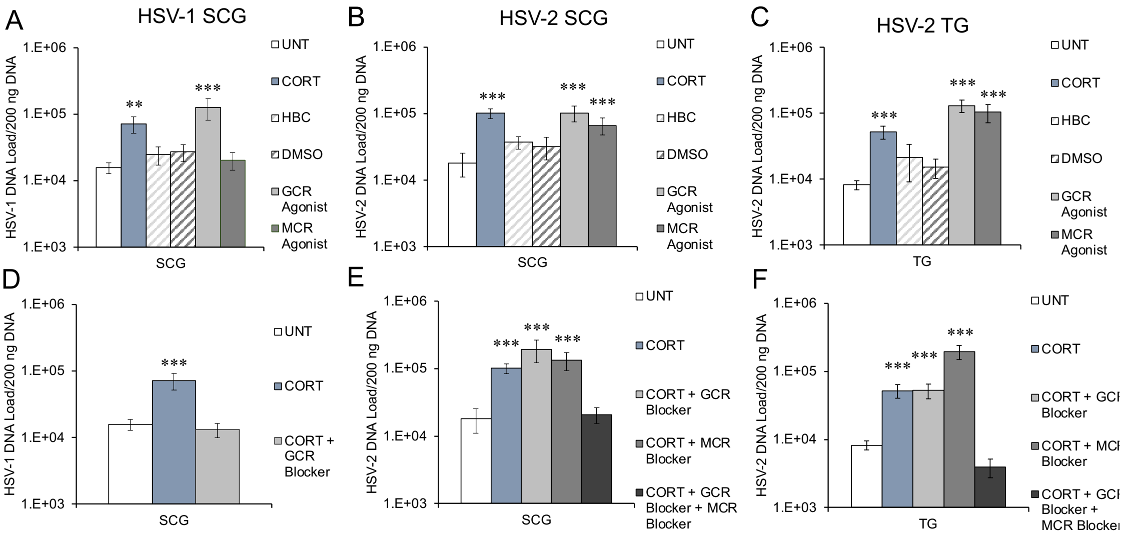

Figure 2.3. GR is required for CORT-induced HSV-1, but GR and MR are redundant for HSV-2 reactivation.

Primary adult neuronal cultures from sensory trigeminal (TG) or sympathetic superior cervical (SCG) ganglia were infected with HSV-1 (**A**,**D**) or HSV-2 (**B**,**C**,**E**,**F**) in the presence of acyclovir for 7 days, followed by treatment with corticosterone (CORT), receptor agonists, or receptor antagonists. (**A**) HSV-1 or (**B**) HSV-2 latently infected sympathetic SCG neuronal cultures were treated with CORT, CORT vehicle HBC, agonist vehicle DMSO, GR agonist (dexamethasone), or MR agonist (aldosterone). (**C**) HSV-2 latently infected sensory TG neuronal cultures were treated with CORT, CORT vehicle HBC, agonist vehicle DMSO, GR agonist (dexamethasone), or MR agonist (aldosterone). (**D**) HSV-1 latently infected SCG neuronal cultures were treated with CORT, either alone or with GR antagonist (mifepristone). HSV-2 latently infected SCG (**E**) or TG (**F**) neuronal cultures were treated with CORT, either alone, with GR antagonist (mifepristone), with MR antagonist (eplerenone), or with both the GR and MR antagonists. Viral DNA was quantified in A–F by DNA collected 24 h posttreatment measured by qPCR. Data are means ± SEM, n > 6; results were compared to untreated cultures by ANOVA and post hoc Tukey's HSD; ** $p < 0.01$; *** $p < 0.001$.

Our results show that CORT induces HSV-1 reactivation only in sympathetic neurons through activation of the GR, while CORT induces HSV-2 reactivation in sensory and sympathetic neurons through either the GR or MR. The GR and MR are cytosolic ligand-activated receptors that can positively or negatively modulate gene expression by binding directly to hormone response elements and recruiting transcription regulatory complexes, or by interacting with other transcription factors. The specificity of GR and MR occurs through binding affinities of the receptors for CORT and ALDO, hormone response element sequences, and posttranslational modifications. Thus, CORT-induced reactivation is selective, and the mechanisms likely differ for HSV-1 and HSV-2.

Kinetics of HSV Gene Expression Differ after EPI and CORT Treatment, but also Depend on Neuron Type

As HSV-1 reactivates in sympathetic SCG neurons using different neuronal receptors in response to EPI and CORT treatment, theoretically, viral gene expression may also differ between EPI and CORT treatments. Kim et al., 2012, categorized HSV reactivation into a biphasic pattern that was time-dependent, in which Phase I transitioned into Phase II at approximately 25 h post-reactivation, using phosphatidyl inositol 3-kinase (PI3K) inhibitor LY294002 as a reactivation stimulus. As our focus was on early events post-stimulus, we quantified the HSV-1 immediate early (IE) gene expression of ICP0, ICP4, and ICP27 during latency (0 h timepoint, prior to hormone treatment) and between 1 and 25 h after EPI and CORT treatment, using droplet digital PCR (ddPCR) to evaluate the expression profiles of these IE genes in primary adult neurons. In addition, we also quantified viral protein 16 (VP16) transcript expression over the same time-course, as previous studies have reported the involvement of trans-activator VP16 in facilitating the transition from latency to a productive infection following a stress-induced stimulus [41,42].

During latency, we detected low levels of expression of ICP0, ICP27, and VP16 in both HSV-1- and HSV-2-infected sensory and sympathetic neurons, shown as number of transcripts per neuron (Figure 2.4). Although expression level varied depending upon HSV type and the neurons in which the viruses established latency, the general pattern of expression was similar (Figure 2.4). ICP0 was the most highly expressed (4.5–16.5 transcripts/neuron), followed by VP16 (3.4–9.1 transcripts/neuron). ICP27 was more highly expressed by HSV-2 (2.7–5.6 transcripts/neuron; Figure 2.4 C,D) than HSV-1 (0.5–1.3 copies/neuron; Figure 2.4 A,B). In contrast, ICP4 showed negligible expression in both TG and SCG neurons during latency (Figure 2.4A–D). For comparison and to demonstrate latency, we also detected up to 70.65 copies of the latency-associated transcript (LAT) per neuron.

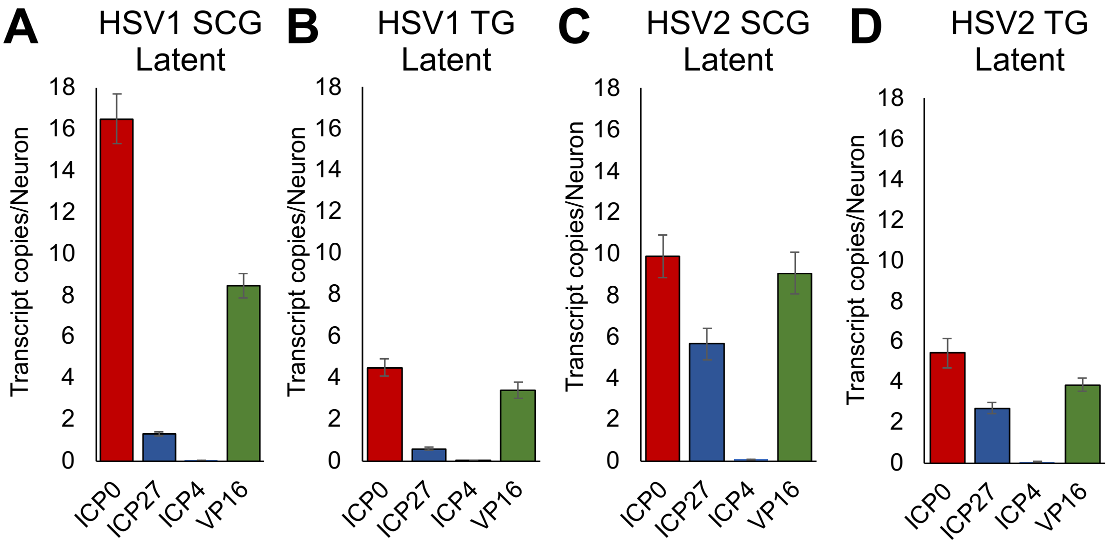

Figure 2.4. HSV-1 and HSV-2 ICP0, ICP27, ICP4, and VP16 gene expression in SCG and TG neurons during latency.
Gene expression was quantified by droplet digital PCR (ddPCR) 7 days after infection in the presence of acyclovir (300μM). Data is shown as gene transcripts per neuron. (**A**) HSV-1 in SCG neuronal cultures. (**B**) HSV-1 in TG neuronal cultures. (**C**) HSV-2 in SCG neuronal cultures. (**D**) HSV-2 in TG neuronal cultures. Data are means ± SEM, n > 6.

Following treatment with EPI and CORT, we show gene expression as both transcript copies per neuron, as well as fold-change over latent data (Figure 2.5), as neither alone provides a complete picture of expression kinetics. HSV-1 reactivates in sympathetic SCG neurons in response to either CORT or EPI treatment (see Figure 2.1). In SCG neurons latently infected with HSV-1, CORT treatment reduced expression of ICP0 and VP16, but ICP27 expression remained stable over 25 h (Figure 2.5A). Although the change in ICP4 expression cannot be discerned in Figure 2.5A (transcript copies per neuron), Figure 2.5B (fold-change in expression) better illustrates the significant increase in ICP4 expression following CORT treatment compared with expression levels during latency. Following EPI treatment, gene expression was also reduced for ICP0 and VP16, although expression increased between 10 h and 20 h posttreatment (Figure 2.5C). Similarly, ICP4 expression increased almost immediately following treatment, fell to below-latency levels, and then increased again between 10 and 20 h (Figure 2.5D). ICP27 increased transiently at 20 h posttreatment, but otherwise, expression remained relatively stable throughout the 25 h time period.

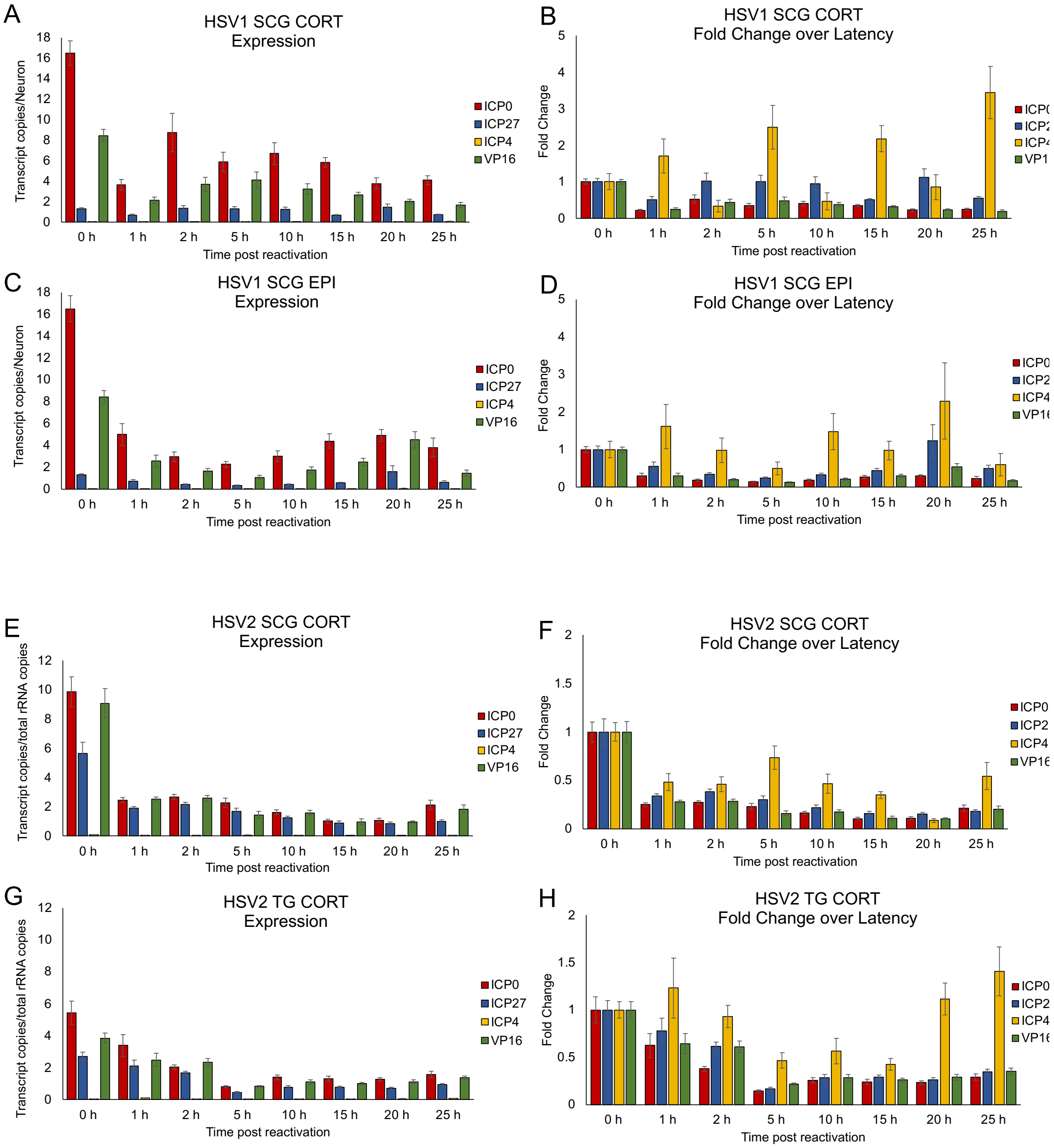

Figure 2.5. HSV gene expression in primary SCG and TG neuronal cultures during EPI- and CORT-induced reactivation.

Primary adult neuronal cultures from sympathetic SCG and sensory TG infected with HSV-1 or HSV-2 for 7 days in the presence of acyclovir to establish latency were treated with epinephrine (EPI) or corticosterone (CORT) to induce reactivation. Total RNA was extracted at the designated timepoints post-reactivation, and viral gene transcripts for ICP0, ICP27, ICP4, and VP16 were quantified by ddPCR. (**A**) HSV-1 in SCG treated with CORT, presented as gene transcript copies per neuron. (**B**) HSV-1 in SCG treated with CORT, presented as fold-change compared to each gene expressed during latency with no treatment and no removal of acyclovir (0 h). (**C**) HSV-1 in SCG treated with EPI, as gene transcripts per neuron. (**D**) HSV-1 in SCG treated with EPI, as fold-change compared to each gene expressed during latency (0 h). (**E**) HSV-2 in SCG treated with CORT, as gene transcript copies per neuron. (**F**) HSV-2 in SCG treated with CORT, as fold-change compared to each gene expressed during latency (0 h). (**G**) HSV-2 in TG treated with CORT, as gene transcripts per neuron. (**H**) HSV-2 in TG treated with CORT, as fold-change compared to each gene expressed during latency (0 h). Data are means ± SEM, n > 6 experiments.

HSV-2 reactivates in both sensory TG and sympathetic SCG neurons in response to only CORT treatment (see Figure 2.1) through either the GR or the MR (see Figure 2.3). Our lab has already established that GR expression differs between sensory TG neurons and sympathetic SCG neurons [16]. Following CORT treatment in SCG or TG neurons, expression of ICP0, VP16, and ICP27 decreased within 1 h compared to latency, and expression remained relatively constant over the next 25 h (Figure 2.5E,G). ICP4 expression also remained low in SCG neurons, below the level of expression during latency (Figure 2.5F), but fluctuated in TG neurons, increasing above latency levels at 25 h posttreatment (Figure 2.5H).

Overall, the temporal profiles of IE gene and VP16 expression varied by virus and neuron type posttreatment with EPI and CORT. Taken together, our data suggest HSV-1 and HSV-2 gene expression during the initial phase of reactivation, immediately following the stimulus, differs depending on the reactivation stimulus and the type of neuron in which the virus is latent.

Inhibition of Protein Kinases and Transcription Factors Selectively Block Stress Hormone-Induced Reactivation of HSV-1 and HSV-2

As G-protein-coupled receptors, adrenergic receptors (α1, α2, β1, β2) are linked to different G-protein alpha (Gα) subunits. Thus, EPI binding to these adrenergic receptors can activate $G\alpha_s$, $G\alpha_{i/o}$, or $G\alpha_q$ proteins, which, in turn, can stimulate or inhibit adenylate cyclase and subsequent activation of cAMP or activate phospholipase C (PLC) to activate the mitogen-activated protein kinase (MAPK) pathway. Ultimately, these signaling pathways either activate or inhibit protein kinases and transcription factors, such as cAMP response element binding protein (CREB), c-Jun N-terminal kinase (JNK), protein kinase B (Akt), and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) (see a simplified signaling diagram in Figure 2.7). Upon activation by CORT, the GR and MR transport into the nucleus to directly bind the DNA of target genes, recruiting the p300/CBP coactivator complex to activate gene transcription through histone acetylation [43]. However, the GR also regulates other signaling pathways that include CREB, JNK, and β-catenin (see Figure 2.7). Previously, JNK- and CREB-responsive elements were shown to be important in stress-induced reactivation of HSV-1 [44–46]. The transcription factor β-catenin plays a role in bovine herpesvirus 1 (BoHV-1) latency and reactivation [47,48], and induction of β-catenin expression decreased HSV-1 replication in vitro, suggesting a possible role in the establishment and maintenance of latency [49]. Therefore, we sought to determine if CREB, JNK, and β-catenin are important in adrenergic and glucocorticoid-induced reactivation of HSV-1 and HSV-2.

To determine if we could prevent EPI- or CORT-induced reactivation by inhibiting activation of CREB, latently infected neurons were treated with EPI or CORT and the

CREB inhibitor 666–15. This inhibitor prevents the phosphorylation of CREB at serine 133 by various cellular serine/threonine protein kinases, such as protein kinase A (PKA) or mitogen-activated protein kinases (MAPKs). Phosphorylation allows CREB to interact with CREB-binding protein (CBP) and p300, which are essential for CREB-mediated gene transcription [50]. Inhibition of CREB activity blocked both EPI- and CORT-induced reactivation of HSV-1 in sympathetic SCG neurons (Figure 2.6A,B). In contrast, inhibition of CREB did not prevent CORT-induced reactivation of HSV-2 in either sympathetic SCG (Figure 2.6C) or sensory TG (Figure 2.6D) neurons. Thus, CREB activation is an essential event during stress hormone-induced HSV-1 reactivation, but not for HSV-2.

SP600125, which competitively inhibits ATP binding to JNK, thus reducing JNK’s ability to phosphorylate c-Jun [51], was previously shown to effectively inhibit HSV-1 reactivation induced by nerve growth factor (NGF) deprivation in embryonic sympathetic SCG neuronal cultures [46]. In latently infected adult SCG neurons, the JNK inhibitor SP600125 effectively prevented both EPI- and CORT-induced reactivation of HSV-1, as expected (Figure 2.6A,B). In contrast, inhibition of JNK was unable to prevent CORT-induced reactivation of HSV-2 in either sympathetic SCG (Figure 2.6C) or sensory TG (Figure 2.6D) neurons. Thus, JNK activity is an essential component of HSV-1 reactivation in response to several different reactivation stimuli, including EPI and CORT treatment in our studies and NGF deprivation in previous studies [46], but it does not appear to be essential for HSV-2 reactivation in either sympathetic or sensory neurons. Alternatively, another signaling cascade may serve as a redundant pathway if JNK is inhibited during HSV-2 reactivation.

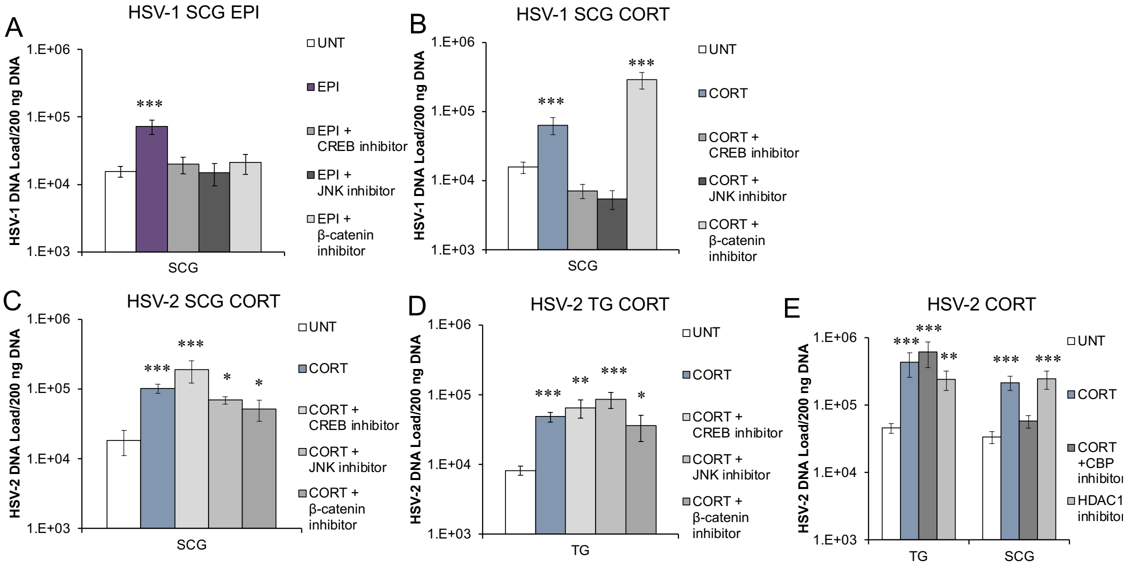

Figure 2.6. Inhibition of transcription factors and protein kinases differentially affect EPI- and CORT-induced reactivation.
Primary adult neuronal cultures from sympathetic SCG or sensory TG were infected with HSV-1 or HSV-2 in the presence of acyclovir for 7 days to establish latency, prior to treatment with EPI or CORT and inhibitors. HSV-1 in SCG neuronal cultures treated with EPI (**A**) or CORT (**B**), either alone or with inhibitors for CREB (666-15), JNK (SP600125), or β-catenin (iCRT14). HSV-2 in SCG (**C**) or TG (**D**) cultures treated with CORT either alone or with CREB, JNK, or β-catenin inhibitors. (**E**) HSV-2 in SCG and TG cultures treated with CORT, CORT with p300/CBP inhibitor C646, or HDAC1 inhibitor pyroxamide alone. Viral DNA was quantified in A–E by viral DNA collected 72 h posttreatment, measured by qPCR. Data are means ± SEM, n > 3; * $p < 0.05$; $p < 0.01$; *** $p < 0.001$.

Under resting conditions, cytoplasmic β-catenin is bound to its destruction complex, which results in its ubiquitination and degradation to maintain a basal level of β-catenin in the cell [52]. In canonical Wnt signaling, β-catenin is released from the destruction complex, enters the nucleus, and binds T cell factor (TCF) to activate the transcription of target genes [52]. A β-catenin inhibitor, iCRT14, inhibits the interaction between nuclear β-catenin and the N-terminal domain of TCF to block the transcriptional targets of β-catenin signaling [53]. In our studies, administration of iCRT14 prevented adrenergic reactivation of HSV-1 in sympathetic SCG neurons (Figure 2.6A). Thus, β-catenin interaction with TCF in the nucleus appears to play a role in adrenergic reactivation of HSV-1 in sympathetic neurons, likely through activation of specific viral or cellular target genes.

In contrast, β-catenin inhibition did not prevent CORT-induced reactivation of HSV-1 (Figure 2.6B), nor did it block HSV-2 reactivation in either sympathetic SCG or sensory TG neurons (Figure 2.6C,D). These results suggest that the mechanism of glucocorticoid-induced reactivation is not dependent on β-catenin–TCF activation of gene transcription. Since activated GR also binds directly to DNA and recruits CBP/p300 to acetylate histones, we also used a p300/CBP inhibitor (C646) to determine if we could prevent CORT-induced HSV-2 reactivation by inhibition of the histone acetyltransferase (HAT) activity of p300/CBP. Although CORT-induced reactivation was not prevented in sensory TG neurons, inhibition of p300/CBP HAT activity effectively prevented CORT-induced reactivation of HSV-2 in sympathetic SCG neurons (Figure 2.6E). Since histone deacetylase 1 (HDAC1) and p300 can directly bind to overlapping regions of the histone H3 tail and compete for histone binding [54], we attempted to induce reactivation of HSV-2 with an HDAC1-specific inhibitor, pyroxamide. In sympathetic SCG neurons, pyroxamide alone induced HSV-2 reactivation similarly to CORT-induced reactivation (Figure 2.6E). Thus, recruitment of p300/CBP and its associated HAT activity are required for CORT-induced reactivation of HSV-2 in adult sympathetic neurons, while deacetylation by HDAC1 assists in the maintenance of the latent state. Although we speculate that chromatin regulation is also the primary regulator for HSV-2 in sensory TG neurons, further studies are needed to determine the precise signaling pathway that induces the necessary changes for HSV-2 reactivation to occur in sensory TG neurons. While

CORT induces HSV-2 reactivation in sensory TG neurons, we were unable to identify a specific inhibitor that could effectively block CORT-induced reactivation in these neurons, demonstrating that multiple signaling pathways can induce reactivation of HSV-2. Further studies are also needed to clarify the role of β-catenin in reactivation, since this factor appears to be a key mediator in differentiating cellular signaling events activated by EPI and CORT.

Discussion

In our studies, we demonstrate that the stress hormones epinephrine (EPI) and corticosterone (CORT) induce reactivation of HSV-1 selectively in sympathetic, but not in sensory or parasympathetic primary adult neurons. We also demonstrate that activation of more than one adrenergic receptor is necessary for EPI to induce HSV-1 reactivation, while only the glucocorticoid receptor is required for CORT-induced reactivation of HSV-1. In contrast, CORT induces HSV-2 reactivation in both sensory and sympathetic neurons, but EPI has no significant effects on HSV-2 latent infection. Furthermore, CORT can induce HSV-2 reactivation through either the glucocorticoid or mineralocorticoid receptors. The temporal kinetics of ICP0, ICP27, ICP4, and VP16 indicate that different reactivation stimuli induce neuron-specific patterns of viral gene expression. Thus, our studies demonstrate that reactivation mechanisms for HSV are neuron-specific, stimulus-specific, and virus-specific.

HSV-1

Previously, we demonstrated that EPI treatment during productive infection enhanced HSV-1 DNA replication and infectious virus titer production in sympathetic, but not sensory, primary adult neurons [16]. Our current studies correlate with these findings, demonstrating that EPI also induces reactivation of HSV-1 in the same neurons. However, CORT *decreased* HSV-1 DNA replication and infectious virus titer during productive infection [16], while our current studies show that CORT *induces* reactivation from latency. Previous research has demonstrated that glucocorticoid treatment during productive infection can reduce the expression of both viral lytic genes and host inflammatory genes, and that the activated GR can bind to one of the origins of replication in the HSV-1 genome, potentially limiting viral gene expression, genome replication, and severity of acute disease [55,56].

During reactivation from latency, the viral genome exists under epigenetic repression and must respond to a reactivation stimulus. Kim et al. (2012) categorized HSV-1 reactivation into a biphasic pattern, in which Phase I served as the priming phase 15–24 h post-reactivation (hpr), characterized by the dysregulated expression of all classes of lytic transcripts, while Phase II (from ~25 hpr onward) resulted in genome amplification and viral progeny similar to productive infection. Their studies utilizing embryonic sympathetic SCG neurons and PI3-K inhibition as a reactivation stimulus demonstrated that ICP27 and VP16 were expressed at 20 hpr and decreased at 25 hpr. We observed a similar pattern in HSV-1 latently infected adult sympathetic SCG neurons treated with EPI, although we observed a greater increase in ICP4 than VP16 at 20 hpr compared to expression levels during latency. Our data show that EPI and CORT induced gene expression for ICP0, ICP27, ICP4 and VP16 with different temporal kinetics. However, both treatments resulted in viral reactivation from latency. The contrasting viral transcript expression kinetics between EPI and CORT treatment suggest that these two reactivation stimuli activate different signaling mechanisms that result in HSV-1 reactivation in sympathetic neurons. Our inhibition studies support this finding as well, demonstrating that inhibiting β-catenin interaction with TCF can prevent EPI-induced reactivation of HSV-1 but not CORT-induced reactivation. Although CREB and JNK are common factors downstream of both EPI and CORT, adrenergic and glucocorticoid receptor stimulation clearly result in the activation of different signaling cascades.

We also show that EPI must activate multiple adrenergic receptors to induce the reactivation of HSV-1 from sympathetic neurons (Figure 2.7A). Since the ARs couple with different G-protein α subunits, EPI can activate or inhibit adenylate cyclase and downstream cyclic AMP (cAMP) production, while also activating Src [35] or phospholipase C (PLC) pathways (Figure 2.7B) [36,38]. Additionally, different Gα proteins modulate β-catenin signaling, with $G\alpha_s$ potentiating and $G\alpha_q$ suppressing β-catenin signaling [39]. The common denominators in the signaling pathways that induce adrenergic reactivation are the activation of CREB and JNK, modulation of β-catenin, and $G\alpha_{i/o}$ inhibition of the adenylate cyclase pathway, which, in turn, inhibits downstream factors NFκB and Akt. In our studies, CREB, JNK, and β-catenin inhibition blocked EPI-induced reactivation, demonstrating that each of these factors can independently play a role in the reactivation process. However, a complex network of signaling events must occur simultaneously for EPI to induce HSV-1 reactivation in adult sympathetic neurons.

Stimulation of the glucocorticoid receptor (GR) by CORT also induced HSV-1 reactivation in sympathetic neurons, but not sensory neurons, although the GR is expressed in both sensory TG [15] and sympathetic SCG neurons [16]. In sensory neurons, activation of either the GR or the ARs was not sufficient to activate or disrupt the necessary signaling pathways to reach the threshold required for the reactivation of HSV-1. Iontophoresis of epinephrine or administration of the GR agonist dexamethasone (DEX) have been shown to reactivate HSV-1 in the rabbit and mouse models of ocular infection [11,12,57–59], although our results suggest that reactivation may have occurred in sympathetic SCG rather than sensory TG neurons in these animal models. DEX can stimulate the expression of a reporter gene from the HSV-1 ICP0 promoter by activating several host transcription factors [22,24]. In cooperation with Krüppel-like transcription factors (KLF15 and KLF4), the GR has also been shown to transactivate ICP27 and ICP4 [25,26], which are the viral genes that increased early in response to CORT treatment in our studies. Both JNK and CREB activity were required for CORT-induced HSV-1 reactivation in sympathetic SCGs, but in contrast to EPI-induced HSV-1 reactivation, β-catenin did not appear to be involved.

Reactivation of HSV-1 from sympathetic neurons depends upon downstream transcription factors and protein kinases. While adrenergic reactivation requires the stimulation of multiple adrenergic receptors, CORT-induced reactivation only required activation of the GR, not the MR (Figure 2.7A). Reactivation from both stress hormone pathways was blocked when CREB and JNK were inhibited, suggesting that there are commonalities between the mechanisms of reactivation by these stress hormones. However, the pathways are not necessarily equivalent, considering that β-catenin is only implicated in adrenergic reactivation, and the kinetics of IE and VP16 gene expression differed between the two

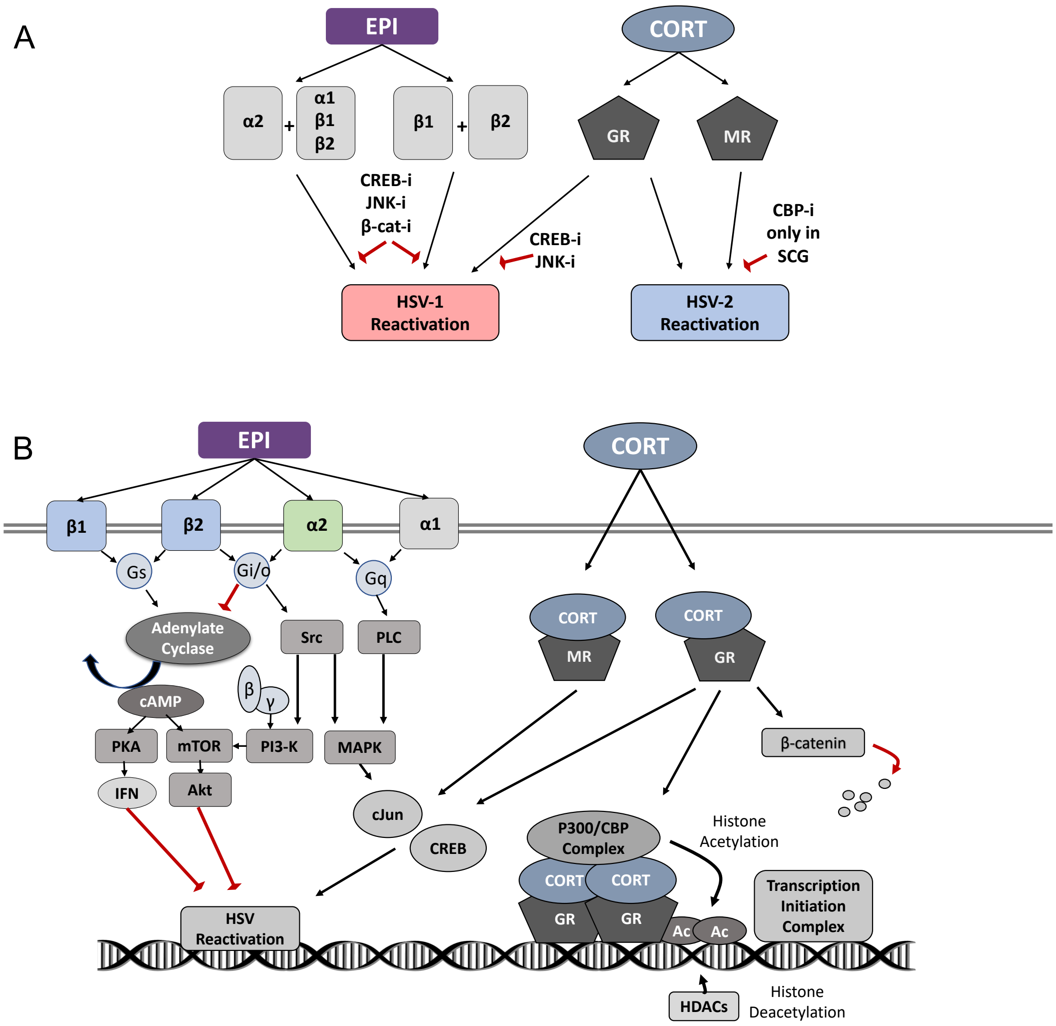

Figure 2.7. Adrenergic and glucocorticoid signaling pathways and HSV reactivation pathways.

(A) Epinephrine (EPI) induces HSV-1 reactivation through the activation of α2 and any other AR, or through the activation of β1 and β2, which can be blocked by the inhibitors of CREB, JNK, or β-catenin. Corticosterone (CORT) induces HSV-1 reactivation through the glucocorticoid receptor (GR), which can be blocked by the inhibitors of CREB or JNK. CORT induces HSV-2 reactivation through either glucocorticoid (GR) or mineralocorticoid (MR) receptors; the only factor we identified able to block HSV-2 reactivation was a CBP inhibitor, and only in SCG neurons. (**B**) Epinephrine (EPI) activates multiple adrenergic receptors on peripheral neurons (α1, α2, β1, β2), which couple with different G-protein α subunits (Gq, Gi/o, Gs), which can activate different signaling cascades. Corticosterone (CORT) activates signaling cascades through cytosolic glucocorticoid (GR) and mineralocorticoid (MR) receptors. Cytosolic effector proteins and transcription factors mediated by EPI and CORT signaling include adenylate cyclase, cyclic AMP (cAMP), protein kinase A (PKA), interferons (IFN), the mechanistic target of rapamycin (mTOR), protein kinase B (Akt), protein kinase Src, phosphoinositide 3-kinase (PI3-K), phospholipase C (PLC), mitogen-activated protein kinases (MAPKs), c-Jun, cAMP response element-binding protein (CREB), CREB binding protein (CBP), p300/CBP Complex, histone deacetylases (HDACs), and β-catenin. Black arrows indicate stimulation or activation; red arrows indicate inhibition.

stress hormones. Considering that neither EPI nor CORT induced HSV-1 reactivation above untreated levels in primary adult sensory neurons, and we have previously demonstrated that HSV-1 reactivates in these neurons in response to the deprivation of the neurotrophic factor neurturin (NTN) [32], we conclude that different mechanisms are responsible for HSV-1 reactivation, depending on the type of neuron in which the latent virus resides and the nature of the reactivation stimulus.

HSV-2

In contrast to HSV-1, EPI does not induce HSV-2 reactivation, regardless of neuron type. Furthermore, the GR and the MR are redundant pathways for CORT-induced reactivation, with activation of either receptor being sufficient to induce HSV-2 reactivation. However, inhibition of the potential downstream effectors of GR and MR that we tested, including CREB, β-catenin, and JNK, did not block HSV-2 reactivation in either sympathetic SCG or sensory TG neurons. These results suggest that the mechanism by which CORT induces HSV-2 reactivation likely involves either the chromatin remodeling properties of the GR or GR's ability to recruit transcription factors that activate genes needed for reactivation. Alternatively, multiple signaling pathways are responsible for HSV-2 reactivation and a combinatorial blockade would be necessary to prevent it.

Our ability to block CORT-induced HSV-2 reactivation with a p300/CBP histone acetylase (HAT) inhibitor supports a mechanism regulated by histone modifications in sympathetic

SCG neurons. Activated GR transports into the nucleus to directly bind the DNA of target genes, recruiting the p300/CBP initiation complex, which acetylates histones and initiates gene transcription [60]. The histone acetylation of immediate early (IE) gene promoters has been associated with HSV-1 reactivation [61,62]. CBP and p300 are recruited to ICP4, ICP0, and ICP27 promoters during HSV-1 productive infection [63], suggesting that activated GR could potentially induce reactivation of HSV-2 through the histone acetylation of IE gene promoters.

We found changes in HSV-2 gene expression of ICP4, ICP0, ICP27, and VP16 after CORT treatment in both sensory and sympathetic neurons, although the specific pattern of gene expression varied by neuron type. However, the kinetic profile of these IE genes and VP16 did not increase beyond untreated levels in HSV-2-infected neurons in the first 25 h posttreatment, although ICP4 expression was trending upwards. These results suggest that HSV-2 latency is not a completely dormant state, but a dynamic process that maintains a basal level of viral gene expression, which maintains the neuronal environment and the viral genome in a ready-state to respond to a reactivation stimulus. In our studies, ICP0 was expressed at low levels during latency; as an E3 ubiquitin ligase, basal expression of ICP0 during latency could potentially target specific neuronal proteins for degradation to maintain the latent state. Upon a reactivation stimulus, ICP0 expression decreased, suggesting that, in the event of a neuronal stressor, the loss of ICP0 may permit the expression of neuronal proteins required for productive infection within the neuron.

Trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor that targets HDACs 1, 3, 4, 6, and 10, has previously been used to induce HSV-1 reactivation in quiescently infected rat pheochromocytoma cells (PC-12) and primary adult sensory and sympathetic neurons

[30,33,64]. In our studies, TSA induced HSV-2 reactivation in sensory and sympathetic neurons, but not in parasympathetic neurons. We previously showed that, following ocular infection in guinea pigs, HSV-2 was unable to fully establish latency in parasympathetic ciliary ganglia, either in vivo or in vitro, and instead maintained a persistent lytic infection [65]. The CBP inhibitor was able to prevent CORT-induced reactivation in sympathetic SCG neurons but not in sensory TG neurons. Our findings support the hypothesis that different types of neurons may maintain the HSV-2 viral genome in different histone configurations under the control of different histone modifiers, although the latent state may not be fully suppressed and as well-controlled by some neurons compared to others, or compared to HSV-1. A less repressed viral genome, responsive to multiple reactivation stimuli in both sensory and autonomic neurons, would lead to more frequent clinical recurrences and episodes of viral shedding, as occurs in humans infected with HSV-2.

Both glucocorticoids and epinephrine are crucial hormones required to maintain homeostasis of the body’s key physiological functions. CORT, being a homeostatic anticipatory hormone, is produced on activation of the hypothalamic–pituitary–adrenal (HPA) axis to regulate neuronal and hormonal systems, respond to stress, and maintain central and peripheral circadian rhythm. Traumatic physiological and emotional distress can also activate the adrenal medulla, inducing the secretion of epinephrine, which then acts as a neurotransmitter for the sympathetic system to restore homeostasis [66,67]. The opposing activity of natural CORT and EPI maintains homeostatic balance, but also causes adverse pathophysiological conditions when that balance is disrupted. Thus, EPI and CORT, from a broader physiological perspective, might not elicit a harsh stimulus for reactivation compared to more acute and direct stressors, such as sunburn, UV radiation, or nerve trauma, which is reflected in our results, showing a relatively low level of reactivation. Nonetheless, the hormones did indeed selectively induce reactivation, as exemplified by the production of viral progeny following treatment.

Conclusions

Stress hormones have differential effects on HSV-1 and HSV-2, demonstrating that the mechanism of reactivation is not the same for the two viruses. Therefore, clinical interventions developed to treat HSV-1 in humans may not work as effectively for HSV-2. Our studies also show that the mechanism of reactivation is not the same between different types of neurons, as exemplified by stress hormone-induced HSV-1 reactivation occurring in sympathetic neurons, but not sensory neurons. Therefore, characteristics of HSV reactivation cannot be generalized to all types of neurons in which HSV-1 and HSV-2 may establish latency. Similarly, mechanisms regulating HSV-1 cannot be generalized to HSV-2. Furthermore, latently infected sympathetic neurons represent an important reservoir of reactivating HSV, particularly in response to hormone stimuli, contributing to differences in recurrence characteristics of HSV-1 and HSV-2. Taken together, reactivation mechanisms for HSV are neuron-specific, stimulus-specific, and virus-specific.

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Chapter 3 Herpes simplex virus mediated regulation in primary adult sensory and sympathetic neurons during latency and post reactivation in response to stress hormone and nerve trauma

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Abstract

Stress stimuli have a strong association with the recurrence of latent herpes simplex viruses (HSV-1 and HSV-2) from peripheral neurons. Our studies show that the stress hormone corticosterone (CORT) and nerve trauma, through neurotrophic factor (NTF) deprivation, selectively reactivate HSV-1 and HSV-2 from adult sensory and sympathetic neurons. However, it is not clear how neuronal pathways respond to HSV-1 and HSV-2 infection during latency and post reactivation with either CORT treatment or NTF deprivation. Studies have implicated association of canonical β-catenin signalosome and PI3K-Akt signaling cascade in HSV mediated neuronal infections. Our studies demonstrate that the mechanism regulating Akt-GSK3β-β-catenin axis differently responds in HSV-1 and HSV-2 infected neurons. In sensory and sympathetic neurons, the signaling cascade attempts to keep the pathway active as a survival instinct during HSV-1 latency and immediately following reactivation with stress hormone and NTF deprivation, though the expression is reduced in sympathetic neurons. In contrast, HSV-2 eliminates the entire signaling cascade in the adult neurons. Latency-associated transcript (LAT) was abundantly present in latent HSV-1 infected sensory TG and sympathetic SCG neurons, compared to HSV-2 infected neurons and decreased following reactivation with NTF deprivation over 25-hour time period. We have previously published our work on temporal kinetics of HSV immediate-early gene in response to CORT treatment. In this study, we evaluated the kinetics of HSV-1 and HSV-2 immediate early (IE) genes and trans activator gene VP16 post reactivation with NTF deprivation. Transcript expression significantly increased in HSV-1 infected sensory and sympathetic neurons over 25 hours, compared to HSV-2 after NTF deprivation. Thus, our study demonstrates how HSV-1 and HSV-2

preferentially reactivate from different types of neurons under different stress responses. This has the potential to expand our knowledge about HSV pathogenesis and neuron-specific mechanisms, impacting the current paradigm of the herpes virology field.

Introduction

Responsible for causing lifelong recurrent infections and debilitating disease, herpes simplex viruses (HSVs) are ubiquitous pathogens in the human population (1). Herpes simplex virus is categorized into two serotypes, with HSV-1 most commonly being transmitted through oral contact routes and causing orolabial lesions and herpes keratitis. HSV-2 is more commonly associated with sexually transmitted infection, responsible for genital herpes. Severe cases of HSV infection can lead to systemic spread to the central nervous system (CNS), causing necrotizing encephalitis or disseminated disease in neonates. After primary lytic infection in epithelial cells, HSVs spend the majority of their life cycle 'in hiding' by establishing latency in peripheral neurons that innervate the site of primary infection. In response to various triggers, the viruses can reactivate from latency to cause recurrent disease episodes throughout the life of the host. Stress hormones or nerve trauma are crucial triggering factors responsible for reactivating HSV in neurons, although the molecular mechanism guiding viral reactivation in response to these triggers is not yet completely understood (1, 2).

Stress is a somatic stimulus that in regular conditions maintains homeostasis, but under intense pressure becomes a threat to our body's normal response. Any stress response, depending upon the stimuli, can elicit social, physiological, pathological, or an infectious response (3). Cortisol, a glucocorticoid, is a vital hormone synthesized and released by the adrenal cortex in response to physiological or psychological stress. The circadian manner of glucocorticoid expression maintains homeostasis during normal and stressful periods (4). The pleiotropic physiological kinetics of glucocorticoid are mediated by glucocorticoid receptors (GR). Studies from our lab have demonstrated that GRs are expressed in sympathetic neurons in superior cervical ganglia (SCG) and sensory neurons in the trigeminal ganglia (TG), and CORT plays a differential role in modulating HSV-1 and HSV-2 infections in these neurons through its cognate receptor (5). We have also shown that CORT differentially reactivates HSV-1 and HSV-2 in primary adult neurons. HSV-1 reactivates only from sympathetic neurons, while HSV-2 reactivates from sympathetic and sensory neurons in response to CORT (6). TG explants from HSV-1 latently-infected mice show increased viral shedding and expression of HSV regulatory proteins when treated with dexamethasone, a GR agonist. β-catenin is differentially expressed in sensory TG neurons during the HSV-1-mediated latency-reactivation cycle in explants from mice and *in-vivo* (7). In neuronal stem cells, the canonical Wnt-β-catenin pathway helps to maintain neuronal survival, proliferation and differentiation, and CORT can impair these functions. Excess production of CORT can also increase Wnt-inhibitors such as dickkopf-1 (DKK-1), which can eventually deactivate the Wnt signaling (8, 9).

Neurotrophic factors (NTFs) are target derived diffusible peptides (growth factors) that travel retrograde through the axons to neuronal soma, supporting neuronal survival, proliferation and cellular homeostasis, and is disrupted following a nerve trauma or axonal injury (10). A nerve injury or trauma can also elicit a herpetic recurrence within the connected dermatome (11). Since nerve injuries disrupt continuous neurotrophic factor support, this has led to the rationale that trophic support helps maintain HSV latency (11-

14). Nerve growth factor (NGF) is required by sensory and sympathetic neurons during embryonic development (15). NGF binding to tyrosine kinase A (TrkA) internalizes the ligand into signaling endosomes and transports retrograde to neuronal soma, activating the downstream PI3K-Akt cascade to ensure transcriptional requirements for cellular survival (10). Certain populations of sensory neurons, during post-natal growth, switch their preferences towards glial cell-derived neurotrophic factors (GDNF) family ligands that include GDNF and neurturin (NTN). GDNF and NTN signal by binding to their respective receptors, GFRα1 and GFRα2. These receptors dimerize with the tyrosine kinase component RET, which auto-phosphorylates and activates multiple downstream signaling cascades, depending on the site of phosphorylation on RET. One of the downstream signaling cascades is the PI3K/Akt pathway that is also activated by NGF binding to TrkA (12, 16, 17). Studies from our lab has demonstrated that different neurotrophic factors mediate HSV-1 and HSV-2 latency and reactivation in different population of adult neurons. In sensory neurons, GDNF and NTN are required for maintaining HSV-1 and HSV-2 latency. However, NGF and GDNF deprivation reactivates HSV-1 from sympathetic neurons (12). One of the key effector cytosolic molecule downstream in this pathway is glycogen synthase kinase-3 beta (GSK3β), which is directly phosphorylated by Akt. GSK3β also feeds into the canonical Wnt signaling pathway, being a key component of β-catenin destruction complex (18).

The neuronal signaling pathway cardinal to HSV latency and reactivation remains largely unknown. Since different exogenous stimuli induce HSV-1 and HSV-2 reactivation within neurons, we wanted to determine whether Akt-GSK3β-β-catenin signaling axis induced by different stress stimuli led to a common mechanism that regulate HSV-1 and HSV-2 latency and early reactivation events, in both sensory and autonomic neurons. Using adult primary neuronal cultures from murine sensory and sympathetic ganglia, we previously determined that CORT differentially reactivates HSV-1 and HSV-2 between sensory and sympathetic neurons, whereas different NTFs support HSV-1 and HSV-2 latency in adult neurons (6, 12). Here, we show that HSV-1 and HSV-2 show distinct mechanisms for regulating latency in adult sensory and sympathetic neurons and in response to different exogenous stimuli that result in viral reactivation.

Materials and Methods

Virus strains and cell lines

HSV-1 (17+) strain was originally obtained from John Hay (SUNY Buffalo, NY) and HSV-2 (333) strain from Gary Hayward (Johns Hopkins, MD) to Krause Lab (FDA, Bethesda, MD). Both HSV-1 and HSV-2 strain was propagated in Vero cells, and first stock passage were given to Margolis lab (UCSF, CA). Virus was again propagated through Vero cells, and the first passage stocks was transferred to Bertke Lab (Virginia Tech, Blacksburg, VA). The virus strains were titrated in Vero cells using Plaque assay to determine the concentrations. During infections, the primary adult neurons were inoculated with 30 multiplicity of the infection (MOI) of the stock virus diluted in Neurobasal A medium (Gibco).

Primary adult neuronal cultures

Six-week-old female Swiss Webster mice (Hilltop Laboratories, Scottsdale, PA, USA) were euthanized using CO2 and perfused with calcium-magnesium-free phosphate buffer saline. The trigeminal (TG) and superior cervical ganglia (SCG) were dissected and digested with papain (Worthington Biochemical, Lakewood, NJ, USA) at 37°C for 20 minutes. The ganglia were then further digested with 4mg/ml collagenase type 1 and 4.67mg/ml dispase (Worthington Biochemical, Lakewood, NJ, USA) for another 20 minutes. TG neurons were enriched in a 4-step Optiprep gradient following mechanical trituration with a 1000µL pipette because of higher volume of ganglionic debris compared to SCGs. SCGs were triturated using a 200µL pipette without a gradient. Both TGs and SCGs were plated on Matrigel Matrix Basement Membrane (Corning) coated 24 well plates. The neurons were maintained with media containing Neurobasal A media supplemented with 2% B27 (Thermo Fisher), 1% Penicillin-Streptomycin (Corning), 10µL/ml Gluatamax (Thermo Fisher), anti-mitotic agent 5-fluorodeoxyuridine (FUDR) (Sigma), along with neurotrophic factors with concentrations of 10 µg/mL nerve growth factor (NGF), 10 µg/mL glial-derived neurotrophic factor (GDNF), 10 µg/mL neurturin (NTN) from Peprotech. The mice were euthanized and tissues dissected in accordance with guidelines of Virginia Tech Institutional Care and Use Committee (IACUC# 15-237 and 21-244).

Infection, Establishment of Latency and Reactivation

Four days post neuronal cultures, the media was removed and the neurons were infected with HSV-1 (17+) and/or HSV-2 (333) at 30 MOI at 37°C with 5% CO2 for 1 hour for adsorption by the neurons. The viral inoculum was removed and the infected neurons were fed with fresh Neurobasal A media without FUDR, and with NGF, GDNF, NTN and 300µM acyclovir to establish viral latency and prevent replication *in-vitro.* 7 days post infection, the media was removed and fresh Neurobasal A media containing different combinations of reactivation stimuli with no acyclovir was added to infected neurons. For stress-induced reactivation, 10μM corticosterone-HBC was added to the Neurobasal media and for neurotrophic factor deprived reactivation, anti-NGF (#AF-556-NA), anti-GDNF (#AF-212-NA) and anti-NTN (#AF387) antibodies from R&D Systems were added to the media to ensure complete deprivation of neurotrophic factors.

Quantification of HSV viral load

24 hours post reactivation (hpr), the HSV infected neurons were harvested by mechanical scraping. DNA was isolated using Trizol method followed by ethanol precipitation. The DNA was resuspended in nuclease free H2O and quantified using a nanodrop. Viral DNA load was then quantified through quantitative polymerase chain reaction (qPCR) on a Viia7 Real-Time PCR system using HSV-1 or HSV-2 thymidine kinase (TK) gene-specific primers and probes and iTaq Universal Probes Supermix (Biorad). The HSV DNA load was normalized to 18S rRNA (Applied Biosystems, Waltham, MA, USA) and viral genome was shown as number of viral DNA copies in 200ng of total DNA.

Quantification of HSV gene expression

HSV infected neurons were collected at different time points between 1 hour and 25 hours post CORT treatment and neurotrophic factor deprivation, and RNA extraction was performed using Trizol method followed by ethanol precipitation, according to manufacturer's instructions. The RNA was then converted to cDNA using iScript Mix (Biorad). HSV-1 and HSV-2 gene expression was determined by quantifying HSV immediate early genes ICP0, ICP4, ICP27 and trans-activator gene VP16 using digital droplet PCR (ddPCR) with HSV-1 and HSV-2 specific primers and Evagreen QX200 Master mix (Biorad) on a QX200 ddPCR system (Biorad). The assay was normalized to 18S rRNA and reported as fold change of treated samples to untreated latent ones.

Protein expression Analysis

HSV infected TG and SCG neurons were collected at different time based on reactivation timepoints in RIPA buffer with 1:100 HALT protease/phosphatase inhibitors (Thermo Fisher) and total protein concentration was determined using BCA assay (Thermo Fisher). Protein samples were run on 8% SDS-PAGE gel followed by transfer to a PVDF membrane using electrophoresis. Blots were incubated with 5% bovine serum albumin (BSA) (Sigma) overnight. Rabbit primary anti β-catenin antibody (Abcam #ab32572), total GSK3β antibody (CST #12456), p-GSK3α/β (CST #8566), total Akt (CST #4691), p-Akt S-473 (CST #4058), and p-Akt (CST #4056) at 1:1000 were added and incubated for 3-4 hrs at 4°C. A secondary Goat HRP conjugated anti-rabbit antibody (Abcam #ab6721) was used for detecting protein bands using chemiluminescence. Densitometry was performed on western blots (n=3) and each protein was normalized to total protein measured using trichloroethanol in the SDS-PAGE gel. A-431 whole cell lysate (Abcam #ab7909) from Abcam was used as a positive control.

Statistical Analysis

All of the experiments were carried out with a minimum of three different biological replicates, that is from different neuronal cultures with each set containing at least 2-3 technical replicates for each treatment. Statistical analysis using ANOVA and students t-test was carried out using MS Excel and JMP Pro version. Statistical significance has been denoted with asterisk following the stated convention: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ where ‘p’ indicates the p-value.

Results

To understand selective latency and reactivation mechanisms of HSV-1 and HSV-2 in adult neurons, we established a primary adult murine neuronal culture model (Figure 3.1) (19). Sensory trigeminal ganglia (TG) and autonomic sympathetic superior cervical ganglia (SCG) were excised, dissociated and cultured, maintaining characteristics found in adult neurons *in vivo*. 5-fluorodeoxyuridine (FUDR) was added to halt the replication of non-neuronal cells. Four days post cultures, the TG and SCG neurons were infected with HSV-1 or HSV-2 and a latent infection was established using acyclovir (300µM) for seven days, which inhibits viral replication (20). Following removal of acyclovir, cultures were treated with CORT or were deprived of neurotrophic factors (NTFs).

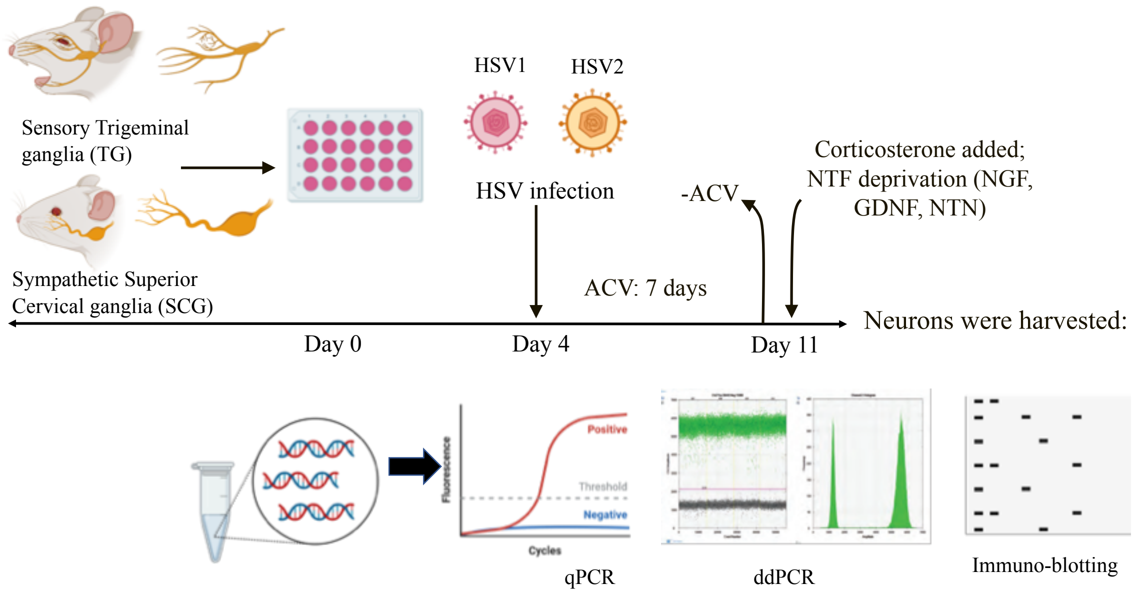


Figure 3.1. Schematic showing primary neuronal culture model from sensory trigeminal ganglia (TG) and sympathetic superior cervical ganglia (SCG) for investigating HSV latency and reactivation in neurons.
The ganglia were excised and cultured into single cell neuronal populations through enzymatic digestion and mechanical trituration. Neurons were infected with HSV-1 or HSV-2 at four days post neuronal cultures, and latency was established using acyclovir

(300μM) for 7days. The virus was then reactivated using either stress hormone, CORT, or through NTF deprivation simulating a nerve trauma. Image source: Individual images were taken from Biorender and Biorad (ddPCR image) and collated using MS PowerPoint.

Different stress stimuli preferentially reactivate HSV-1 and HSV-2 in adult sensory and sympathetic neurons

Previous studies from our lab have determined that CORT selectively modulates HSV-1 and HSV-2 productive infection. CORT treatment significantly reduces HSV-2 replication in sympathetic SCG neurons but not in sensory TG neurons (5). Studies of HSV-1 in rat embryonic and neonatal sympathetic neurons showed that NGF is crucial for maintenance of HSV latency in these neurons (14, 21). Our lab demonstrated, using primary adult murine neurons, that deprivation of NGF and GDNF caused HSV-1 to reactivate from sympathetic SCG neurons. However, in sensory TG neurons, NTN and GDNF are crucial for maintaining HSV-1 and HSV-2 latency respectively, showing a preferential reactivation mechanism depending on the trophic factor (12). In this study, we wanted to determine whether the stress hormone CORT or NTF deprivation (mimicking a nerve injury where axon terminals are deprived of local target derived NTFs) reactivate HSV-1 and HSV-2 from latency in different adult neurons. We have already published that CORT differentially induces HSV-1 and HSV-2 reactivation. Only HSV-2 is reactivated in sensory neurons, whereas both HSV-1 and HSV-2 are reactivated in sympathetic SCG neurons post treatment with CORT (6) . In contrast, we found NTF deprivation induces reactivation of HSV-1 and HSV-2 in both sensory TG and sympathetic SCG neurons (Figure 3.2A and Figure 3.2B). Using primary neurons constrains our access to a specific number of neurons we can harvest at a given time, hence a log increase in HSV copy number post treatment with reactivation stimuli is significant to us, and is considered a reactivation event. Both reactivation treatments have been compared to latent HSV infected untreated neurons supplemented with NTF factors. A more robust HSV-1 and HSV-2 reactivation (almost 10^6 copies/200ng total DNA) is observed in both TG and SCG neurons post reactivation with NTF deprivation compared to CORT treatment.

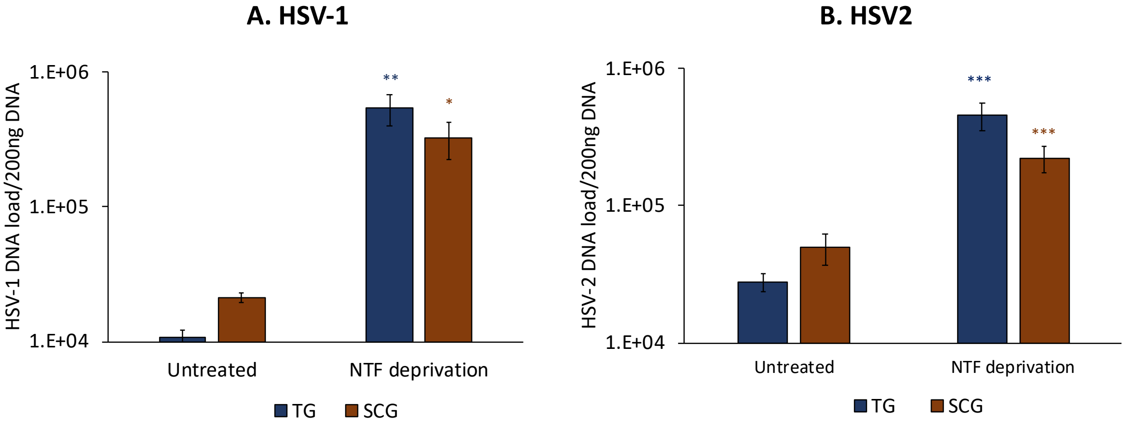
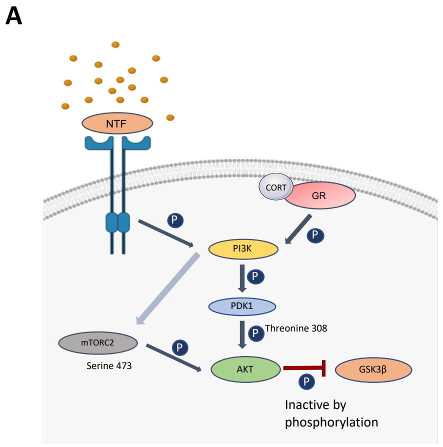


Figure 3.2. Neurotrophic factor deprivation reactivates both HSV-1 and HSV-2 in adult neurons.
After establishment of latency, NTF deprivation induced HSV-1 and HSV-2 reactivation in TG and SCG neurons. Viral DNA was quantified by qPCR 24 hours post reactivation.
(A) HSV-1 viral DNA load in sensory TG and sympathetic SCG neurons.
(B) HSV-2 viral DNA load in sensory TG and sympathetic SCG neurons.
Data are mean ± S.E.M with n>6. Results were compared by unpaired t-test for overall significance showing * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

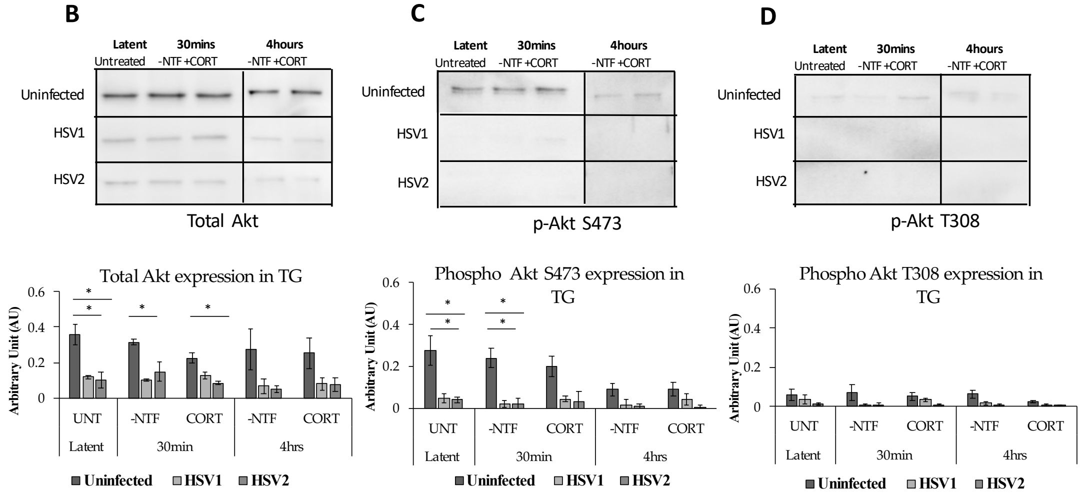
HSV-1 and HSV-2 modulate Akt-GSK3β-β-catenin signaling axis differently in sensory and sympathetic neurons during latency and post reactivation with NTF deprivation and CORT treatment

Regulation of Akt expression by HSV in adult neurons

When phosphatidylinositol 3-kinase (PI3K) is activated, downstream protein kinase B (Akt or PKB) becomes activated through phosphorylation at two key residues, at threonine 308 (T308) in the catalytic protein kinase core by pyruvate dehydrogenase kinase 1 (PDK1) and at serine 473 (S473) in the C-terminal hydrophobic motif by mammalian target of rapamycin complex 2 (mTORC2) (22) (Figure 3.3A). In embryonic sympathetic neurons, HSV-1 requires the mitogen-activated protein (MAP) kinase pathway, PI3K and axonal mTOR signaling to maintain latency and suppress reactivation in the presence of trophic support (17, 21). Nerve injury, simulated by neurotrophic factor deprivation, reactivates HSV-1 from embryonic sympathetic neurons requiring c-Jun N-terminal kinase (JNK) activation through an accumulation of JNK interacting protein 3 (JIP3) and dual-leucine-zipper kinase (DLK), disrupting Akt signaling, which is a negative regulator of DLK proteins (23, 24). To determine how Akt is regulated by HSV during latency and reactivation in adult neurons, we analyzed the expression of total Akt and phosphorylated Akt at both S473 and T308 in primary adult neurons infected with HSV-1 and HSV-2. We analyzed both sensory and sympathetic neurons to determine if signaling responses were similar in different types of neurons. Expression of total Akt and phosphorylated Akt levels was normalized to total protein expression in neurons for quantitative analysis and also used as a loading control (Supplementary Figure 3.1). Results were compared to kinetics of uninfected sensory TG (Figure 3.3B-D) and sympathetic SCG (Figure 3.3E-G) neurons with exact treatments at the same timepoints.



Sensory TG neurons



Sympathetic SCG neurons

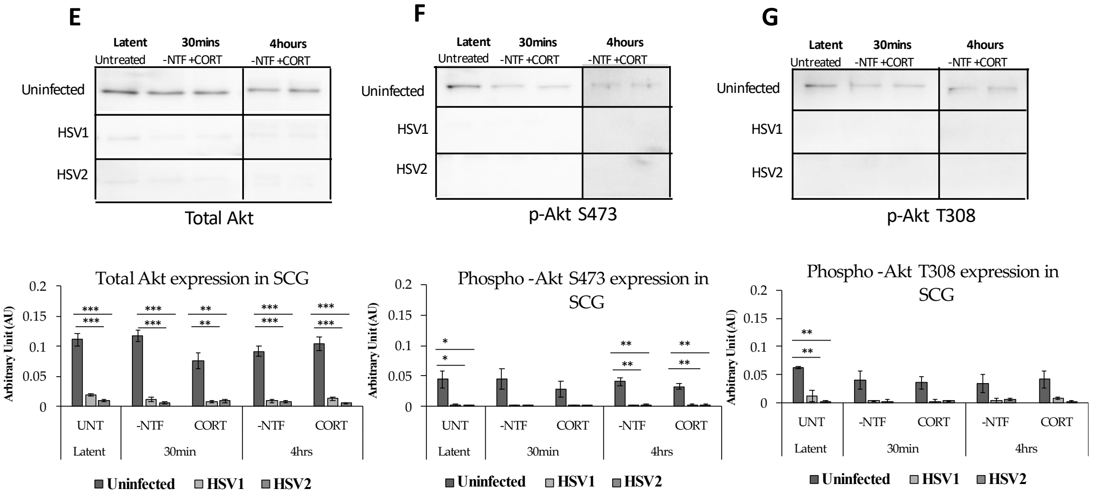


Figure 3.3. Akt expression profile is regulated by HSV-1 and HSV-2 in primary adult neurons during latency and immediately following reactivation.
(A) Schematic showing the Akt signaling pathway. When the pathway is active, AKT is phosphorylated at threonine-308 by PDK1 and at serine-473 by mTORC2 through downstream signal transduction.
(B-D; Top panel) Western blot showing expression of total Akt and phosphorylated Akt at S473 and T308 residue in uninfected **sensory TG neurons,** untreated and at 30 mins and 4 hours post treatment with CORT or NTF deprivation, and neurons latently infected with HSV-1 or HSV-2, untreated (latent) and at 30 mins and 4 hours post treatment with CORT or NTF deprivation. **(Bottom panel)** Quantitative analysis of the total Akt, p-Akt S473 and p-Akt-T308 between uninfected TG neurons and TGs infected with HSV-1 and HSV-2 during latency, and 30 mins and 4 hours following treatment.
(E-G; Top panel) Western blot showing expression of total Akt and phosphorylated Akt at S473 and T308 residue in uninfected **sympathetic SCG neurons,** untreated and at 30 mins and 4 hours post treatment with CORT or NTF deprivation, and neurons latently infected with HSV-1 or HSV-2, untreated (latent) and at 30 mins and 4 hours post treatment with CORT or NTF deprivation. **(Bottom panel)** Quantitative analysis of the total Akt, p-Akt S473 and p-Akt-T308 between uninfected SCG neurons and SCGs infected with HSV-1 and HSV-2 during latency, 30mins and 4hrs following treatment. Data are mean ± S.D with n=3. Results were compared using ANOVA and post hoc Tukey's HSD for overall significance showing * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

In uninfected neurons, total Akt shows minimal variation in expression between untreated neurons and those treated with CORT or deprived of NTFs at 30 mins or 4 hours post treatment with these reactivation inducers, in either sensory or sympathetic neurons (Figure 3.3B and Figure 3.3E). However, overall expression of total Akt was higher in TG compared to SCG neurons, with the same loading concentration of proteins (15μg/well). Phosphorylation of Akt on both S473 and T308 residues are needed to maintain Akt signaling activity; maximal activation is reported to be through phosphorylation at the S473 residue (22). In uninfected adult neurons, Akt phosphorylation kinetics diverged between sensory TG (Figure 3.3C-D) and sympathetic SCG (Figure 3.3F-G) cultures. NTF deprivation and CORT treatment did not produce differences in phosphorylation of Akt at

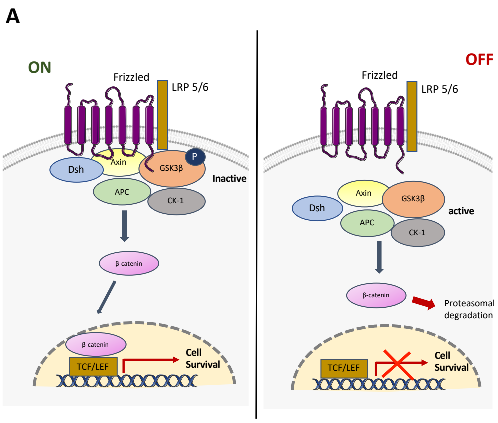
S473 by 30 mins post treatment, compared to untreated TG neurons, although phosphorylation was significantly reduced by the 4 hr timepoint with both of the treatments (Figure 3.3E). Expression of p-Akt T308 is notably decreased compared to p-Akt S473 in uninfected TG with almost no change in expression between latent through 4 hrs post treatment (Figure 3.3D). In contrast, a very different phosphorylation profile is evident in uninfected SCG neurons in which p-Akt S473 and p-Akt T308 are both decreased at 30 mins and 4 hrs post treatment with NTF deprivation or addition of CORT (Figure 3.3F-G). Hence, it is evident that cellular stressors alone can differentially modulate signaling kinetics of Akt between the two types of neurons.

Infection with HSV-1 or HSV-2 completely modifies the neuronal environment. Total Akt expression significantly decreases with both HSV-1 and HSV-2 infection during latency in TGs (67 and 71%, respectively) and SCGs (82 and 91%) in comparison to uninfected neurons (Figure 3.3D and Figure 3.3G). Although reduced overall, expression profiles in TG neurons are similar to uninfected neurons following NTF deprivation or CORT treatment (Figure 3.3D). In contrast, total Akt expression is almost undetectable in SCG neurons following reactivation treatments (Figure 3.3G). Phosphorylation of Akt at S473 and T308 is depleted during latent infection in HSV-1 and HSV-2 infected TG and SCG neurons, and treatment with either of the stress inducers does not alter the phosphorylation events, which are mostly undetectable (Figure 3.3C-D and Figure 3.3F-G).

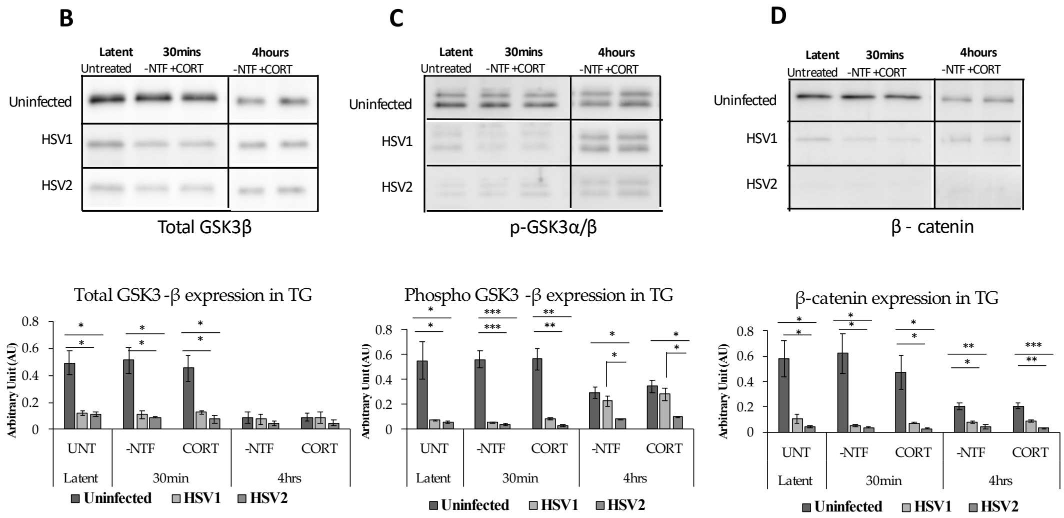
Expression of GSK3β-β-catenin axis in HSV infected neurons

Glycogen synthase kinase 3 (GSK3) is a multifunctional serine-threonine protein kinase having two isoforms, GSK3α and GSK3β, which share 85% homology and functional redundancy. GSK3 acts as an integrator of signals from multiple signal pathways. Serine-21 residues on GSK3α and serine-9 on GSK3β are phosphorylated by numerous kinases, including Akt (18, 22). GSK3β is a component of the destruction complex in the canonical-Wnt signaling pathway consisting of axin, adenomatous polyposis coli (APC), and casein kinase 1 (CK1) in addition to GSK3β. In the absence of Wnt ligand, GSK3β is not phosphorylated, and β-catenin is degraded by the active destruction complex. When GSK3β is phosphorylated, β-catenin is not degraded and acts as a transducer of signal to the nucleus, aiding in cell survival, maintenance and homeostasis (9) (Figure 3.4A). Akt can repress GSK3-mediated β-catenin degradation. Glucocorticoid in excess can lead to β-catenin destabilization and degradation through accumulation of Wnt inhibitors (22, 25).

We systemically analyzed the GSK3β-β-catenin axis during HSV-1 and HSV-2 latency and immediately following reactivation with NTF deprivation and CORT treatment (concurrent with the timepoints for total Akt expression and phosphorylation) in adult sensory TG neurons (Figure 3.4B-D) and sympathetic SCG neurons (Figure 3.4E-G). GSK3β and β-catenin expression was normalized to total protein expression in neurons (Supplementary Figure 3.1) for quantitative analysis. In uninfected neurons, NTF deprivation and CORT treatment did not alter expression of total GSK3β 30 min after treatment, compared to untreated neurons, in either TG or SCG neurons. By 4 hrs post treatment, total GSK3β was reduced in TG but not in SCG neurons (Figure 3.4B and Figure 3.4E). Phosphorylation status of GSK3β remained relatively stable, regardless of treatment or neuron type, in uninfected neurons (Figure 3.4C and Figure 3.4F). Regardless of total GSK3β, maintenance of a consistent level of phosphorylated GSK3β likely compensates for the persistent cellular stress signal, supporting neuronal survival.



Sensory TG neurons



Sympathetic SCG neurons

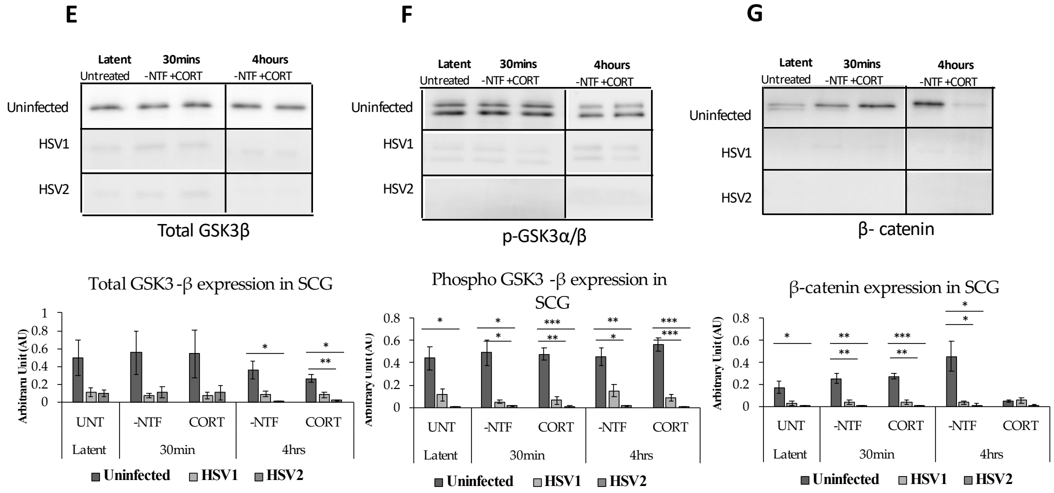


Figure 3.4. GSK3β-β-catenin expression profile is regulated by HSV-1 and HSV-2 in primary adult neurons during latency and immediately following reactivation.
(A) Schematic showing GSK3β-β-catenin signaling cascade. Inactive β-catenin destruction complex, in which GSK3β is phosphorylated, allows β-catenin to accumulate in the cytoplasm and translocate to the nucleus. Binding of β-catenin to transcription factors TCF/LEF results in cell survival gene transcription. Alternately, an active destruction complex, in which GSK3β is not phosphorylated, causes targeted degradation of β-catenin in the proteasome.
(B-D; Top panel) Western blot showing expression of total GSK3β, phosphorylated GSK3α/β and total β-catenin in **sensory TG neurons** in uninfected neurons and neurons latently infected with either HSV-1 or HSV-2, untreated and at 30 mins and 4 hours post treatment with CORT or NTF deprivation. **(Bottom panel)** Quantitative analysis of the total GSK3β, phosphorylated GSK3α/β and β-catenin between uninfected TG neurons and TGs infected with HSV-1 and HSV-2 during latency, 30 mins and 4 hrs following treatment.
(E-G; Top panel) Western blot showing expression of total GSK3β, phosphorylated GSK3α/β and β-catenin in **sympathetic SCG** neurons in uninfected neurons and neurons latently infected with either HSV-1 or HSV-2, untreated and at 30 mins and 4 hours post treatment. **(Bottom panel)** Quantitative analysis of the total GSK3β, phosphorylated GSK3α/β and β-catenin uninfected SCG neurons and SCGs infected with HSV-1 and HSV-2 during latency, 30mins and 4hrs following treatment. Data are mean ± S.D with n=3. Data are mean ± S.D with n=3. Results were compared using ANOVA and post hoc Tukey's HSD for overall significance showing * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Latent infection with HSV-1 or HSV-2 significantly decreased total GSK3β expression compared to uninfected TG (reduced 75% and 77%, respectively) and SCG (reduced 77% and 81%) neurons. In TG neurons, CORT treatment or NTF deprivation produced no significant changes between latency and either 30 mins or 4 hrs post reactivation (Figure 3.4D). In SCG neurons, however, both NTF deprivation and CORT treatment significantly reduced total GSK3β expression 4 hrs after treatment, becoming undetectable in HSV-2 infected neurons (Figure 3.4E). In TG neurons, NTF deprivation and CORT treatment increased phosphorylated GSK3β by 4 hr post treatment (Figure 3.4C) in HSV-1-infected neurons but had minimal effects on HSV-2-infected neurons. In sympathetic SCG neurons, HSV-1 suppressed GSK3β phosphorylation, compared to uninfected neurons, although a small increase was noted following NTF deprivation 4 hr post treatment, which did not reach statistical significance (Figure 3.4F). Phosphorylated GSK3β could not be detected in HSV-2-infected SCG neurons (Figure 3.4F). In uninfected neurons, NTF deprivation and CORT treatment significantly reduced β-catenin protein levels by 4 hrs post treatment in TG neurons. Similar to HSV effects on GSK3β, HSV-1 significantly reduced total β-catenin in TG neurons and HSV-2 depleted β-catenin to nearly undetectable levels (Figure 3.4D). Uninfected SCG neurons were more responsive to CORT treatment and NTF deprivation. NTF deprivation caused β-catenin to increase at 30 min post treatment and a greater increase by 4 hrs post treatment. However, CORT treatment caused a transient increase at 30 min post treatment, followed by a significant decrease by 4 hr post treatment (Figure 3.4G). Similar to HSV effects on GSK3β, HSV-1 reduced β-catenin, compared to uninfected neurons, and HSV-2 depleted β-catenin to nearly undetectable levels (Figure 3.4G), with greater reductions in SCG neurons compared to TG neurons. Neither NTF deprivation nor CORT treatment had significant effects on these low levels of β-catenin remaining in the neurons.

Pleiotropic effects of glucocorticoid signaling impacts the canonical Wnt signaling pathway. Excess glucocorticoids can shut down β-catenin expression by aiding activation of Wnt antagonists. One of the important Wnt pathway inhibitors is Dikkopf-1 (DKK-1) (9). DKK-1 binds to LRP5/6, and prevents the Frizzled-LRP5/6 complex association. This action inhibits the downstream Wnt signal transduction and the pathway becomes inactive, leading to β-catenin degradation (26). To address whether the decrease in β-catenin response with CORT treatment in uninfected SCG neuron after 4 hrs is mediated through DKK-1, we inhibited DKK-1 using gallocyanin (Abcam ab145230) (27). However, there was no difference in expression of β-catenin with CORT treatment in the presence or absence of gallocyanin (Supplementary Figure 3.2). Since DKK-1 is a part of Wnt responsive pathway, the result suggests that β-catenin signaling in response to CORT stimuli might be independent of Wnt-responsive genes.

HSV-1 and HSV-2 show different temporal kinetics of LAT and immediate-early (IE) gene expression in sensory and sympathetic neurons following reactivation with NTF deprivation

Our data shows that neuronal signaling cascades are modulated in neurons latently infected with HSV-1 and HSV-2, in the absence of stimuli (Figure 3.3 and Figure 3.4). The latent state of HSV is marked by the persistence of viral genome without production of infectious viral progeny. Expression of long non-coding RNAs, known as latency-associated transcript (LAT) along with micro RNAs (miRNA), are key molecular signatures of HSV latency. LAT is upregulated during HSV latency, represses HSV lytic transcripts and promotes histone accumulation and heterochromatin modification on viral genome (24, 28, 29). Studies have suggested that immediate early (IE) gene ICP0 mediates establishment of latency *in-vitro* and *in-vivo* in the mouse model of HSV infection by promoting LAT expression and other lytic gene expression (29). Post reactivation, LAT expression has been shown to decrease over time in HSV infected neurons (30, 31). In order to assess LAT expression post reactivation with NTF deprivation, we quantified LAT expression over 25 hours post reactivation in TG and SCG neurons. In sensory TG neurons infected with HSV-1, LAT expression is significantly decreased at 1 hr post reactivation and maintains basal expression over the next 24 hours (Figure 3.5A). Sympathetic SCG neurons latently infected with HSV-1 show a reduction in expression by 2 hrs post treatment, which is maintained over the next 24 hours with a marginal increase at 15 hr timepoint (Figure 3.5C). Alternatively, LAT expression in HSV-2 infected TG does not drastically decrease when compared with latent expression except at 15 hours and 25 hours post reactivation (Figure 3.5B). Sympathetic SCG neurons latently infected with HSV-2 fluctuate LAT transcript expression over 25 hours post reactivation (Figure 3.5D).

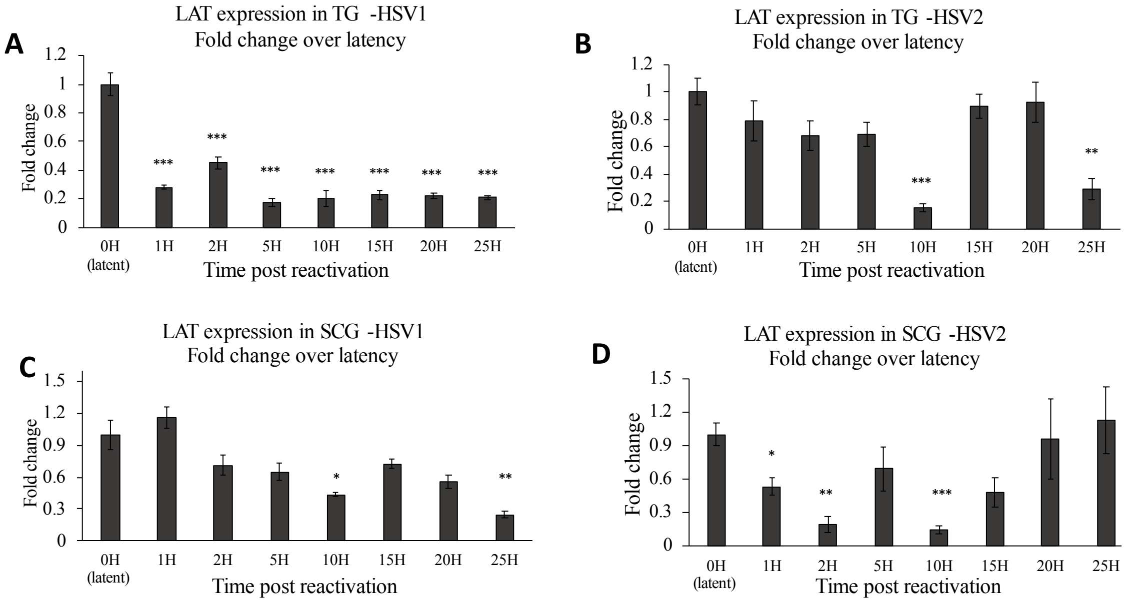


Figure 3.5. Latency-associated transcript (LAT) expression varies between HSV-1 and HSV-2 post reactivation with NTF deprivation.
After seven days of latency, TG and SCG neurons infected with HSV-1 and HSV-2 were reactivated through NTF deprivation. LAT expression was quantified by ddPCR over a 25 hour time period and compared to LAT expression during latency.
(A-B) LAT in **sensory TG neurons** infected with HSV-1 and HSV-2 respectively.
(C-D) LAT in **sympathetic SCG neurons** infected with HSV-1 and HSV-2 respectively.
Data are mean ± S.E.M with n>6. Results were compared by unpaired t-test for overall significance showing * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

In our primary neuronal cultures, we detected low levels of Immediate-Early (IE) gene transcripts for ICP0, ICP4, and ICP27, as well as trans-activator VP16, in both HSV-1 and HSV-2 infected neurons during latency. Following reactivation with stress hormones epinephrine and corticosterone, the expression of these genes did not increase beyond transcripts detected during latency (6). To analyze the effects of NTF deprivation on expression of these viral genes, we quantified the temporal kinetics of ICP0, ICP4, ICP27, VP16 over a 25-hour time period following deprivation of NTF in both HSV-1 and HSV-2 latently infected TG and SCG neurons. LAT expression was also included to compare with the kinetics of IE genes. NTF deprivation resulted in a significant increase in expression of ICP0, ICP27 and VP16 1 hr post reactivation and remained significantly expressed over the next 24 hours (Figure 3.6A and Figure 3.6C). The increased gene expression was more prominent in HSV-1 infected sensory TG neurons, with a higher fold-change over latency that is sustained through 25 hours, compared to sympathetic SCG neurons, in which the transcripts reduced in expression by 15 hours post reactivation. Although ICP4 expression increased in HSV-1 infected TG neurons in response to NTF deprivation, as in response to CORT treatment (6), ICP4 expression did not exceed expression levels of ICP0, ICP27, and VP16 in either TG or SCG neurons (Figure 3.6A and Figure 3.6C). IE and VP16 gene expression in HSV-2 latently infected TG neurons rapidly increased within 1 hr of NTF deprivation, with ICP4 expression exceeding ICP0, ICP27 and VP16, but expression ceased by 5 hours post reactivation (Figure 3.6B). In SCG neurons, NTF deprivation only induced increased expression of ICP4 above latency levels of expression, 5 hrs, 15 hrs, and 20 hrs post reactivation (Figure 3.6D). As LAT expression is a hallmark of latency, we quantified LAT expression. As expected HSV-1 expressed abundant LAT transcripts, although less in SCG than TG (70.65 transcripts/neuron in TG and 17.96 transcripts/neuron in SCG). LAT expression from HSV-2 was detected at significantly lower levels.

Altogether, our data shows that establishment of latency is dynamic in nature and is different between HSV-1 and HSV-2. Additionally, HSV-1 and HSV-2 respond with different temporal kinetics of LAT, immediate-early and VP16 gene expression in response to NTF deprivation in sensory and sympathetic neurons, which also varied from latently infected neurons treated with stress hormones EPI and CORT in our previous studies (6).

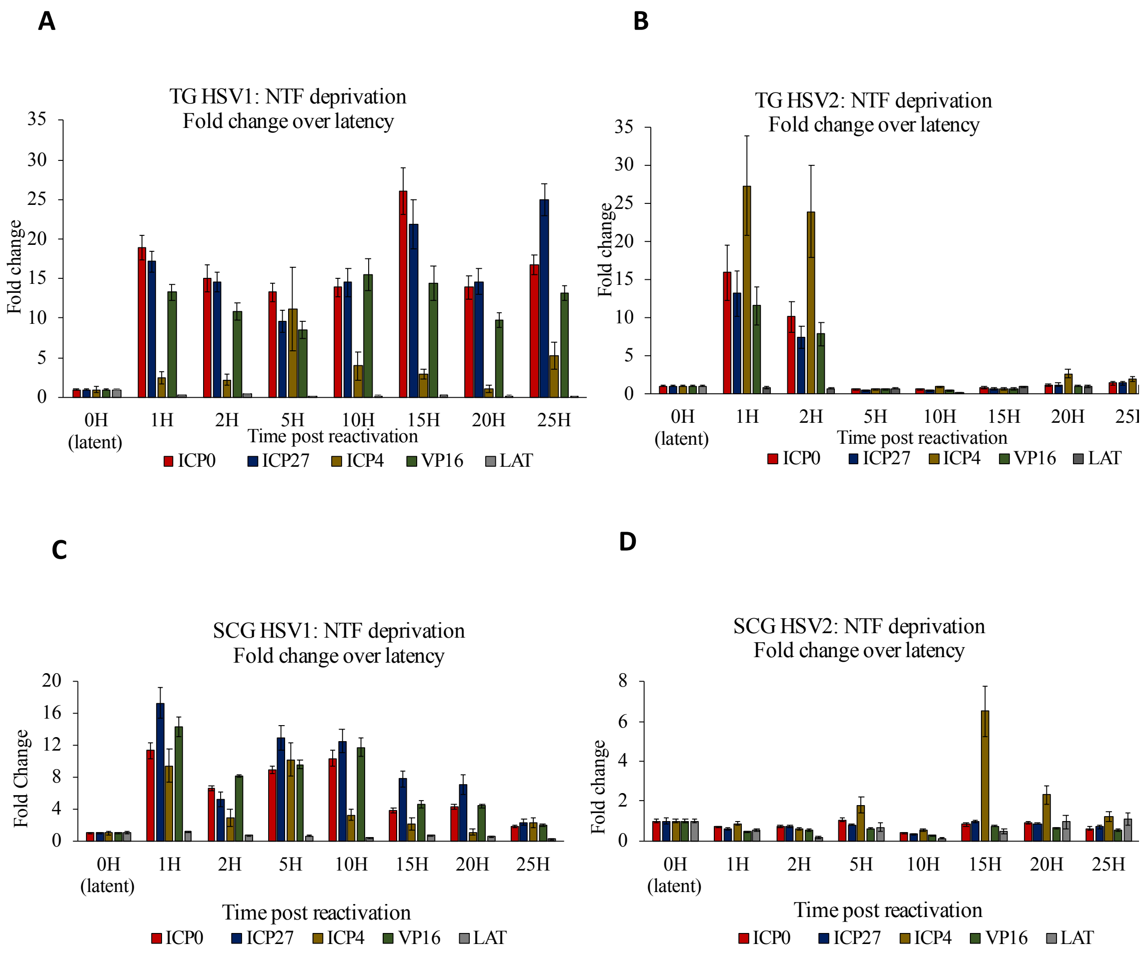


Figure 3.6. HSV-1 and HSV-2 immediate-early and VP16 gene expression varies post reactivation with NTF deprivation.

TG and SCG neurons latently infected with HSV-1 and HSV-2 were reactivated through NTF deprivation. Immediate-early (IE) transcripts of ICP0, ICP4, ICP27 and trans activator VP16 were quantified by ddPCR over a 25 hour time period. LAT expression was also included for comparison. The IE transcripts and VP16 were quantified as fold-change over expression during latency.

(A-B) Gene expression in **sensory TG neurons** latently infected with HSV-1 and HSV-2, respectively.

(C-D) LAT in **sympathetic SCG neurons** latently infected with HSV-1 and HSV-2, respectively.

Data are mean ± S.E.M with n>6.

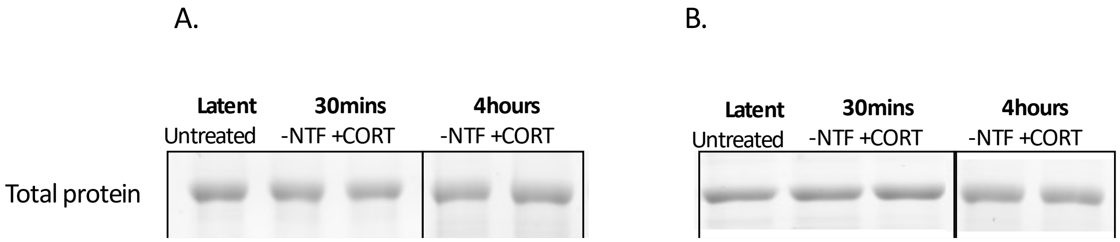
Discussion

It is long known that HSV establishes latency in peripheral neurons after infection and neuronal stress reactivates the virus (24). Cortisol (in humans) and corticosterone (in rodents), collectively called CORT, is a homeostatic anticipatory hormone secreted through the Hypothalamus-Pituitary Axis (HPA) and has diverse regulatory functions involving metabolism, circadian rhythm and the immune system (3, 32). However, under adverse physiological conditions, CORT can trigger a range of unfavorable events. Studies with corticosterone (CORT), the rodent variant of human cortisol, showed that CORT concentration increases in plasma during cold restraint stress in rats and hypothermic stress in mice, thereby reactivating HSV-1 (33). CORT can also reactivate HSV-1 during the lactation period in mice through suppression of immune system (34). Dexamethasone, an agonist for CORT, acts as a stress trigger for HSV and BoHV-1 reactivation from latency *in vivo* and from explants of trigeminal ganglia (7, 35). Studies from our lab have demonstrated that CORT selectively modulates HSV-1 and HSV-2 infection in primary adult sensory and sympathetic neurons during productive infection as well as post reactivation from latency (5, 6). Neurotrophic factor (NTF) deprivation acts as a more direct and acute stimulus towards HSV reactivation. Neurotrophic factors (NTF) are target-tissue derived ligands that aide in neuronal survival, synaptic plasticity and axonal growth, and are crucial in regulating HSV latency and reactivation (11-14). Loss of nerve growth factor (NGF) support reactivates HSV-1 in embryonic sensory and sympathetic neurons (14, 21, 36). Apart from NGF, GDNF and NTN deprivation also contribute to HSV-1 and HSV-2 reactivation from adult neurons (12). We determined that CORT selectively reactivates HSV-1 and HSV-2 from primary adult neurons, whereas NTF deprivation reactivates both HSV-1 and HSV-2 from the neurons. The accepted paradigm of HSV latency is the expression of the latency-associated transcript (LAT) and repression of lytic transcripts. Reactivation stimuli induce the expression of lytic viral genes and suppress LAT expression. In our primary neuronal cultures, LAT was abundantly present in latent HSV-1 infected sensory TG and sympathetic SCG neurons, compared to HSV-2 infected neurons, and decreased following reactivation with NTF deprivation. We have already shown that post reactivation with stress hormones epinephrine and corticosterone (CORT), the expression of IE genes and VP16 fluctuates over 25 hours following the treatment but does not increase beyond their respective latent transcripts (6). We have argued that hormones, being a physiological stressor, acts as a restrained stimulus compared to a direct stressor such as a nerve trauma or a nerve injury. Kinetics of these IE genes increased rapidly after reactivation with NTF deprivation, which simulates a nerve injury, and displayed a different kinetic profile than that observed with stress hormones, particularly with HSV-1.

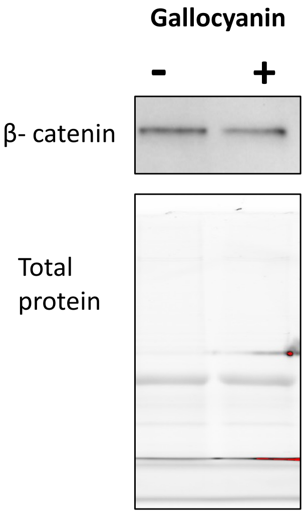
The Akt-GSK3β-β-catenin signaling axis contributes to neuronal survival and maintenance of homeostasis. CORT treatment and NTF deprivation serve as stressful stimuli to the neurons, even in the absence of virus. We observed different signaling kinetics between uninfected sensory TG and sympathetic SCG neurons in response to CORT treatment and deprivation of NTFs. In addition, the Akt-GSK3β-β-catenin axis response vastly differs between neurons infected with HSV-1 and HSV-2 during latency and post reactivation with CORT treatment or NTF deprivation. Total Akt signaling expression is sharply reduced and Akt proteins are not phosphorylated during latency in both HSV-1 and HSV-2 infected TG and SCG neurons. Reactivation with different stimuli does not alter the phosphorylation state. GSK3β regulates downstream β-catenin expression, whose degradation is mediated through GSK3β-dependent phosphorylation. GSK3β, when phosphorylated, inactivates the signaling cascade and prevents degradation of β-catenin (22). HSV-1-infected TG and SCG neurons attempt to maintain the GSK3β-β-catenin pathway through increased expression of P-GSK3β and β-catenin 4 hrs post reactivation, though β-catenin is still degraded in SCGs. In contrast HSV-2 infection completely depletes β-catenin expression in the neurons irrespective of the survival response by P-GSK3β. GSK3β is an important cytosolic protein kinase, and integrates signaling pathways controlling cellular metabolism, lineage commitment, neuronal physiology and cell survival. This effector protein is also a substrate of Akt and feeds into the downstream β-catenin pathway (18). However, redundancy among the signaling pathway that independently regulates GSK3β and functionally insulated pools of GSK3β in the cell helps to maintain its phosphorylated state and regulate activity of its downstream substrates including β-catenin, independent of inputs from Akt (22, 37). We observed that both HSV-1 and HSV-2 eliminate Akt phosphorylation, but P-GSK3β is still expressed in HSV infected neurons and attempts to keep the GSK3β-β-catenin signalosome active for neuronal homeostasis through a definitive survival response which is independent of Akt signaling.

Overall, our data show that HSV infection in adult neurons completely re-arranges the neuronal signaling machinery to its advantage during latency and post reactivation. HSV-2 infection particularly demonstrates a less repressed genome as evident through lower LAT expression in both sensory and sympathetic neurons when compared to HSV-1. Both

HSV-1 and HSV-2 express different temporal kinetics of immediate-early gene (IE) and VP16 transcript in response to CORT treatment (6) or NTF deprivation. Additionally, the presence of HSV in the neurons even in the absence of any reactivation stimuli completely alters the environment of that neuron. However, gaps in knowledge still exist. In our primary neuronal culture model, we look at the cellular proteins and viral gene transcripts after establishment of latency for seven days. By this time HSV has altered and modified the neuronal milieu and its viral transcripts. To understand the dynamic nature of latency post infection, a more in-depth analysis of the process of the establishment of the latent phase is required. In this study, we analyzed the Akt-GSK3β-β-catenin signalosome, but cellular proteins are constantly turned over in the neurons, and the signaling pathways are highly intersecting and compensate for redundancy to protect the host from stress and viral pathogens. Further research is required to comprehensively understand HSV mediated latency and reactivation under different stress condition in neurons. In this study, we infer that HSV-1 and HSV-2 does not have a 'one for all' approach. HSV preferentially reactivate in response to 'different' stimuli showing 'different' viral gene transcript, and using 'different' neuronal mechanism in 'different' types of primary adult neurons.



Supplementary Figure 3.1. Total protein expression (using trichloroethanol in 8% SDS-PAGE) for (a) *sensory TG neurons and* (b) *sympathetic SCG neurons* during latency, and post reactivation at 30mins and 4hrs timepoint.



Supplementary Figure 3.2. DKK-1 antagonist does not alter CORT induced β-catenin expression in uninfected SCG neurons.

Glucocorticoids can inhibit canonical Wnt signaling pathway and suppress β-catenin expression by aiding activation of Wnt inhibitor Dickkopf-1 (DKK-1). We observed a decrease in expression of β-catenin in uninfected SCG neurons at 4hours post treatment with CORT (Figure 3.4G). Treating neurons with gallocyanin (ab145230), an inhibitor of DKK-1, we wanted to observe whether inhibiting DKK-1 can compensate the low levels of β-catenin in CORT treated SCG neurons. The Western blot data shows there is no difference in the β-catenin protein expression between Gallocyanin treated and untreated SCG neurons at 4hour timepoint post treatment with CORT. This is suggestive that CORT might change β-catenin expression in the neurons through pathway independent of Wnt-responsive genes.

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Chapter 4 Summary Discussion

Herpes simplex virus (HSV) employs contrasting infection mechanisms comprising productive *de novo* replication in epithelial cells at the site of infection and establishment of latency through retrograde axonal transport in peripheral neurons that innervate the site of primary infection. HSV is able to maintain a lifelong reservoir in these neurons, but stressful stimuli can induce reactivation from latency and a secondary lytic infection cycle as the virus travels back down the axon to the epithelium to cause recurrent lesions (1, 7, 8). Stress has been conceptualized as a natural stimulus that under intense pressure elicits a non-specific response from our body. For our studies, we investigated two orthogonal stress stimuli responsible for HSV reactivation from latency. Stress hormones epinephrine (EPI) and glucocorticoid (CORT) are required to regulate physiological kinetics of our body. Excess of these hormones initiates a cascade of adverse effects to imbalance homeostasis (19, 35, 36). Both EPI and CORT have been shown to be strongly correlated with stress-induced HSV reactivation (14, 15, 37). We have shown that EPI and CORT selectively modulate HSV-1 and HSV-2 primary acute infection and reactivation from latency in sensory and sympathetic neurons (18, 20). Target-derived neurotrophic factors (NTF) are produced in tissues of the body innervated by neuronal axon terminals. These NTFs travel along the axons to the neuronal cell body (soma) and provide support to maintain survival, health, and homeostasis of individual neurons, particularly during a neuronal injury or disease. Extensive studies have demonstrated that depriving HSV-infected latent neurons of neurotrophic factors reactivates the virus (7, 24-28, 38).

HSV-1 tropism is more commonly associated with orolabial lesions while HSV-2 is involved with genital herpes. However, there exists a redundancy between two serotypes. Around 30% of primary genital herpes is caused by HSV-1 infection. However, HSV-1 and HSV-2 have differences in their recurrence frequency after genital infection. Historically, HSV-1 and HSV-2 were considered to have similarity genetically, as well as in their mechanisms in establishing latency or reactivating, resulting in few laboratories directly investigating mechanistic differences between these viruses (8). Over the years, studies have confirmed that HSV-1 and HSV-2 transcriptomics and proteomics expression, along with infection mechanism in peripheral neurons, are distinctly different and contrasting from each other (18, 20, 28, 39, 40). Overall, we infer that the mechanisms of establishing latency and reactivation of herpes simplex virus (HSV) is ***virus specific, stimulus specific and neuron specific.***

Comparing between the stress stimuli: Stress hormone (EPI and CORT) and neurotrophic factors (NTF) deprivation

EPI and CORT are metabolic hormones involved in the stress response and induce HSV-1 and HSV-2 reactivation. Treatment with EPI increases DNA replication and infectious viral progeny production in HSV-1-infected sympathetic superior cervical ganglia (SCG), but not in sensory trigeminal ganglia (TG) neurons. CORT differentially reactivates HSV-1 from SCG neurons and HSV-2 from both SCG and TG neurons. The stress hormones directly regulate HSV reactivation in the neurons through their specific cognate receptors. EPI primarily utilizes combination of $\alpha2$, $\beta1$ and $\beta2$ adrenergic receptors, while CORT signals through glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) (18). Neurotrophic factors (NTF) are crucial for maintaining HSV latency *in-vivo* as well as *in-*

vitro. We have previously demonstrated that apart from nerve growth factor (NGF), the glial cell line-derived neurotrophic factor (GDNF) family selectively modulates HSV-1 and HSV-2 productive infection and reactivation from adult neurons (28). In our study, combined NTF deprivation (depriving of NGF, GDNF and neurturin) increased DNA replication of both HSV-1 and HSV-2 in TG and SCG neurons. Temporal kinetics of HSV-1 and HSV-2 immediate early (IE) genes and trans activator gene VP16 post reactivation with EPI and CORT shows fluctuating transcript expression over 25 hours following the reactivation stimulus, but remained below the level of expression during latency. Conversely, NTF deprivation significantly increased the immediate early gene temporal kinetics in both sensory and sympathetic neurons with HSV-1 infection, but not so much with HSV-2.

The primary activity of EPI and CORT is to maintain homeostatic balance in our body. Even with disruption, these two hormones do not elicit a strong stress response compared to NTF deprivation. In contrast, NTF deprivation, resembling a nerve trauma or injury, serves as a more intense and acute stimulus in regulating HSV-1 and HSV-2 viral transcripts following reactivation, even though the end point of both stress responses results in HSV reactivation.

Comparing HSV-1 and HSV-2

Even though HSV-1 and HSV-2 have different transmission routes, and in most cases affect different anatomical sites, the two viruses are thought to have similar infection mechanisms due to their overlapping disease symptoms and sequence similarity at the genomic level. In the herpes virology field, most of the work has focused on HSV-1 research, and general concepts and observations have been extended to both viruses. Studies have shown that HSV-1 and HSV-2 do not conform to a ‘one for all approach’, and instead employ differential mechanisms of infection during latency and following reactivation, and are further dependent on the peripheral ganglia they infect (12, 13, 18, 28, 41). It has been demonstrated that in mice sensory ganglia, HSV-1 prefers to establish latency in the A5 positive subpopulation of neurons, and KH10 positive neurons are the preferred reservoir for the establishment of latency by HSV-2. In guinea pigs *in-vivo*, HSV-2 was involved in more severe acute ocular disease, but HSV-1 had more spontaneous recurrences after the establishment of latency (12). Expression of latency associated transcript (LAT), a long non-coding RNA, is a hallmark of latency and is expressed by both HSV-1 and HSV-2 in sensory and sympathetic ganglia. However, in parasympathetic ganglia, LAT was expressed by HSV-1 only (13). *In-vitro* studies with primary adult neurons show that LAT is highly expressed by HSV-1 in both sensory TG and sympathetic SCG neurons, compared to HSV-2. Reactivation from latency following a stress stimulus varies between HSV-1 and HSV-2, and also between the neurons where the HSVs establish latency. Following treatment with stress hormones or NTF deprivation, there is a preferential reactivation of HSV depending on stimuli. HSV-1 reactivates mostly from sympathetic SCG neurons. HSV-2 DNA replication post reactivation increases in both sensory and sympathetic neurons. Further, compared to HSV-2, LAT expression in HSV-1-infected TG and SCG neurons decreases over time following reactivation through NTF deprivation with increases in immediate early genes. These observations provide compelling evidence that HSV-2 genes are not completely repressed, and HSV-2 is unable to establish complete latency in the neurons. Neuronal signaling mechanisms regulating the Akt-GSK3β-β-catenin axis expression in HSV-infected neurons showed variation between HSV-1 and HSV-2 infection. In sensory TG neurons and sympathetic SCG neurons, the signaling cascade attempts to keep the pathway active as a survival instinct during HSV-1 latency and immediately following reactivation with stress hormone CORT and NTF deprivation, though the expression is reduced in sympathetic neurons. HSV-2 alternately eliminates the entire signaling cascade in both sympathetic and sensory neurons. This dissertation demonstrates that HSV-1 and HSV-2 express different temporal kinetics of viral genes in adult neurons and re-arrange the neuronal signaling cascades to their advantage during latency and following reactivation. We show that the establishment of latency is more dynamic than previously thought and the reactivation mechanisms of HSV are selective. Different stress stimuli exert preferential levels of reactivation between HSV-1 and HSV-2 in different neurons by altering the signaling kinetics of the neuronal pathways and viral gene response.

Overall, our studies highlight that HSV-1 and HSV-2 infection dynamics do not demonstrate a ‘one size fits all’ approach, and there is a complex interplay between different HSV serotypes, different stress stimuli and the different peripheral neurons where they establish latency. Understanding the HSV-1 and HSV-2 latency and reactivation process through different neuronal pathways will help us develop more intelligent and targeted therapeutics to prevent HSV recurrent disease episodes, and will have the potential to expand our knowledge neuron specific mechanisms regulating HSV pathogenesis and can change the current paradigm of the field.

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