# Stress hormones epinephrine and corticosterone modulate herpes simplex virus 1 and 2 productive infection and reactivation primarily in sympathetic, not sensory, neurons

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**ABSTRACT (academic)**

Herpes simplex viruses 1 and 2 (HSV-1 and HSV-2) infect and establish latency in peripheral neurons, from which they can reactivate to cause recurrent disease throughout the life of the host. Stress is associated with exacerbation of clinical symptoms and induction of recurrences in humans and animal models. The viruses preferentially replicate and establish latency in different subtypes of sensory neurons, as well as in neurons of the autonomic nervous system that are highly responsive to stress hormones. To determine if stress-related hormones modulate productive and latent HSV-1 and HSV-2 infection within sensory and autonomic neurons, we analyzed viral DNA after treatment of primary adult murine neuronal cultures with the stress hormones epinephrine and corticosterone. Both sensory trigeminal (TG) and sympathetic superior cervical ganglia (SCG) neurons expressed adrenergic receptors and glucocorticoid receptor. In productively infected neuronal cultures, epinephrine treatment significantly increased HSV-1 DNA replication and production of viral progeny in SCG neurons, but no significant differences were found in TG neurons. In contrast, corticosterone significantly decreased HSV-2 DNA replication and production of viral progeny in SCG neurons, but not in TG neurons. In quiescently infected neuronal cultures, epinephrine and corticosterone significantly increased HSV-1 reactivation from sympathetic SCG neurons, but not sensory TG neurons. In contrast, corticosterone increased HSV-2 reactivation from both SCG and TG neurons, but

epinephrine had no effect. Adrenergic or epinephrine-induced reactivation of HSV-1 in SCG neurons involved activation of several adrenergic receptors, the cyclic AMP response element binding protein (CREB), the transcription factor β-catenin, and the c-Jun N-terminal kinase (JNK). Corticosterone-induced reactivation of HSV-1 in SCG neurons required activation of glucocorticoid receptor (GCR) and transcription factors CREB and JNK. In contrast, corticosterone-induced reactivation of HSV-2 in TG and SCG neurons could utilize either the GCR or mineralocorticoid receptor (MCR) and most likely involves the chromatin remodeling properties of those receptors. Thus, stress-related hormones, epinephrine and corticosterone, selectively modulate productive and quiescent HSV-1 and HSV-2 infections primarily in sympathetic, but not sensory, neurons through different mechanisms. These results have implications for describing a mechanism by which stress-induced reactivation may occur in humans.

# Stress hormones epinephrine and corticosterone modulate herpes simplex virus 1 and 2 productive infection and reactivation primarily in sympathetic, not sensory, neurons

Angela Marie Ives

**ABSTRACT (public)**

Herpes simplex virus type 1 and 2 (HSV-1 and HSV-2) are major human pathogens, which establish latency in neurons of the peripheral nervous system and reactivate to cause recurrent disease in humans. Physiological stress, which includes the secretion of the stress hormones epinephrine and cortisol, has been associated with increases in severity of clinical signs and increased recurrent disease in humans and animal models of herpetic disease. The mechanism by which physiological stress induces HSV reactivation has been assumed to be through suppression of the immune system. In addition, it has been assumed that sensory neurons harboring latent HSV are the primary source of reactivating virus for recurrent HSV disease. However, my dissertation provides evidence that the stress hormones epinephrine and corticosterone (the rodent equivalent of cortisol) can act on peripheral neurons in which the virus is latent, rather than through immune system suppression. In addition, my dissertation provides evidence that the autonomic nervous system, which modulates the physiological stress response, is an important source of reactivating virus to cause recurrent disease. The molecular pathway by which epinephrine and corticosterone induce HSV reactivation in primary adult murine neurons involves specific receptors, transcription factors, and protein kinases that could potentially be targeted in humans for inhibition of HSV reactivation and prevention of herpetic recurrent disease.

## DEDICATION

This dissertation is dedicated to my father, Robert Ives, and my brother, Ethan, for their love and support.

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Rebecca Powell-Doherty, Ph.D., MPH (Department of Population Health Sciences) was a Post-doctoral student in the Bertke Lab and is a co-author on these manuscripts. She helped with interpreting and analyzing data, editing of the manuscripts, and overall mentorship for these projects.

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# Chapter 1: Introduction

Herpes simplex virus 1 (HSV-1) and 2 (HSV-2) infect mucosal cells and establish a life-long latent infection in the sensory and autonomic neurons innervating the site of entry, leading to recurrent human disease throughout the life of the host. Approximately 70% of Americans are seropositive for HSV-1 [1], while approximately 20% of Americans are seropositive for HSV-2 [2]. HSV-1 most often causes disease "above the waist," such as herpes simplex keratitis and orolabial lesions (cold sores), and is responsible for 30% of new genital herpes cases [3]. HSV-2 most often causes disease "below the waist," and is the major cause of genital herpes. HSV-1 is the leading preventable cause of blindness worldwide and more rarely can cause viral encephalitis, while HSV-2 can cause recurrent sacral meningitis. Even when infection occurs in the same anatomical site, HSV-2 is more likely to reactivate to cause recurrent disease, compared to HSV-1. Mechanisms regulating differences in anatomical preferences for disease and recurrence frequencies between HSV-1 and HSV-2 are not clear. Vaccine development has thus far been unsuccessful, and a vaccine would not help those already latently infected. The current treatment acyclovir or its derivatives, while successful in preventing most cases of human clinical disease, does not prevent asymptomatic reactivation and viral shedding [4], and thus HSV can still be transmitted. Therefore, it is vital to understand how HSV reactivates and which neurons it is reactivating from in order to prevent recurrent human disease, and thus halt transmission of these viruses.

## HSV PRODUCTIVE AND LATENT INFECTIONS

HSV-1 and HSV-2 first enter mucosal cells of either the face or genital region, where they undergo a productive infection and produce infectious virions (Figure 1). Then, they enter the

nerve terminals of sensory or autonomic neurons, undergo retrograde transport to the cell body, and can either undergo a productive infection or shut down and establish latency. Upon reactivation, in which an environmental stimulus such as stress induces the virus to resume a productive infection from a latent state, HSV can either transport back to the mucosal cells of the face or genital region to cause recurring herpes keratitis, fever blisters, or genital lesions, or it can travel towards the brain or spinal cord to cause encephalitis (in the case of HSV-1) or meningitis (in the case of HSV-2).

During ocular infection, HSV can infect sensory neurons of the trigeminal ganglia (TG), postganglionic sympathetic neurons of the superior cervical ganglia (SCG), and postganglionic parasympathetic neurons of the ciliary ganglia (CG) (Figure 2). During genital infection, HSV can infect sensory neurons of the lumbar and sacral dorsal root ganglia (DRG), and postganglionic sympathetic and parasympathetic neurons of the major pelvic ganglia (MPG). Sensory neurons in the TG and DRG are believed to be the primary sites of reactivating HSV-1 and HSV-2, respectively, causing recurrent herpetic disease in humans.

Productive infection in epithelial or mucosal cells is characterized by a temporal cascade of gene expression (Figure 3). Upon entry into a cell, virus protein 16 (VP16), which is carried within the tegument of the virion particle, binds to the host proteins host cell factor 1 (HCF1) and Oct1 to form the VP16-initiation complex. This complex binds to the TAATGARAT sequence in five immediate early gene promoters, infected cell protein 4 (ICP4), 0 (ICP0), 27 (ICP27), 22 (ICP22) and 47 (ICP47). These immediate early genes are transcribed and translated, and in turn stimulate viral DNA replication and expression of proteins required for the building, release, and function of infectious virions. This temporal cascade of gene expression was defined in epithelial cell culture and the pattern of gene expression is not well-characterized in neurons.

In contrast, herpes simplex virus latency is defined as the presence of viral DNA in the nucleus of a cell in the absence of viral DNA replication or production of infectious virus particles. However, the HSV genome is not completely silent during latency. Though low levels of immediate early gene expression have been shown to occur during latency [5], the genes most highly expressed are a family of long non-coding RNAs collectively named the latency-associated transcripts (LATs) [6-8]. However, these LATs are not transcribed in every neuron in which the virus is latent [9, 10]; therefore, the function that the LAT region plays in latency and reactivation of HSV is still not well understood.

Latent HSV has the ability to reactivate or resume a productive infection. Stress, UV light exposure, heat, and chromatin modulators have been demonstrated to reactivate HSV in either humans, animal models, or *in vitro* [11-17]. During reactivation triggered by the phosphatidylinositol-3-kinase (PI3-K) inhibitor LY294002 in neonatal SCG neurons, two phases of viral gene expression occur [18]. First, there is a disordered, transient expression of viral lytic genes, including ICP0, ICP4, ICP27 and VP16 [18], which occurs without the removal of repressive chromatin structures [19]. This initial phase of reactivation lasts approximately 15 to 20 hours post-reactivation (hpr), while the second phase of reactivation occurs between 25 to 30 hpr as viral DNA is replicated and viral proteins are synthesized. This second phase resembles the temporal cascade of gene expression seen during productive infection. VP16, though not required for the initial phase of reactivation, is required for the second phase [18]. However, gene expression following reactivation has not been characterized in adult neurons.

Reactivation of HSV is critical to the virus' ability to cause recurrent disease in humans. Although the mechanism underlying HSV reactivation has not been fully described, the neuron in which the virus is latent does play a significant role as to its ability to reactivate [20, 21].

## STRESS AND HSV DISEASE

Physiological stress, which is characterized by the activation of the sympathetic nervous system and secretion of the stress hormones epinephrine and cortisol, is strongly correlated with exacerbation of HSV acute and recurrent clinical disease in humans [22-25]. The mechanism by which physiological stress induces HSV reactivation has been assumed to be through suppression of the immune system [15, 24, 26, 27]. However, there is some evidence that stress hormones could induce HSV reactivation directly, by acting on the neuron in which the virus is latent [28-36].

### *Epinephrine*

Epinephrine is a catecholaminergic hormone secreted by the adrenal medulla in response to the activation of the sympathetic nervous system and contributes to the "short-term" fight-or-flight response. Epinephrine iontophoresis has been used to induce HSV-1 reactivation in rabbits [14], mice [37] and non-human primates [38] in the ocular model of infection. Adrenergic reactivation in rabbits is significantly reduced if the latency associated transcript (LAT) is deleted or mutated [39]. A cAMP response element in the LAT promoter and a 348 base pair sequence within LAT exon 1 were identified as essential for adrenergic reactivation of HSV-1 [34, 40], but follow-up studies were unable to further map any specific binding sites within exon 1 [30].

Epinephrine binds to five different G-protein coupled receptors, collectively termed the adrenergic receptors, which include two alpha adrenergic receptor subtypes (alpha-1 and alpha-2) and three beta adrenergic receptor subtypes (beta-1, beta-2, and beta-3). Adrenergic receptors are expressed on sensory and autonomic neurons that support HSV productive infection [28, 41]; therefore, epinephrine may mediate its effects on HSV-1 reactivation in animal models through one of the adrenergic receptors. Blockage of the beta-adrenergic receptors decreased HSV-1

reactivation after hyperthermic stress or iontophoresis of epinephrine in the rabbit ocular model [42, 43], supporting the hypothesis that adrenergic receptors may be important in viral reactivation. Blockage of the alpha-adrenergic receptors has also been shown to block epinephrine-induced HSV-1 reactivation in the ocular model of infection in mice and rabbits [44]. However, it is unknown whether adrenergic receptors expressed on neurons in which HSV is latent, or adrenergic receptors expressed on immune cells, are responsible for epinephrine-induced reactivation in these animal models.

***Cortisol***

Cortisol is a glucocorticoid hormone secreted by the adrenal cortex that induces a persistent or "long-term" stress response and regulates metabolism and modulation of the immune system. Hyperthermic stress, which increases plasma concentrations of corticosterone, the rodent equivalent of cortisol in humans, resulted in HSV-1 reactivation in mice [15]. Administration of a glucocorticoid synthesis inhibitor blocked hyperthermic stress-induced reactivation [15]. Cold-restraint stress, which also increases plasma concentrations of corticosterone, induced HSV-1 reactivation in rats, as measured by infectious virus recovered from dorsal root ganglia [45].

Cortisol binds to the glucocorticoid (GCR) and mineralocorticoid receptors (MCR) to mediate its effects on metabolism and the immune system. GCR is expressed in sensory and autonomic neurons that support HSV productive infection [28, 46]. Treatment with dexamethasone, a GCR agonist, has also increased the mortality of mice acutely infected with HSV-1 [47]. Dexamethasone treatment of two different cell lines – immortalized neuronal PC12 cells and human gingival fibroblasts – increased HSV-1 DNA replication and infectious virus yield during productive infection [48, 49].

Dexamethasone can also induce HSV-1 keratitis in humans who have been previously infected with HSV-1 [50]. In combination with cyclophosphamide, an immunosuppressant, dexamethasone induced reactivation of HSV-1 in the rabbit [51, 52] and murine models of ocular infection [53]. Dexamethasone has also been shown to stimulate expression of a reporter gene from the HSV-1 ICP0 promoter by activating several transcription factors [29]. Dexamethasone also consistently induces reactivation of bovine herpesvirus 1 (BHV-1) in calves [54].

Though there is strong evidence that cortisol can affect HSV productive infection and reactivation through the glucocorticoid receptor, it is unclear whether its effects are mediated by its role as an immune system modulator or by acting directly on neurons infected with HSV.

## HSV AND DIFFERENT TYPES OF NEURONS

According to the current paradigm of HSV latency and reactivation, sensory neurons harboring latent HSV are the primary source of reactivating virus for recurrent HSV disease. However, there is ample evidence to suggest that autonomic neurons contribute to HSV pathogenesis and should be further explored [55-57].

### ***Sensory Neurons***

During initial infection, HSV gains entry into the axon terminals of sensory neurons innervating the skin and mucous membranes at the site of infection. Sensory neuron cell bodies reside in distinct structures (ganglia) near the brain and spinal cord, extending long axons to the periphery. The ganglia contain a heterogeneous population of sensory neurons, which can be defined by distinct surface molecules, gene and receptor expression profiles, and responses to endogenous and exogenous factors. Upon entry, HSV can replicate in some types of sensory

neurons while in others, viral replication is inhibited. The types of neurons that support lytic infection or that have the ability to inhibit viral replication differs for HSV-1 and HSV-2.

HSV-1 preferentially establishes latency in sensory neurons recognized by the monoclonal antibody Fe-A5 (A5+), while HSV-2 prefers neurons bound by the isolectin B4 (IB4+) [58, 59]. The sensory A5+ and IB4+ neurons are non-permissive for productive infection of HSV-1 and HSV-2, respectively, in murine adult neuronal cultures [20, 21]. Thus, this leads to a preferential establishment of latency in neurons that do not support productive infection of the virus. In addition, the ratio of A5+ and IB4+ neurons does not differ between the sensory trigeminal ganglia (TG) and the sensory dorsal root ganglia (DRG) [20]. Therefore, differences in anatomical site-specific reactivation of these viruses are not explained by preferential establishment of latency in different types of sensory neurons.

***Autonomic Neurons***

During initial infection, HSV gains entry into the axon terminals of post-ganglionic sympathetic and parasympathetic neurons innervating the face and genital regions. Autonomic neuron cell bodies reside in ganglia where they synapse with preganglionic nerve fibers that originate from the spinal cord or the brain.

HSV-1 and HSV-2 also establish latency in different types of autonomic neurons [60-65]. The autonomic nervous system is divided into three branches: sympathetic, which activates the fight-or-flight stress response; parasympathetic, which inhibits the stress response and promotes "feed and breed" activities; and the enteric nervous system, which regulates digestion.

Characterization of viral gene expression during reactivation following PI3-K treatment has been performed in neonatal sympathetic neurons from the superior cervical ganglia (SCG) [18, 19, 66]. However, adult neurons express different patterns of gene transcription, relative to

immature neurons, are responsive to different types of stimuli, and are less dependent on neurotrophic factor support [67-69]. In the guinea pig ocular model, HSV-1 reactivates from parasympathetic ciliary ganglia to cause recurrent clinical disease independently of sensory trigeminal ganglia (TG) [55]. HSV-1 also reactivates from primary adult murine cultured sympathetic superior cervical ganglia (SCG) at a higher rate, as measured by viral DNA levels, compared to HSV-2 [70]. In addition, a higher percentage of sympathetic SCG neurons express stress hormone receptors compared to sensory TG neurons [28], and are therefore more sensitive to stress factors such as epinephrine and cortisol.

## Hypothesis and Specific Aims

Suppression of the immune system is thought to be the mechanism by which these stress hormones affect HSV reactivation [15, 24, 26, 27]. However, sensory and autonomic neurons that support HSV productive and latent infections selectively express different patterns of the receptors for stress hormones epinephrine and cortisol [28, 41, 46]. Therefore, epinephrine and cortisol may contribute to the ability of the viruses to replicate efficiently in specific types of neurons, which may impact disease severity and the ability to reactivate later to cause recurrent lesions [29-36].

**Hypothesis:** Stress hormones epinephrine and corticosterone differentially regulate HSV1 and HSV2 replication and reactivation through specific endocrine receptors expressed in autonomic and sensory neurons.

**Specific Aim 1.** Determine if epinephrine and corticosterone selectively modulate HSV-1 and HSV-2 productive infection in adult sensory and autonomic neurons. Previous studies have shown that epinephrine (EPI) and corticosterone (CORT) affect productive infection of HSV-1 and HSV-2 [48, 71]. It is also important to consider the effects of stress hormones on HSV primary

infection in neurons, since stress during primary infection could potentially affect HSV-related clinical disease in humans.

**Specific Aim 2.** Determine if epinephrine and corticosterone selectively induce reactivation of HSV-1 and HSV-2 in different types of neurons. Previous studies have shown that epinephrine (EPI) and corticosterone (CORT) can induce reactivation of HSV-1 in animal models of infection, as well as in humans [14, 15, 26, 37, 38, 45, 50]. In humans, stress is strongly correlated with the appearance of fever blisters, which are recurrent lesions caused by reactivating HSV-1, as well as anecdotally correlated with recurrences of genital herpes. The neurons in which HSV establishes latency express receptors for EPI and CORT, suggesting that these endocrine factors may be capable of reactivating latent HSV in the neurons directly, rather than indirectly through suppressive effects on the immune system.

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## FIGURES

**Figure 1. HSV infection of mucosal cells and sensory neurons.** HSV first enters the mucosal cells at the point of entry (grey and yellow circles on the right), where it undergoes a productive infection. Next, the virion undergoes retrograde axonal transport up the axon of the sensory neuron to the nucleus. Depending on the type of sensory neuron, HSV can undergo a productive infection or becomes latent (shown in the figure). During reactivation, an environmental stimulus (represented by the yellow lightning bolt) results in the resumption of a productive infection. HSV can then transport anterograde back to the mucosal cells innervated by the sensory neuron, leading to recurrent herpetic lesions. The virus can also transport anterograde through the central axonal branch of the bifurcated axon towards the central nervous system, which can result in HSV-1 encephalitis or and HSV-2 sacral meningitis. (By permission, Andrea S. Bertke © 2007).

**Figure 1.**

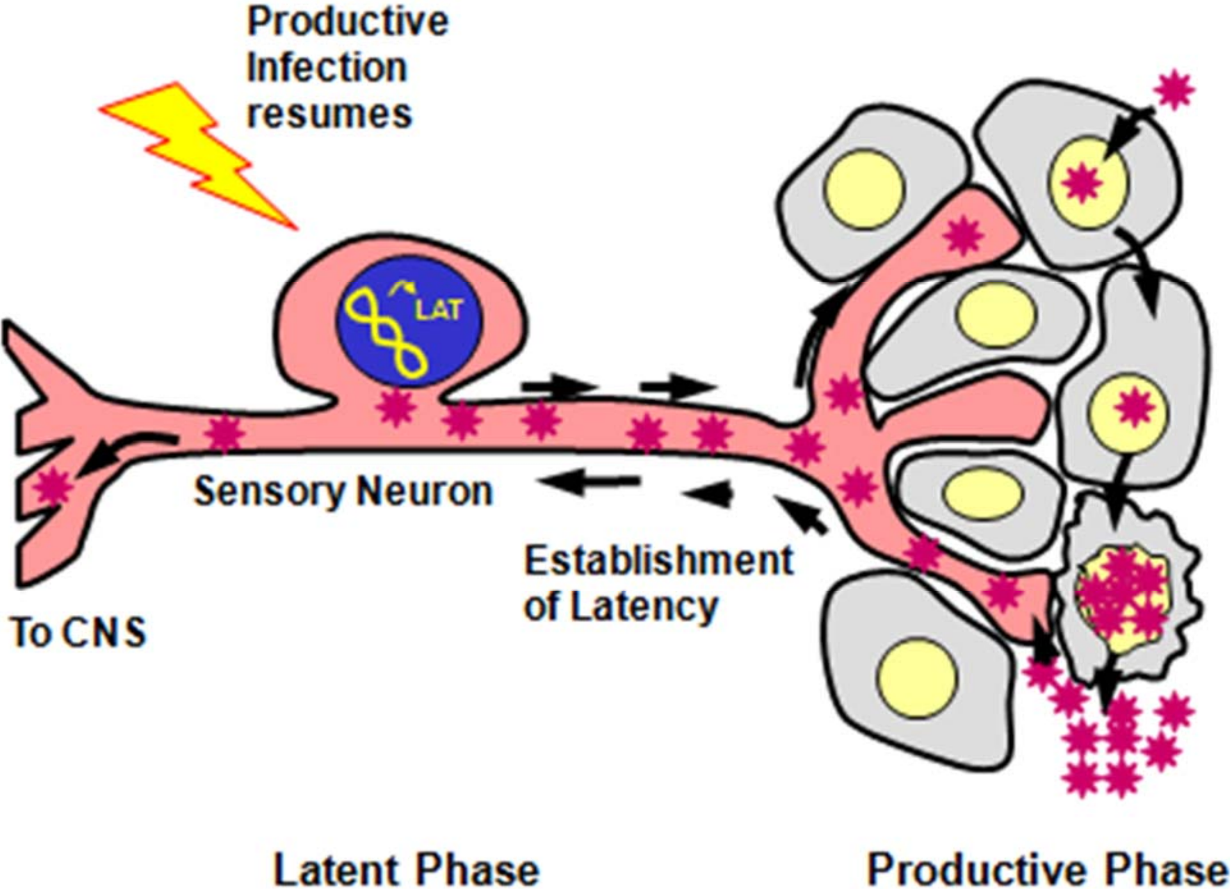


**Figure 2. Approximate location of ganglia of interest for HSV infection in an adult mouse.** During ocular infection, HSV undergoes productive infection in the corneal cells. From there, the virus can enter the sensory trigeminal ganglia, the postganglionic sympathetic neurons of the superior cervical ganglia, and the postganglionic parasympathetic neurons of the ciliary ganglia. During genital infection, HSV undergoes productive infection in the mucosal cells of the vaginal tract in female mice. From there, it can enter the sensory neurons of the lumbar and sacral dorsal root ganglia or the postganglionic sympathetic and parasympathetic neurons of the major pelvic ganglia. For testing my specific aims, I concentrated my studies on the ganglia innervating the eye, since the autonomic ganglia are separated into distinct ganglia in this anatomical region.

**Figure 2.**

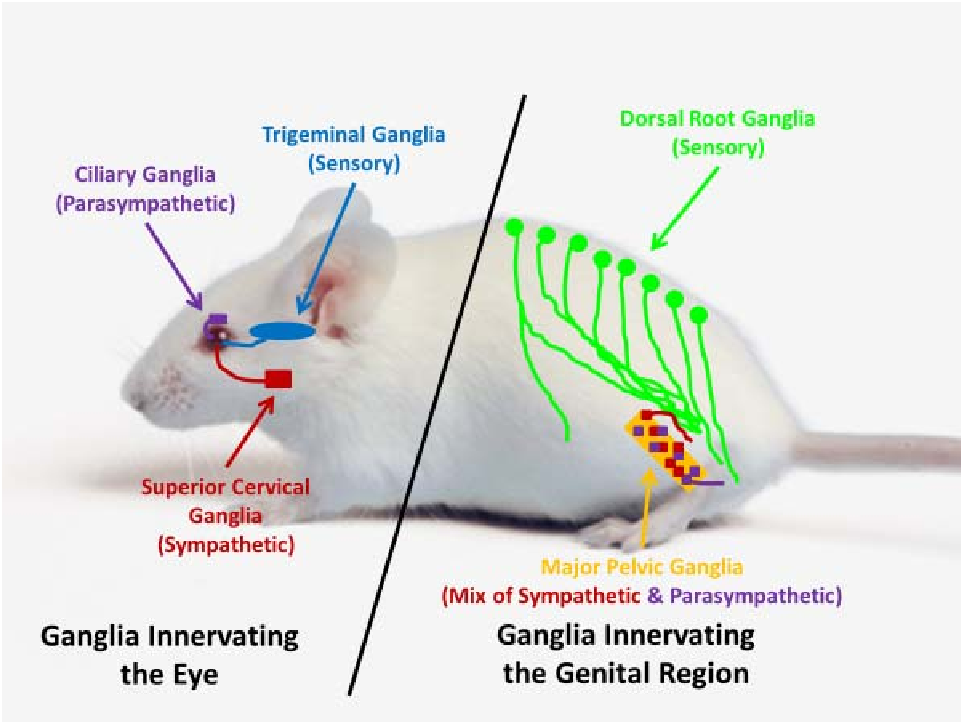
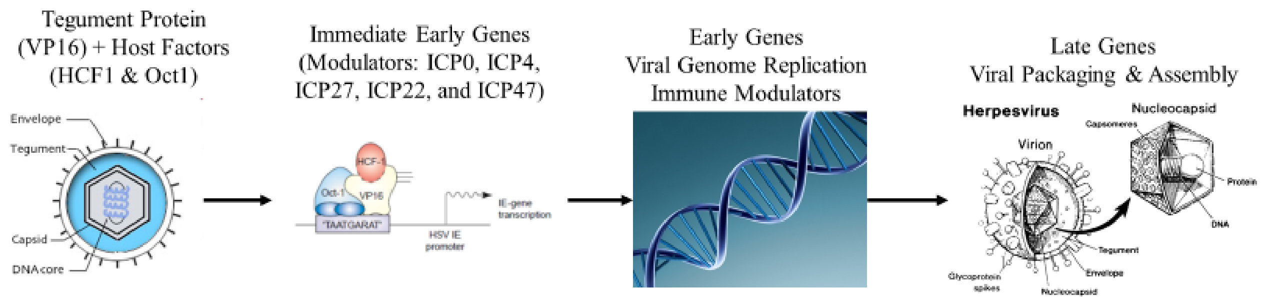


**Figure 3. HSV temporal cascade of gene expression during productive infection.** Upon entering the host epithelial or mucosal cell, the protein VP16 is released from the viral tegument (blue space in the first picture) and binds to two host cell proteins, HCF1 and Oct1. This forms the VP16-initiation complex, which binds to viral immediate early gene promoters, including ICP0, ICP4, ICP27, ICP22, and ICP47. These genes are transcribed, translated in the host cell cytoplasm, and then are transported back into the host cell nucleus, where they stimulate promoters of early genes, which initiate viral DNA replication, and late genes, which are responsible for packaging and assembly.

(Adapted from http://stdgen.northwestern.edu/stdgen/bacteria/hhv2/herpes.html; Wysocka et al., 2003; https://dnatesting.com/wp-content/uploads/2012/09/9-11-2012_IDG-Blog.jpg; and openi.nlm.nih.gov)

**Figure 3.**



# Chapter 2: Stress Hormones Epinephrine and Corticosterone Selectively Modulate HSV-1 and HSV-2 Productive Infection in Adult Sympathetic, but not Sensory, Neurons

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**ABSTRACT**

Herpes simplex viruses 1 and 2 (HSV-1 and HSV-2) infect and establish latency in peripheral neurons, from which they can reactivate to cause recurrent disease throughout the life of the host. Stress is associated with exacerbation of clinical symptoms and induction of recurrences in humans and animal models. The viruses preferentially replicate and establish latency in different subtypes of sensory neurons, as well as in neurons of the autonomic nervous system that are highly responsive to stress hormones. To determine if stress-related hormones modulate productive HSV-1 and HSV-2 infection within sensory and autonomic neurons, we analyzed viral DNA and production of viral progeny after treatment of primary adult murine neuronal cultures with the stress hormones epinephrine and corticosterone. Both sensory trigeminal (TG) and sympathetic superior cervical ganglia (SCG) neurons expressed adrenergic receptors (activated by epinephrine) and glucocorticoid receptor (activated by corticosterone). Productive HSV infection co-localized with these receptors in SCG but not in TG neurons. In productively infected neuronal cultures, epinephrine treatment significantly increased HSV-1 DNA replication and production of viral progeny in SCG neurons, but no significant differences were found in TG neurons. In contrast, corticosterone significantly decreased HSV-2 DNA replication and production of viral progeny in SCG neurons, but not in TG neurons. Thus, stress-related hormones, epinephrine and corticosterone, selectively modulate acute HSV-1 and HSV-2 infections in autonomic, but not sensory, neurons.

*Keywords: Herpes simplex virus, HSV-1, HSV-2, epinephrine, corticosterone, stress, reactivation*

# INTRODUCTION

Herpes simplex viruses 1 and 2 (HSV-1 and HSV-2) infect mucosal cells, then establish a life-long latent infection in sensory and autonomic ganglia innervating the site of infection. HSV-1 is more commonly associated with orolabial lesions (cold sores) and ocular disease (herpes simplex keratitis), while HSV-2 causes genital lesions. Both viruses can reactivate from latency to cause recurrent disease, which also tends to occur in different anatomical patterns. Although HSV-1 is diagnosed in approximately 30% of primary genital herpes cases, HSV-2 is more likely to reactivate to cause recurrent genital lesions [1]. More rarely, HSV-1 causes necrotizing encephalitis with a high mortality rate, and HSV-2 can cause recurrent sacral meningitis. Mechanisms that cause these differences in HSV-1 and HSV-2 acute and recurrent disease patterns are not understood.

Stress is strongly correlated with exacerbation of acute HSV disease symptoms and the appearance of recurrent disease in humans and animal models [2-7]. Psychosocial stress has been correlated with oral herpes recurrences in humans [8]. Psychological stress at the time of infection increases HSV-1 viral titers and pathology following intranasal or vaginal infection in mice [7, 9]. Epinephrine, a catecholaminergic hormone secreted by the adrenal medulla to induce the “short-term” fight-or-flight stress response, is regulated by the sympathetic nervous system. Iontophoresis of epinephrine has been used to induce reactivation of HSV-1 in the rabbit ocular model of infection [5], as well as in mice [10] and non-human primates [11], demonstrating the ability of epinephrine to impact HSV-1. Cortisol, a glucocorticoid secreted by the adrenal cortex that induces a “long-term” stress response, binds to the glucocorticoid receptor (GCR), regulating metabolism and suppression of the immune system. Persistent stress has been correlated with reactivation

of both HSV-1 and HSV-2 in humans [3, 4]. Cold-restraint stress in rats and hyperthermic stress in mice were reported to increase plasma concentrations of corticosterone, the rodent equivalent of cortisol in humans, resulting in HSV-1 reactivation [6, 12]. In addition, treatment of the immortalized neuronal PC12 cell line and human gingival fibroblasts with dexamethasone, a GCR agonist, resulted in an increase in HSV-1 DNA replication and infectious virus yield during productive infection [13, 14]. Furthermore, dexamethasone treatment increased mortality of HSV-1 acutely infected mice [15], significantly increased HSV-1 ocular shedding and corneal ulceration in latently infected rabbits [16], consistently induces reactivation of bovine herpesvirus 1 in calves [17], and can induce HSV-1 keratitis in humans [18].. Taken together, the stress hormones epinephrine and corticosterone have an impact on HSV-1 and HSV-2 acute and latent infections.

The mechanism through which these stress factors impact HSV disease severity and recurrences is thought to be through suppression of the immune system, permitting the viruses to escape immune surveillance [2, 7, 12, 19]. However, the receptors for the two major stress hormones, epinephrine and cortisol, are expressed selectively by the different types of neurons infected by HSV, including sensory and autonomic neurons [20, 21]. Therefore, epinephrine and cortisol may contribute to the ability of the viruses to replicate efficiently in specific types of neurons, which may impact disease severity and the ability to reactivate later to cause recurrent lesions [22-29].

HSV-1 and HSV-2 preferentially establish latency in different types of sensory neurons, with HSV-1 preferring sensory neurons recognized by the monoclonal antibody Fe-A5 (A5+), while HSV-2 prefers neurons bound by the isolectin B4 (IB4+) [30, 31]. In murine adult neuronal cultures, A5+ and IB4+ neurons are non-permissive for productive

infection of HSV-1 and HSV-2, respectively, leading to preferential establishment of latency in these neurons [32, 33]. Thus, latency is established in neurons that do not support efficient productive infection. The viruses also establish latency in autonomic neurons [34-39], which are highly responsive to endocrine factors. Therefore, we hypothesized that stress hormones, acting through their cognate receptors, could potentially modulate HSV-1 and HSV-2 productive infection by acting directly on neurons, rather than indirectly through their effects on the immune system.

Although stress, and the stress-related endocrine factors epinephrine and cortisol, have a strong correlation with HSV-1 and HSV-2 reactivation, their effects on primary infection are not clear. Since these viruses infect sensory and autonomic neurons, the latter of which are exquisitely sensitive to endocrine factors, we sought to determine if stress hormones differentially modulate HSV-1 and HSV-2 lytic infections at a cellular level in primary adult sensory and autonomic neurons. Characterization of stress hormone receptor expression and analysis of viral replication in primary adult sensory and autonomic neurons showed that the stress hormones, epinephrine and corticosterone, have differential effects on HSV-1 and HSV-2. Furthermore, these differential effects occurred only in autonomic neurons, rather than sensory neurons.

## MATERIALS AND METHODS

**Virus strains.** HSV-1 strain 17+ was originally transferred from John Hay (SUNY Buffalo, NY) to the Krause lab (FDA, Bethesda, MD). Viruses were propagated in Vero cells (ATCC) and first passage stocks were transferred to the Margolis lab (UCSF, San Francisco, CA). Viruses were propagated in Vero cells and first passage stocks were

transferred to the Bertke lab (Virginia Tech, Blacksburg, VA). Viruses were propagated in Vero cells and titrated in quadruplicate by plaque assay in Vero cells. Stock viruses were diluted in Neurobasal A medium supplemented with penicillin-streptomycin and B27 supplement for inoculation of primary adult murine neuronal cultures.

**Neuronal cultures**. Sensory trigeminal ganglia (TG), sympathetic superior cervical ganglia (SCG), and parasympathetic ciliary ganglia (CG) were removed from 6 week old female Swiss Webster mice, dissociated, and plated on Matrigel-coated Lab-Tek II chamber slides (ThermoScientific) as previously described [32, 40]. Briefly, ganglia were digested in papain, collagenase, and dispase (Worthington), followed by mechanical trituration. TG were enriched for neurons using a multi-step Optiprep gradient (BD Biosciences), while SCG and CG were plated without a gradient step since they contain less ganglionic debris after dissociation. Cells were maintained in Complete Neuro media, consisting of Neurobasal A media supplemented with B27, penicillin-streptomycin, L-glutamine, neurotrophic factors, and mitotic inhibitors (Life Technologies). All studies were approved by and conducted in accordance with the Virginia Tech Institutional Care and Use Committee (IACUC# 13-008-CVM and 15-237).

**Infection and hormone treatment.** Four days after plating, media was removed and neurons were inoculated with 30 multiplicity of infection (moi) of HSV-1 (strain 17+) or HSV-2 (strain 333). After a one hour adsorption period, inoculum was removed and replaced with Complete Neuro media with no mitotic inhibitors, with or without epinephrine or corticosterone-HBC (Sigma) at the concentrations indicated in the figures. 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 alone was used as a control to ensure any effects were due to corticosterone, rather than the HBC carrier molecule.

**Plaque assay.** Twenty-four hours post-infection, neurons and media were collected and stored at -80°C until assayed. The suspension was serially diluted from $10^{-1}$ to $10^{-4}$ and dilutions were inoculated onto 70-80% confluent Vero cells (ATCC). After a one hour adsorption period, inoculum was removed and replaced with Dulbecco's Modified Essential Medium (DMEM) with 2% fetal bovine serum, 1% penicillin-streptomycin, and 4 μL/mL human IgG for 48 hours. Vero cells were fixed and stained with crystal violet and plaques were counted on a light microscope (Olympus).

**Immunofluorescence.** Nine hours post-infection, neuronal cultures were fixed with 2% paraformaldehyde and immunostained. Individual neurons positive for immunofluorescence were counted to determine the percentage of HSV-positive neurons and receptor-positive neurons. Neuronal subpopulations were labeled with the isolectin IB4 conjugated to FITC or rhodamine (Vector, 1:500) and/or the following antibodies: Fe-A5 IgM (DSHB, undiluted supernatant), anti-HSV-FITC (Dako, 1:40, polyclonal reacts with antigens common to HSV-1 and HSV-2, including all major glycoproteins in the viral envelope and at least one core protein as determined by crossed immunoelectrophoresis) [30, 41], anti-HSV (Abcam, 1:750), anti-TH (Abcam, 1:500), anti-GCR (Thermo, 1:250), anti-AR α-2A (Abcam, 1:300), anti-AR α-2B (Sigma Aldrich, 1:300), anti-AR α-2C (Thermo, 1:300), anti-AR β-2 (Abcam, 1:500). Secondary antibodies were AlexaFluor-labeled species-specific antibodies (Life Technologies, 1:1000). Number of dual-labeled neurons (receptor+ and neuronal marker+) were compared to total number of neurons positive for neuronal marker to calculate percentages of neuronal populations expressing

specific hormone receptors (Figure 1 and Table 1). N = the number of cryosectioned murine ganglia or number of primary adult murine cultures, and all marker-positive neurons were counted in each well. In uninfected neurons stained using immunofluorescence, both from *in vitro* primary murine neuronal cultures and *in vivo* cryosectioned murine ganglia, percentages of stress hormone receptor expression were calculated by counting the number of dual-labeled neurons co-expressing both the stress hormone receptor and the neuronal marker divided by all of the neurons expressing the neuronal marker. In infected neurons stained using immunofluorescence *in vitro*, percentages of co-localization of stress hormone receptors with HSV antigen were calculated by counting the number of neurons co-labelled for stress hormone receptor and HSV antigen divided by all of neurons positive for HSV antigen, designated as "AR α-2+/AR β-2+/GCR+ neurons infected with HSV-1 or HSV-2" in Figure 2. Percentages of infected neurons were calculated by counting the number of neurons positive for HSV antigen divided by all of the neurons in the well, designated as "Total HSV-1-infected or HSV-2-infected neurons" in Figure 2. Immunostained, uninfected adult murine neuronal cultures were imaged on an inverted fluorescence Olympus IX71 microscope using CellSens Dimension software at 20X magnification.

**Quantitation of HSV viral load and gene expression**. Viral DNA was extracted from neuronal cultures 10 hours post-infection (hpi) with TRIzol reagent (ThermoScientific), according to the manufacturer's instructions. Viral DNA load was determined by quantifying viral DNA by qPCR using HSV-1 and HSV-2 thymidine kinase (TK) gene-specific primers and probes [42, 43]. All assays were normalized to 18s rRNA (Applied Biosystems) and reported as viral copy number in 200 ng of total DNA.

**Statistics.** Statistics were performed using parametric analyses with JMP Pro version 12, including analysis of variance with contrast tests. Comparisons of percentage expression, viral DNA load, and infectious virus titer were performed using analysis of variance. Error bars represent standard errors of the mean.

## RESULTS

**Stress hormone receptor expression in sensory and sympathetic neurons.** Cell type-specific differences in adrenergic (epinephrine) and glucocorticoid (cortisol) receptor expression have been previously reported [20, 21]. To identify differences in receptor expression by different populations of sensory and autonomic neurons relevant to HSV infection, we determined expression profiles of glucocorticoid receptor (GCR) and adrenergic receptors (AR) in cultured primary adult murine neurons from sensory trigeminal (TG) and sympathetic superior cervical ganglia (SCG) by immunofluorescence.

In sensory TG neurons, a significantly greater percentage of IB4+ neurons (78.6%), which support HSV-1 productive infection, expressed the GCR compared to A5+ sensory neurons (25.3%), which support HSV-2 productive infection ($p < 0.0001$) (Figure 1A). Therefore, IB4+ sensory neurons are likely more responsive to glucocorticoids, including corticosterone (the rodent equivalent of human cortisol), than are A5+ neurons.

Adrenergic receptors (ARs) α-2 and β-2 are expressed by 34.4% and 33.0% of total cultured adult sensory TG neurons (Figure 1A). A higher percentage of A5+ neurons (63.3% and 80.7%) expressed ARs compared to the total population of TG neurons ($p < 0.0001$) (Figure 1A). Similarly, a higher percentage of IB4+ TG neurons (68.1%) also expressed adrenergic α-2 receptors, compared to the total population ($p < 0.0001$).

However, AR β-2 receptors were expressed in fewer IB4+ neurons (22.5%), compared to other TG neurons (33.0%). Therefore, while TG sensory neurons are likely responsive to epinephrine through adrenergic receptors, the neuronal populations in which HSV-1 and HSV-2 preferentially replicate express different patterns of adrenergic receptors, suggesting possible differences in responsiveness to epinephrine.

Nearly half (41.5%) of cultured sympathetic SCG neurons expressed the GCR (Figure 1B). In contrast, 83.3% and 72.6% of SCG neurons expressed ARs α-2 and β-2 ($p = 0.0001$) (Figure 1B). Therefore, sympathetic SCG neurons are capable of being stimulated by both epinephrine and glucocorticoids.

Immunofluorescence detection of the sensory neuronal markers A5 and IB4, as well as the sympathetic marker tyrosine hydroxylase (TH), produced distinct staining patterns of the neuronal membrane and axons (Figure 1C). Glucocorticoid and adrenergic receptor immunofluorescence was also detectable in distinct staining patterns, with GCR present in the nucleus and AR α-2 and AR β-2 present on the cell surface and perinuclear region (Figure 1C) [21, 44, 45]. These distinct patterns were readily identifiable to determine percentages of neuronal subpopulations that expressed each receptor type.

To validate that the percentage of primary adult cultured neurons expressing adrenergic and glucocorticoid receptors was similar to percentages expressed *in vivo*, ganglia from mice were cryosectioned and immunostained for co-localization of stress hormone receptors and neuronal markers. There were no significant differences in the percentages of each neuronal subpopulation that expressed GCR and ARs between the cultured neurons and the sectioned ganglia (Table 1). We detected no expression of stress

hormone receptors in satellite glial cells present in immunostained sensory TG or sympathetic SCG primary adult murine cultures (Figure 1C).

**Neuron specificity of HSV productive infection.** To determine if HSV-1 or HSV-2 demonstrate a preference for productive infection in neurons expressing specific receptors, we co-immunostained infected TG and SCG adult neuronal cultures for HSV antigen and receptors AR α-2, AR β-2, and GCR (Figure 5).

In sensory TG neuronal cultures, neurons expressing AR α-2 restricted productive HSV-1 compared to the total TG neurons ($p = 0.0172$) (Figure 2A). In contrast, sympathetic SCG neurons that expressed AR α-2 selectively supported productive infection of HSV-1 and HSV-2, compared to other SCG neurons ($p < 0.0001$) (Figure 2A).

Similarly, AR β-2+ sensory TG neurons restricted HSV-1 ($p = 0.0434$) and HSV-2 ($p = 0.0074$) productive infection, compared to other TG neurons (Figure 2B). However, AR β-2+ SCG neurons selectively supported HSV-1 productive infection ($p = 0.042$ compared to total neurons infected with HSV-1), but not HSV-2 productive infection (Figure 2B).

In sensory TG neurons, there were no significant differences between GCR+ neurons and total neurons infected with HSV-1 or HSV-2 (Figure 2C), demonstrating that GCR+ sensory neurons support productive infection of HSV-1 and HSV-2 as well as sensory neurons that do not express the GCR. In SCG, however, HSV-1 and HSV-2 antigens were detected in a greater percentage of GCR+ neurons compared to total SCG neurons ($p < 0.0001$ and 0.0012, respectively).

**Stress hormones modulate HSV DNA replication during productive infection *in vitro*.** To determine if stress hormones affect HSV-1 and HSV-2 replication during productive infection, epinephrine (EPI) or corticosterone (CORT, conjugated to (2-Hydroxypropyl)-β-cyclodextrin in the water-soluble form) were added to productively infected neuronal cultures one hour post inoculation (hpi). The water-soluble carrier molecule (2-Hydroxypropyl)-β-cyclodextrin (HBC) was also used as a control. Viral DNA was isolated 10 hpi, representing a single cycle of viral replication, and quantified by qPCR for the thymidine kinase (TK) gene.

HSV-1 DNA was significantly increased in sympathetic SCG neurons treated with 0.01, 0.1, and 10 μM EPI compared to untreated (UNT) control SCG neurons ($p < 0.004$) (Figure 3A). In contrast, there were no significant differences in HSV-1-infected sensory TG or parasympathetic CG neurons. No significant differences were detected after EPI treatment of sensory or autonomic neuronal cultures infected with HSV-2 (Figure 3B). Therefore, epinephrine increases HSV-1 DNA replication during productive infection in sympathetic SCG neurons in a non-monotonic dose-dependent manner but has no significant effect on HSV-2.

In contrast, HSV-1 viral DNA was significantly decreased in sympathetic SCG neurons treated with 10 μM CORT ($p = 0.02$), showing a linear dose response from 0.1 μM to 10 μM CORT treatments (Figure 3C). HSV-2 viral replication was also significantly decreased in sympathetic SCG neurons treated with CORT, regardless of the concentration from 0.01 to 10 μM, also reflecting a linear dose response from CORT treatment ($p < 0.0001$), although HSV-2 appears to be more sensitive to CORT treatment than HSV-1. No significant effect of CORT treatment was detected for either HSV-1 or HSV-2 DNA

replication in sensory TG or parasympathetic CG neurons (Figure 3C and D). There were also no significant effects of the HBC carrier molecule on HSV-1 or HSV-2 DNA replication in the sensory or autonomic neurons (Figure 3C and D).

Furthermore, both HSV-1 and HSV-2 replicated more efficiently in sympathetic SCG neurons, compared to sensory TG neurons, shown by the significantly greater viral DNA loads in infected untreated SCG neurons compared to infected untreated TG neurons ($p < 0.0001$) (Figure 3).

**Stress hormones affect HSV infectious virus titers *in vitro*.** Since EPI increased HSV-1 viral DNA in sympathetic SCG neurons, we next determined if EPI also increased the production of viral progeny. Using the same four concentrations of EPI treatment, neurons and media were collected 24 hpi and analyzed for viral titers by plaque assay on Vero cells. The quantity of HSV-1 infectious virus progeny was significantly increased in sympathetic SCG neurons treated with 0.01 μM, 0.1 μM, and 10 μM EPI ($p < 0.02$) (Figure 4A), correlating with the increased viral DNA in response to the same EPI treatments in a similar non-monotonic dose response (Figure 3A). Parasympathetic CG neurons also produced significantly higher HSV-1 titers in untreated neurons compared to sensory TG neurons ($p = 0.014$), showing that autonomic neurons support more efficient production of HSV-1 infectious virus progeny compared to sensory neurons.

Since CORT decreased HSV-2 viral DNA in sympathetic neurons, we determined if CORT also decreased the production of viral progeny. CORT or its water-soluble carrier molecule, HBC, were added to HSV-2-infected neuronal cultures one hpi and infectious viral progeny were assessed by plaque assay on Vero cells. CORT significantly decreased HSV-2

infectious virus titers in sympathetic SCG neurons compared to untreated SCG neurons ($p = 0.0115$) (Figure 4B). HBC had no significant effect on viral titer (Figure 4B). There were no significant differences in virus titers between CORT treated and untreated TG or CG neurons. In addition, sympathetic SCG produced significantly greater quantities of HSV-2 viral progeny compared to parasympathetic CG and sensory TG ($p < 0.0001$) (Figure 4B).

## DISCUSSION

Previous studies have shown that epinephrine (EPI) and corticosterone (CORT) can induce reactivation of HSV-1 in animal models of infection, as well as in humans [5-7, 10-12, 18]. In humans, stress is strongly correlated with the appearance of fever blisters, which are recurrent lesions caused by reactivating HSV-1, as well as anecdotally correlated with recurrences of genital herpes. The neurons in which HSV establishes latency express receptors for EPI and CORT, suggesting that these endocrine factors may be capable of modulating HSV infection in the neurons directly, rather than indirectly through suppressive effects on the immune system. It is also important to consider the effects of stress hormones on HSV primary infection in neurons, since stress during primary infection could potentially affect HSV-related clinical disease in humans.

In our studies, in which we tested the effect of stress hormones on productive infection rather than reactivation from latency, EPI increased HSV-1 replication and production of infectious virus progeny. However, the effects occurred in sympathetic neurons, not sensory neurons. Although sensory neurons express adrenergic receptors similar to sympathetic neurons, HSV-1 productive infection was inhibited in AR+ sensory neurons, but enhanced in AR+ sympathetic neurons. While HSV-2 showed a preference

for productive infection in AR α-2+ sympathetic neurons, EPI had no impact on HSV-2 productive infection. Thus, the mechanism by which EPI modulates productive infection is exclusive to HSV-1 and only occurs in sympathetic neurons.

EPI also increased HSV-1 replication and release of infectious virus progeny during productive infection at two different concentrations. While non-monotonic dose response curves are relatively common for endocrine factors, specific mechanisms remain elusive [46]. It is possible that this inverted bell-curve is due to EPI's property as a nonselective agonist for the various classes of adrenergic receptors. Sympathetic neurons express two different subtypes of adrenergic receptors that differ in their intrinsic receptor properties [47, 48]. AR α-2 receptors inhibit forskolin-mediated cyclic AMP (cAMP) accumulation at norepinephrine concentrations of 100 pM to 100 nM but potentiate cAMP accumulation at concentrations of 1 μM and above [47]. This is due to the ability of AR α-2 receptors to recruit different G-proteins [49]. In contrast, the binding of EPI to the β-2 adrenergic receptor activates a stimulatory G-protein, which stimulates adenylate cyclase and causes an increase in cAMP in sympathetic neurons of the superior cervical ganglia (SCG) [50]. An increase in cAMP production has been shown to reactivate HSV-1 from quiescence in embryonic SCG neurons [51]. The adenylate cyclase enzymes are optimally stimulated by AR β-2 treated with 100 nM epinephrine [52]. Therefore, the increase in HSV-1 DNA replication at 0.01 μM and 0.1 μM of epinephrine most likely represents stimulation of AR β-2 receptors, while the increase in HSV-1 DNA replication at 10 μM epinephrine is a result of stimulation of AR α-2 receptors. The lack of response at 1 μM epinephrine is most likely due to inhibition of adenylate cyclase by AR α-2 receptors. Furthermore, pre-treatment with cAMP results in an increase in ICP4 and ICP0 expression from an

immortalized neuronal cell line [53]. Therefore, it is possible that epinephrine is acting through a similar mechanism to increase HSV-1 replication and release of infectious virus progeny from sympathetic neurons during productive infection. Although further studies are necessary to determine the specific mechanism, it is clear that sympathetic neurons are significantly more responsive to EPI-induced enhancement of productive HSV-1 infection than are sensory neurons.

Corticosterone treatment had minimal effects on HSV-1 productive infection in primary adult neurons, regardless of neuron type. We observed a decreasing trend in HSV-1 viral DNA at the highest concentrations of CORT, suggesting a potential effect at concentrations beyond biologically relevant range, even under stress [54]. However, CORT decreased HSV-2 DNA replication and release of infectious virus progeny. Again, these effects occurred in sympathetic neurons, rather than sensory neurons, further implicating the autonomic nervous system in HSV pathogenesis. Previous research has shown that the origin of replication in the unique long region of the HSV-1 genome (oriL) contains a glucocorticoid response element that can bind GCR; the GCR agonist dexamethasone (DEX) was able to increase oriL-dependent HSV-1 DNA replication in rat pheochromocytoma (PC12) cells [13]. In addition, pre-treatment with DEX was also reported to increase HSV-1 infectious virus progeny in human gingival fibroblasts [14]. Serum and glucocorticoid-regulated protein kinases (SGK) induced by stress have also been implicated in stimulation of HSV-1 viral replication in Vero cells [54]. We observed no such increases in HSV-1 replication in response to CORT treatment, which may be due to the types of cells utilized, since PC12 cells, human gingival fibroblasts, and Vero cells are not analogous to adult, differentiated neurons [55]. One report found that the effect of

glucocorticoids on HSV-2 productive infection *in vitro* differed depending on the type of cell utilized and the characteristics of the viral strain used [56]; addition of dexamethasone and cortisol increased the infectious virus titer of HSV-2 in 3T3 cells, but decreased plaque size of HSV-2 in embryonic mouse fibroblast cells [56]. Effects of glucocorticoids on HSV-2 in neurons remain largely unexplored. However, our studies show that CORT has a profound impact on HSV-2 viral replication in adult sympathetic neurons, but not in adult sensory neurons, during productive infection.

Because we studied productive HSV infection in primary adult sensory TG and sympathetic SCG neuronal cultures, it is possible that the satellite glial cells present within the cultured ganglia contributed to viral DNA quantities and infectious virus titers. However, there is currently no evidence to suggest that there are significant phenotypic differences between the satellite glial cells in sensory and autonomic ganglia [44, 57, 58]. Satellite glial cells exhibit a similar morphology, function, and pharmacology in both sensory and autonomic ganglia, and there has been no report that satellite glial cells from sensory or autonomic ganglia express adrenergic or glucocorticoid receptors, which correlate with our findings. One report demonstrated uptake of radiographically tagged dexamethasone, but not corticosterone, into satellite glial cells of rat superior cervical ganglia, but did not demonstrate the presence of a receptor, or why there was a difference between uptake of the two glucocorticoids [59]. Therefore, we conclude that while replication of HSV in satellite glial cells may contribute to levels of viral DNA and infectious virus titer during productive infection, the current literature and our findings suggest that the differences found between sensory and sympathetic ganglia are due to differences between the neurons, not the satellite glial cells supporting them.

In summary, the present study demonstrates that stress hormone receptors are differentially expressed on sensory and sympathetic neurons relevant to HSV-1 and HSV-2 infection. The stress hormones, EPI and CORT, have differential effects on HSV-1 and HSV-2 DNA replication and release of infectious virus progeny in sympathetic neurons, but not in sensory neurons, during productive infection, suggesting that autonomic neurons play a distinctive role in stress-induced modulation of HSV productive infection.

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## FIGURES

**Figure 1. Expression of stress hormone receptors in sensory and sympathetic neurons.** Sensory trigeminal (TG) and sympathetic superior cervical ganglia (SCG) adult murine neuronal cultures immunostained for neuronal markers (Fe-A5, IB4, and tyrosine hydroxylase (TH)) and glucocorticoid (GCR), adrenergic alpha-2 (AR α-2), and adrenergic beta-2 receptors (AR β-2). **A)** Trigeminal ganglia (TG): GCR was differentially expressed in Fe-A5+ ($p=0.0023$) and IB4+ ($p=0.0024$) neurons compared to total neurons ($n>5$); AR α-2 was expressed in a higher percentage of Fe-A5+ ($p<0.0001$) and IB4+ ($p<0.0001$) neurons compared to total neurons ($n>6$); AR β-2 was expressed in a greater percentage of Fe-A5+ neurons compared to IB4+ ($p<0.0001$) and total neurons ($p<0.0001$) ($n>5$). **B)** Superior cervical ganglia (SCG): a greater percentage of neurons expressed AR α-2 ($p<0.0001$) and β-2 ($p<0.0001$) compared to those expressing GCR ($n>3$). (n = number of cultures, counting all neuronal marker positive neurons present) * $p < 0.05$, ** $p < 0.01$, and *** $p<0.001$. **C)** Representative fluorescent microscopy images of TG and SCG co-localization between neuronal markers (A5, IB4, and TH) with stress hormone receptors (GCR, AR α-2, and AR β-2), and phase contrast (PC) to show neuronal morphology.

**Figure 1.**

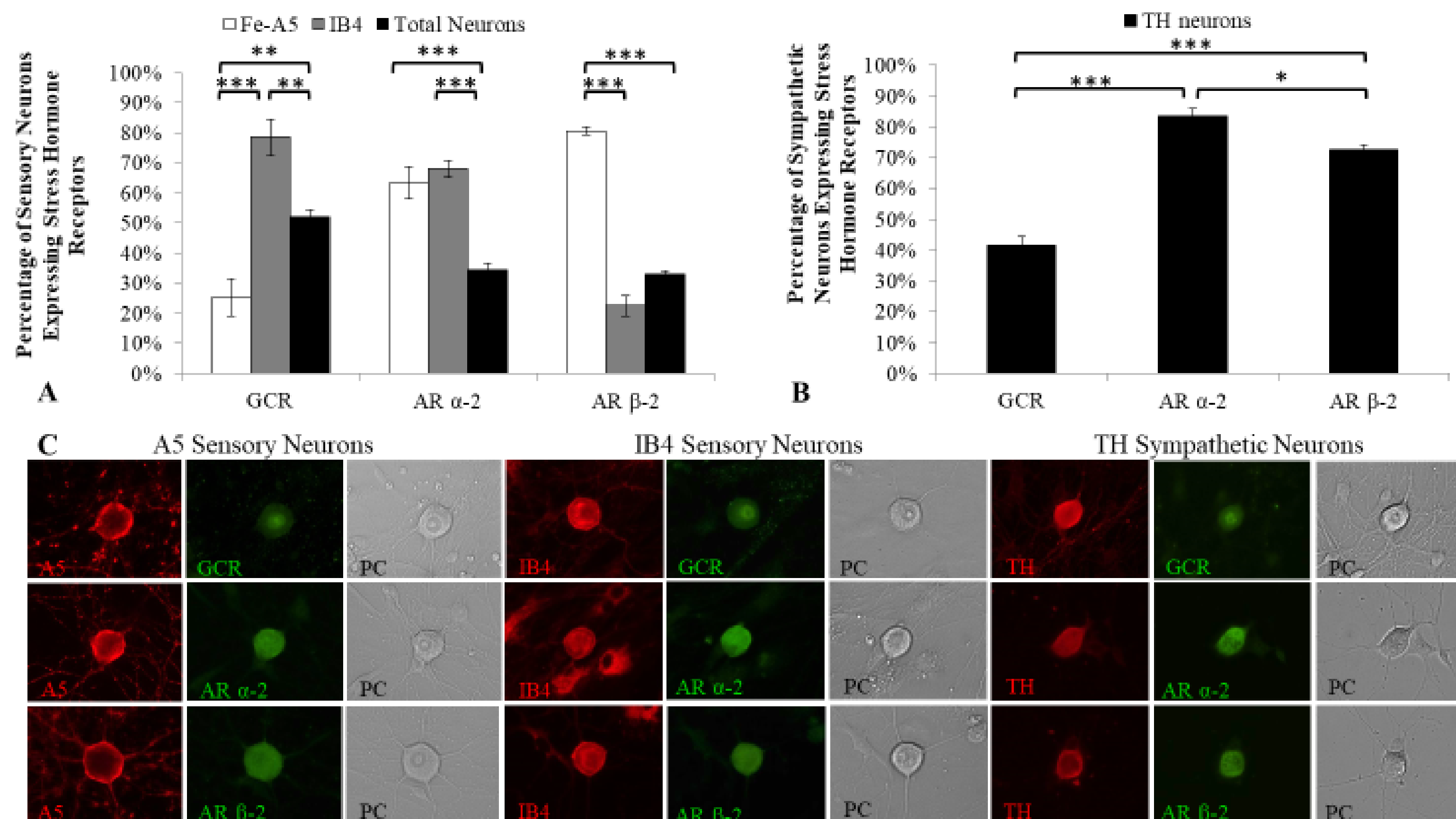


**Figure 2. HSV productive infection in neurons expressing stress hormone receptors.** Sensory trigeminal ganglia neurons (TG) and sympathetic superior cervical ganglia (SCG) adult murine neuronal cultures immunostained 9 hpi for HSV antigen and adrenergic alpha-2 receptors (AR α-2), adrenergic beta-2 receptors (AR β-2), or glucocorticoid receptor (GCR). **A)** AR α-2+ sensory TG neurons restricted HSV-1 productive infection ($p=0.0172$ compared to total infected neurons), and AR α-2+ sympathetic SCG neurons supported HSV-1 ($p<0.0001$) and HSV-2 ($p<0.0001$) productive infection ($n>6$). **B)** AR β-2+ sensory TG neurons restricted HSV-1 ($p=0.0434$) and HSV-2 ($p=0.0074$) productive infection, and AR β-2+ sympathetic SCG neurons supported HSV-1 productive infection ($p=0.0426$) ($n>3$). **C)** GCR+ sympathetic SCG neurons supported HSV-1 ($p<0.0001$) and HSV-2 ($p=0.0012$) productive infection, but there was no difference between GCR+ and GCR- sensory TG neurons ($n>3$). (n = number of cultures, counting >200 neurons/culture) * designates $p < 0.05$, ** $p < 0.01$, and *** $p<0.001$.

**Figure 2.**

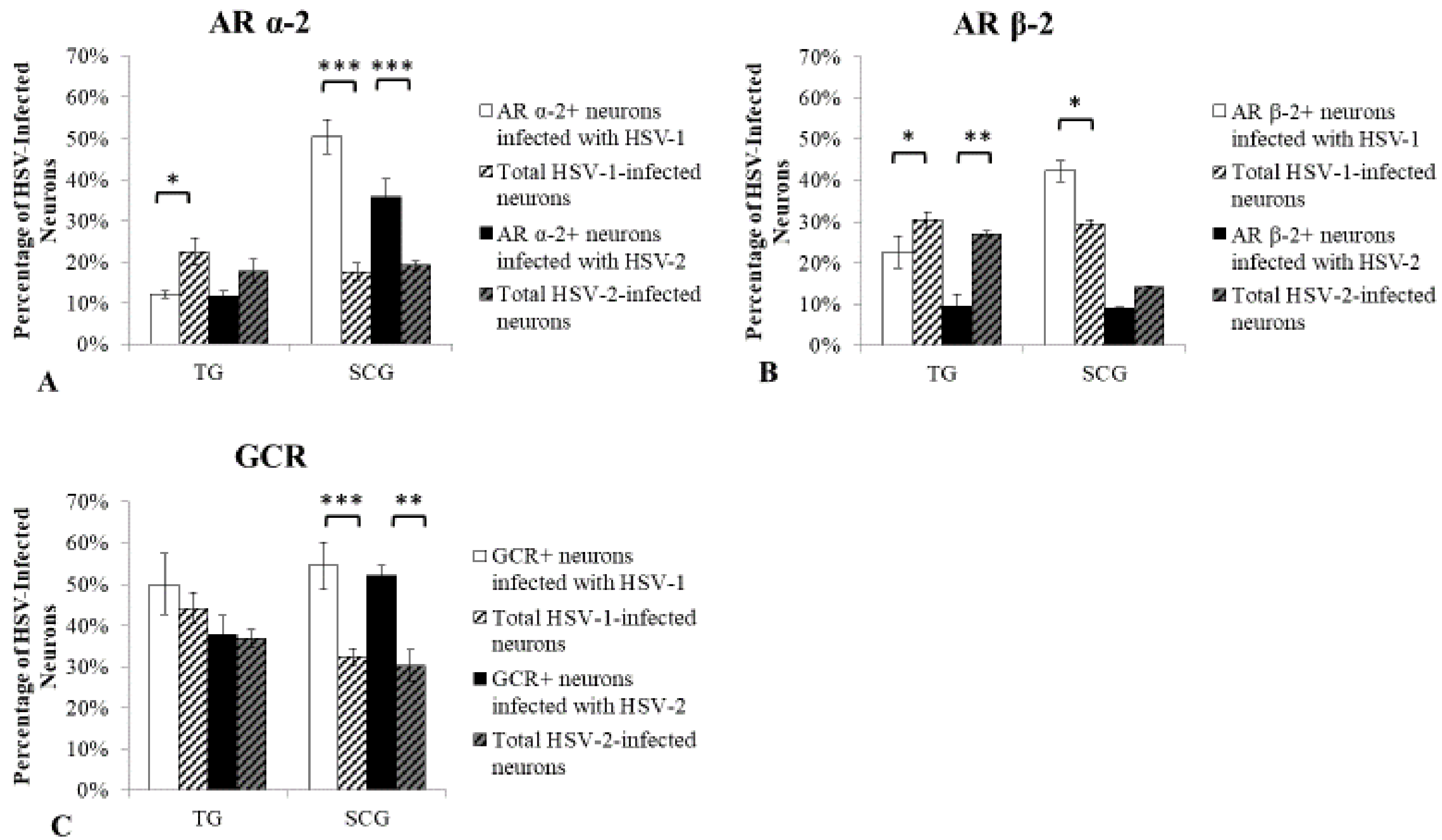


**Figure 3. Effect of EPI and CORT on HSV-1 and HSV-2 replication during productive infection.** Trigeminal (TG), superior cervical (SCG), and ciliary ganglia (CG) primary adult murine neuronal cultures were treated with EPI or CORT during HSV infection (10 hpi), followed by collection of neurons and media for qPCR analysis for viral DNA. **A)** HSV-1 DNA was increased by 0.01 μM ($p<0.001$), 0.1 μM ($p=0.003$), and 10 μM ($p<0.001$) EPI in SCG neurons, but not TG or CG ($n>3$). **B)** HSV-2 DNA was not significantly affected by EPI treatment, regardless of dose ($n>3$). **C)** HSV-1 DNA was decreased by 10 μM CORT ($p=0.02$) in SCG neurons, but not TG or CG ($n>3$), and **D)** HSV-2 DNA was decreased by 0.01 μM ($p=0.006$), 0.1 μM ($p=1.1 \times 10^{-5}$), 1 μM ($p=0.0004$), and 10 μM ($p=0.0001$) CORT in SCG neurons, but not TG or CG ($n>3$). UNT refers to neurons infected with HSV-1 or HSV-2 but not treated with stress hormones. HBC refers to the water-soluble carrier molecule (2-Hydroxypropyl)-β-cyclodextrin that was conjugated to CORT and used as a control. HBC was not significantly different from UNT in C or D ($n>3$). (n = number of cultures) * designates $p < 0.05$, ** $p < 0.01$, and *** $p<0.001$.

Figure 3.

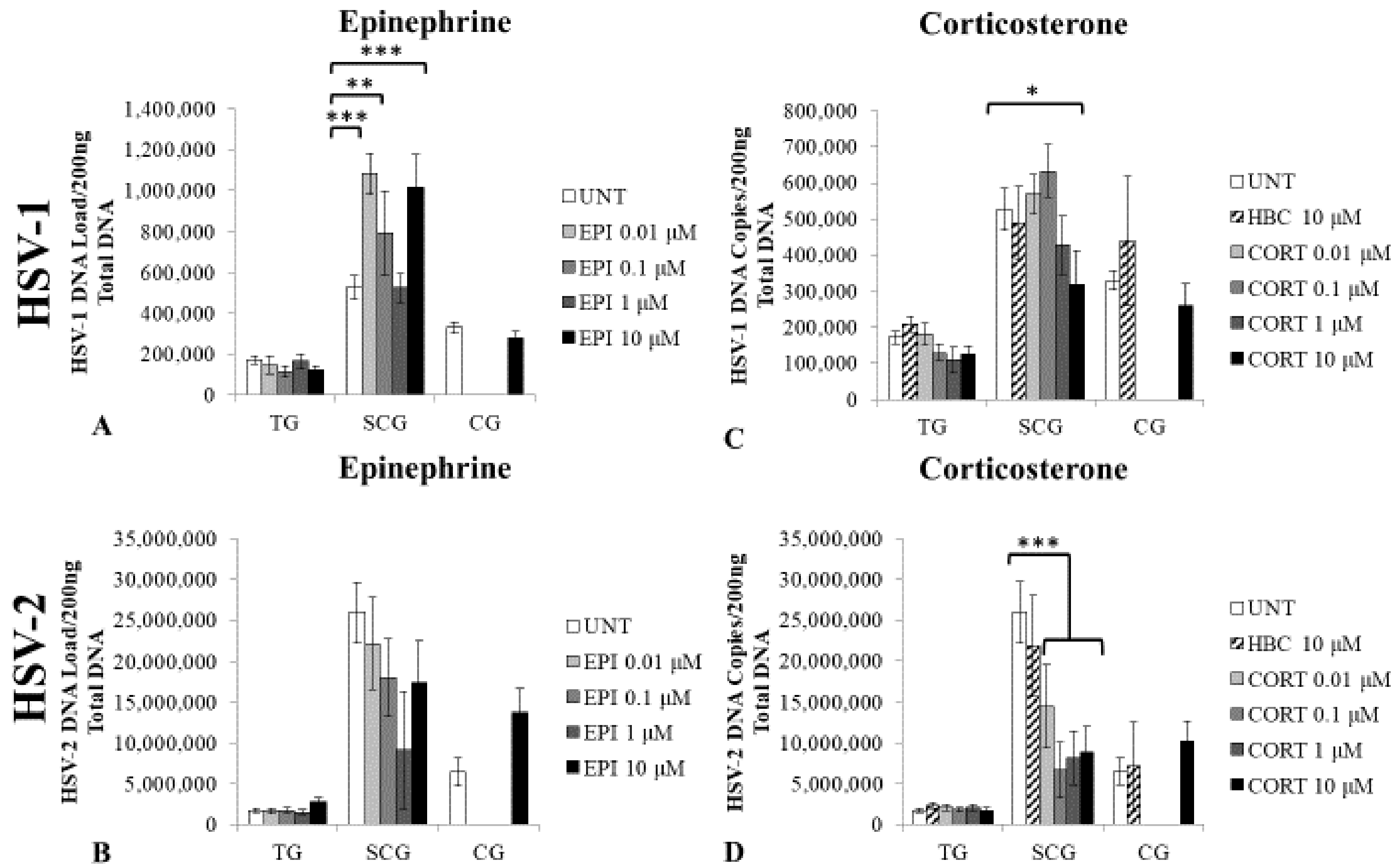
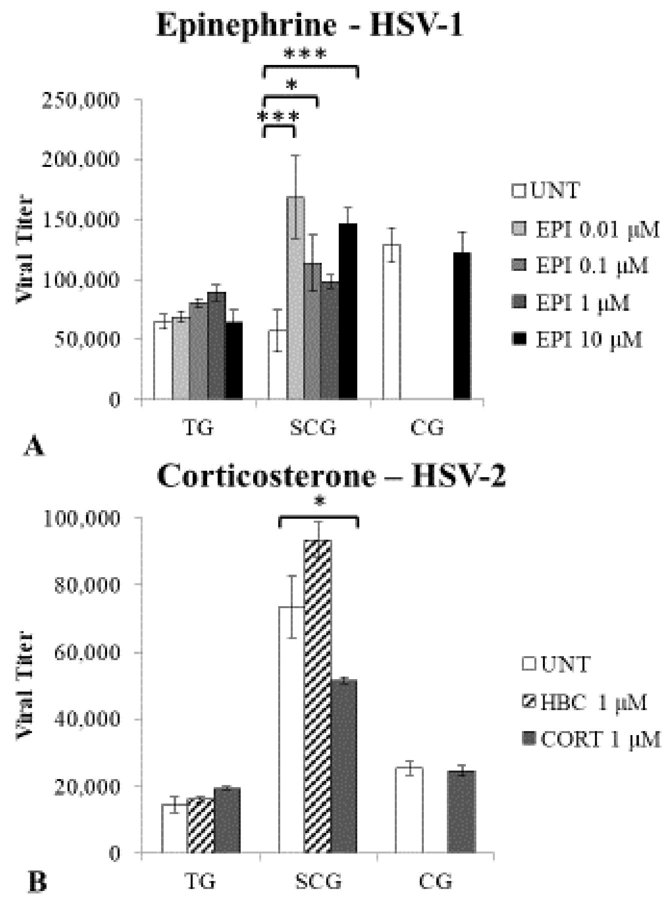


**Figure 4. Effect of stress hormones on infectious virus titers during productive infection.** Virus titers determined by plaque assay in Vero cells for infected neurons and media treated with designated concentrations of EPI or CORT (24 hpi). **A)** Epinephrine treatment of HSV-1 infected neurons increased virus titers compared to untreated HSV-1 infected control neurons: 0.01 μM ($p=0.0001$), 0.1 μM ($p=0.03$), and 10 μM ($p=0.001$) ($n>3$). **B)** Corticosterone treatment of HSV-2 infected neurons decreased virus titers compared to untreated HSV-2 infected control neurons: 1 μM CORT ($p= 0.012$). UNT refers to infected neurons not treated with stress hormones. HBC refers to the water-soluble carrier molecule (2-Hydroxypropyl)-β-cyclodextrin that was conjugated to CORT and used as a control. HBC was not significantly different from UNT ($n>3$). (n = the number of samples collected in which neurons were treated with stress hormones and evaluated for infectious virus titers) * designates $p < 0.05$, ** $p < 0.01$, and *** $p<0.001$.

**Figure 4.**



## TABLES

**Table 1. Expression of stress hormone receptors in sensory TG and sympathetic SCG neurons in vitro and in vivo.**

| Receptor | Neuron Type | Percentage of neuron type that expresses stress hormone receptors | | | |
|---|---|---|---|---|---|
| | | *In vitro* (SEM; no. of dual-labeled neurons/no. of neurons counted) | | *In vivo* (no. of dual-labeled neurons/no. of neurons counted)[1] | |
| GCR | Fe-A5 | 25.31% | (0.0622; 57/220) | 29.79% | (84/282) |
| | IB4 | 78.57% | (0.0606; 851/1080) | 65.88% | (390/592) |
| | Total TG | 52.01% | (0.0234; 536/831) | 49.05% | (304/621) |
| | Total SCG | 41.50% | (0.0300; 166/400) | 45.55% | (225/494) |
| AR α-2 | Fe-A5 | 63.26% | (0.0536; 567/1142) | 69.09% | (509/714) |
| | IB4 | 68.07% | (0.0275; 1535/2358) | 56.97% | (894/1520) |
| | Total TG | 34.35% | (0.0216; 1331/3883) | 37.29% | (490/1316) |
| | Total SCG | 83.33% | (0.0276; 1000/1200) | 87.83% | (758/863) |
| AR β-2 | Fe-A5 | 80.67% | (0.0125; 370/465) | 70.93% | (122/172) |
| | IB4 | 22.52% | (0.0341; 347/1586) | 43.99% | (139/316) |
| | Total TG | 32.95% | (0.0094; 997/3234) | 39.93% | (206/529) |
| | Total SCG | 72.58% | (0.0171; 594/819) | 84.26% | (182/216) |

[1]Counts obtained from *in vivo* receptor expression were from four mice, with the ganglia from all four mice pooled together and not counted separately.

# Chapter 3: Epinephrine and corticosterone induce HSV-1 reactivation through their cognate receptors in sympathetic, but not sensory, neurons

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*Under preparation*

## ABSTRACT

Herpes simplex virus type 1 (HSV-1) infects and establishes latency in sensory and autonomic neurons, from which it can reactivate to cause recurrent disease throughout the life of the host. Stress has been correlated with HSV-1 recurrences in both humans and animal models. To determine whether stress-related hormones reactivate quiescent HSV-1 infection within sensory and autonomic neurons, we analyzed viral DNA and the production of viral progeny after treatment with the stress hormones epinephrine and corticosterone. Both epinephrine and corticosterone induced HSV-1 reactivation from sympathetic superior cervical ganglia (SCG) neurons, but not sensory trigeminal ganglia (TG) neurons. Epinephrine-induced reactivation of HSV-1 in sympathetic neurons required activation of multiple adrenergic receptors (to which epinephrine binds), as well as the transcription factors β-catenin, cAMP-response element binding protein (CREB) and the c-Jun N-terminal kinase (JNK). CORT-induced reactivation required the activation of the glucocorticoid receptor (GCR), CREB, and JNK. EPI induced expression of ICP0 and VP16 within 1 hr post-treatment, while CORT induction of ICP0 and VP16 expression was delayed until 2 hrs post-treatment. Thus, the stress hormones epinephrine and corticosterone can induce reactivation of HSV-1 from sympathetic, but not sensory, neurons, dependent on different receptors and signaling pathways, resulting in expression of ICP0 and VP16 with different kinetics.

## INTRODUCTION

Herpes simplex virus 1 (HSV-1) infects mucosal cells, then establishes a life-long latent infection in sensory and autonomic ganglia innervating the site of infection. The virus can reactivate to cause recurrent orolabial lesions and herpes stromal keratitis (HSK). HSV-1 is also diagnosed as the cause of approximately 30% of primary genital herpes cases. More rarely, HSV-1 causes necrotizing encephalitis with a high mortality rate.

Stress is strongly correlated with recurrent HSV-1 disease in humans and with reactivation in animal models [1-8]. Receptors for the the two major stress hormones, epinephrine and corticosterone, 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) [9-11].

Epinephrine, a catecholaminergic hormone secreted by the adrenal medulla, is regulated by the sympathetic nervous system to induce a "short-term" stress response known as the "flight or flight response." Epinephrine induces HSV-1 reactivation in an *in vivo* model of infection using mice and rabbits [4, 5], although adrenergic reactivation is significantly reduced if the latency associated transcript (LAT) is deleted or mutated [12].

Corticosterone, a glucocorticoid secreted by the adrenal cortex, induces a "long-term" stress response. Corticosterone binds to the glucocorticoid (GCR) and mineralocorticoid receptors (MCR), and its major effects on humans and mammals include regulation of metabolism and suppression of the immune system. Corticosterone induces HSV-1 reactivation *in vivo,* presumably through immune system suppression [7, 13-15]. However, a previous study showed that several stress-induced cellular transcription factors expressed in TG neurons stimulated the HSV-1 ICP0 promoter [16] and ICP0 alone can initiate reactivation of HSV-1 from latency [17-19]. In addition,

we recently demonstrated that corticosterone decreases HSV-1 DNA replication during productive infection in sympathetic, but not sensory, neurons [11]. Thus, corticosterone could potentially induce HSV-1 reactivation by acting directly on neurons that harbor latent virus, rather than acting through immune system suppression to indirectly induce reactivation.

To determine if stress hormones differentially reactivate HSV-1 at a cellular level in different types of mature neurons, we administered either corticosterone (CORT) or epinephrine (EPI) to quiescently infected primary murine adult sensory and autonomic neurons. Quantification of viral replication, production of viral progeny, and gene expression showed that stress hormones differentially induce reactivation depending on the type of neuron. Furthermore, administration of stress hormone receptor agonists and antagonists demonstrated that EPI and CORT exert their effects through specific adrenergic and glucocorticoid receptors to induce HSV-1 reactivation from quiescence *in vitro.*

## MATERIALS & METHODS

**Cell lines and Virus Strains.** HSV-1 strain 17+ was originally transferred from John Hay (SUNY Buffalo, NY) to the Krause lab (FDA, Bethesda, MD). Virus was propagated in Vero cells (ATCC) and first passage stocks were transferred to the Margolis lab (UCSF, San Francisco, CA). Virus was then propagated in Vero cells and first passage stocks were transferred to the Bertke lab (Virginia Tech, Blacksburg, VA). Stocks were titrated in Vero cells in quadruplicate to determine concentration. Stock viruses were diluted in Neurobasal A medium (Gibco) for inoculation of primary adult murine neuronal cultures.

**Mice.** Female 6 week old Swiss Webster mice were purchased from Envigo. Mice were euthanized and tissues processed following delivery, without being housed in the animal facility. All animal

care and handling was in accordance with the Virginia Tech Institutional Care and Use Committee (IACUC# 13-008-CVM and 15-237).

**Primary Adult Murine Neuronal Cultures.** Six-week old Swiss Webster female mice (Envigo) 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 removed, incubated at 37°C for 20 minutes in papain (≥100 units) (Worthington) reconstituted with 5 mL of Neurobasal A medium (Gibco), followed by 20 minutes in 4 mg/mL collagenase and 4.67 mg/mL dispase (Worthington) diluted in Hank's balanced salt solution (HBSS) (Gibco) on a rotator. Following mechanical trituration with a 1 mL pipette, the TG cell suspension was layered on a 4-step Optiprep (Sigma) gradient. Optiprep was first diluted with 0.8% sodium chloride (50.25:49.75 mL) to make a working solution (specific gravity 1.15). Then, cold Optiprep working solution was diluted with 37°C Neurobasal A medium to make each step of the gradient: first or bottom layer 550 µL Neurobasal A medium and 450 µL Optiprep working solution; second layer 650 µL Neurobasal A medium and 350 µL Optiprep working solution; third layer 750 µL Neurobasal A medium and 250 µL Optiprep working solution; and the fourth or top layer 850 µL Neurobasal A medium and 150 µL Optiprep working solution. The gradient with the TG cell suspension was centrifuged at 800 x g for 20 minutes, and the two middle layers containing neuronal cells (~ 2 mL) were collected. SCG and CG were triturated with a 200 µL pipette and did not go through a gradient step because they contain less ganglionic debris after dissociation. TG, SCG, and CG were washed twice with Neurobasal A medium supplemented with 2% SM-1 (StemCell Technologies) and 1% penicillin-streptomycin (Corning). Neurons were counted and plated on Matrigel-coated (Corning) Lab-Tek II chamber slides (ThermoFisher) at a density of 3000 neurons/50µL per well and

incubated in 37°C/5% $CO_2$ incubator in Complete Neuro media (Neurobasal A media supplemented with 2% SM-1 (StemCell Technologies), 10 mg/mL penicillin-streptomycin (Corning), 10 µL/mL Glutamax (ThermoFisher), 10 µg/mL nerve growth factor (Peprotech), 10 µg/mL glial-derived neurotrophic factor (Peprotech), 10 µg/mL neurturin (Peprotech), 10 µg/mL ciliary neurotrophic factor (Peprotech; for CGs only) and 200 mM 5-fluorodeoxyuridine (Sigma) to deplete non-neuronal cells. After one hour, debris were removed by pipette and cells were maintained in 300 µL of fresh Complete Neuro media until infection.

**Establishment of Quiescent Infection.** Four days after plating, media was removed and neurons were inoculated with 30 multiplicity of infection (moi) of HSV-1 (strain 17+). 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) for five days to establish and maintain a quiescent infection *in vitro.*

**Reactivation with Stress Hormones.** Five days after establishment of a quiescent 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. 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.

**Reactivation with Receptor Agonists & Antagonists.** For testing adrenergic receptor specificity, five days after establishment of a quiescent infection, media containing acyclovir was removed and Complete Neuro media with no acyclovir and with one, two, or three adrenergic agonists (10

μM) were added. Adrenergic receptor (AR) agonists included phenylephrine (alpha-1 AR agonist), clonidine (alpha-2 AR agonist), dobutamine (beta-1 AR agonist), or terbutaline (beta-2 AR agonist) (Sigma). Adrenergic receptor antagonists phentolamine (non-specific alpha AR antagonist) or timolol (non-specific beta 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 or mineralocorticoid receptor agonist aldosterone (Sigma) were added to Complete Neuro media (10 μM). The GCR antagonist mifepristone (10 μM) was added to fresh Complete Neuro media with 10 μM corticosterone-HBC.

**Reactivation with Protein Inhibitors.** For testing molecular inhibition of intermediates in signaling cascades, five days after establishment of quiescent 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), or 20 μM JNK inhibitor SP600125 (Sigma) with or without 10 μM epinephrine or corticosterone-HBC were added to primary adult neuronal cultures quiescently infected with HSV-1.

**Quantitation of HSV viral load.** Viral DNA was extracted from neuronal cultures 72 hours post-reactivation (hpr) with TRIsure reagent (Biorad), according to the manufacturer's instructions. Viral DNA load was determined by quantifying viral DNA by qPCR using HSV-1 thymidine kinase (TK) gene-specific primers and probes (Table 2). All assays were normalized to 18s rRNA (Applied Biosystems) and reported as viral copy number in 200 ng of total DNA.

**Quantitation of HSV gene expression.** Viral RNA was extracted from neuronal cultures at 1 hour, 2 hours, and 24 hours hpr with TRIsure reagent (Biorad), according to the manufacturer's instructions, and converted to cDNA with iScript (Biorad). Gene expression was determined by

quantifying the HSV-1 immediate early genes ICP0, ICP4, ICP27, and VP16 by digital droplet PCR using HSV-1 gene-specific primers (Table 2) and EvaGreen QX200 Master mix (Biorad). All assays were normalized to 18s rRNA (Table 2) and reported as fold change of epinephrine or corticosterone treated samples compared to untreated.

**Quantification and Statistical Analysis.** Data are presented as means ± standard errors of the mean. Statistics were performed using parametric analyses with JMP Pro version 12, including analysis of variance with contrast tests. N represents the number of cultures. Where indicated, asterisks denote statistical significance as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

## RESULTS

**Stress hormones induce HSV-1 reactivation *in vitro*.** To determine if stress hormones induce HSV-1 reactivation from quiescent infection, epinephrine (EPI) or corticosterone (CORT, conjugated to (2-Hydroxypropyl)-β-cyclodextrin in the water-soluble form) were added to quiescently infected neuronal cultures maintained in acyclovir (ACV) for five days post-inoculation (dpi). Viral DNA was isolated 72 hours post-reactivation (hpr) and quantified by qPCR for the thymidine kinase (TK) gene.

In sympathetic SCG neurons quiescently infected with HSV-1, both EPI and CORT induced viral reactivation, as shown by increased viral DNA compared to quiescently infected neuronal cultures that had ACV removed but no other treatment (untreated neuronal cultures, UNT) ($p < 0.01$ for both EPI and CORT) (Figure 1). Neither EPI nor CORT had an effect on reactivation in sensory trigeminal ganglia (TG) or parasympathetic ciliary ganglia (CG) neurons. Trichostatin A (TSA), a histone deacetylase inhibitor that has been shown to reactivate HSV-1

reliably from quiescence [20], was used as a positive control. Therefore, EPI and CORT induce HSV-1 reactivation in sympathetic, but not sensory or parasympathetic, adult neurons.

To verify that EPI and CORT-induced reactivation progressed through the complete virus cycle to produce infectious progeny, quiescently infected neurons were treated with the hormones for 24 hrs. Neurons and media were then collected and viral titers were quantified by plaque assay on Vero cells. Neither EPI nor CORT induced an increase in infectious virus titer compared to UNT in sensory TG or sympathetic SCG neurons.

**Stress hormones alter HSV-1 gene expression during reactivation.** To determine which immediate early (IE) HSV genes respond to stress hormone treatment during reactivation, viral RNA was isolated 1 hour, 2 hours, and 24 hours after EPI or CORT treatment to induce reactivation. IE ICP4, ICP0, and ICP27 transcript expression was quantified by digital droplet PCR. Viral protein 16 (VP16) transcript expression was also quantified, since previous reports have demonstrated its involvement in stress-induced reactivation [21, 22].

In HSV-1 infected sympathetic SCG neurons, both EPI and CORT treatment had minimal effect of ICP4 expression in the first two hours following treatment, but ICP4 expression was significantly reduced by 24 hrs post treatment ($p < 0.01$) (Figure 2A). EPI treatment increased expression of ICP0 within 1 hr post-treatment compared to untreated control neurons ($p < 0.01$); CORT treatment also increased ICP0 expression but at a slower rate, increasing expression at 2 hrs post treatment. Similarly, VP16 expression was increased one and two hours after EPI administration ($p < 0.05$) (Figure 2D) and two hrs after CORT administration ($p = 0.04$) (Figure 2D). In contrast, neither EPI nor CORT treatment had a significant effect on ICP27 expression (Fig 2C). Therefore, EPI induces expression of ICP0 and VP16 rapidly after treatment, while

CORT treatment produces a delayed increase of these genes, and both hormones suppress ICP4 expression by 24 hrs post treatment.

**Adrenergic receptor specificity of epinephrine-induced reactivation.** Previously we demonstrated that adrenergic receptor expression is maintained in cultured primary adult murine neurons of sympathetic SCG neurons similarly to expression *in vivo* [11]. Furthermore, we showed that HSV-1 antigens generated during productive infection co-localize with adrenergic receptors [11]. We reasoned that if EPI induced reactivation through a specific adrenergic receptor, an agonist that activates that specific receptor would also induce reactivation. Therefore, to determine whether EPI-induced reactivation of HSV-1 was occurring through binding of epinephrine to specific adrenergic receptors, SCG neurons quiescently infected with HSV-1 were treated with agonists for one of the four different adrenergic receptors: α-1, α-2, β-1, and β-2. Seventy-two hours after treatment, viral DNA was isolated and quantified by qPCR for the TK gene.

In comparison to untreated (UNT) neurons and those treated with EPI, which induced reactivation in these neurons, none of the four adrenergic receptor agonists alone induced reactivation of HSV-1 in sympathetic SCG neurons (Figure 3A). This suggests that activation of a single adrenergic receptor is not sufficient for EPI-induced reactivation of HSV-1 from quiescence in sympathetic SCG neurons.

To determine whether we could block EPI-induced reactivation of HSV-1 in sympathetic SCG neurons, SCG neurons quiescently infected with HSV-1 were treated with adrenergic receptor antagonists to prevent activation of adrenergic receptors. The non-specific β-adrenergic receptor antagonist timolol inhibited EPI-induced reactivation in quiescently infected sympathetic SCG neurons (Figure 3B), demonstrating that β-adrenergic receptor activation is necessary to

induce reactivation following EPI treatment. In comparison, treatment with the non-specific α-adrenergic receptor antagonist phentolamine produced quantities of HSV-1 DNA that were still significantly higher than untreated sympathetic neurons ($p = 0.035$), showing that blockade of α-adrenergic receptors cannot block EPI-induced HSV-1 reactivation in sympathetic SCG neurons.

Since individual receptor agonists were unable to induce HSV-1 reactivation but a non-specific beta blocker was able to block reactivation, we reasoned that activation of a single receptor was insufficient to induce reactivation but β-adrenergic receptors are necessary for EPI-induced reactivation. To determine whether EPI was inducing HSV-1 reactivation in sympathetic SCG neurons by binding to multiple adrenergic receptors, SCG neurons quiescently infected with HSV-1 were treated with agonists for two of the four different adrenergic receptors simultaneously. All treatments containing the α-2 adrenergic receptor agonist clonidine induced reactivation similarly to EPI ($p < 0.05$ for all) (Figure 3C). However, combination of the β-1 and β-2 agonists induced reactivation without clonidine ($p = 0.003$). The α-1 agonist phenylephrine induced reactivation when combined with clonidine ($p = 0.0155$), but not with either of the β-AR agonists. Therefore, the α-2 AR agonist is necessary, but not sufficient alone, to induce HSV-1 reactivation from sympathetic neurons.

To determine whether one specific adrenergic receptor is necessary for HSV-1 reactivation in sympathetic SCG neurons, SCG neurons quiescently infected with HSV-1 were treated with agonists for three of the four different adrenergic receptors, omitting one (Figure 3D). All treatments induced HSV-1 reactivation compared to untreated control neurons ($p < 0.05$). However, the treatment that excluded the α-2 adrenergic receptor agonist, clonidine, induced reactivation similarly to EPI treatment alone, while all treatments that included clonidine induced

HSV-1 reactivation above EPI-induced levels. Thus, activation of AR α-2 is necessary for full reactivation potential of HSV-1 in sympathetic neurons.

**Steroid receptor specificity of corticosterone-induced reactivation.** Previously we demonstrated that glucocorticoid receptors (GCR) are expressed in cultured primary adult murine sensory TG and sympathetic SCG neurons, and HSV antigens generated during productive infection co-localize with receptor expression [11]. CORT can bind to and activate glucocorticoid (GCR) and mineralocorticoid (MCR) receptors [23].

To determine whether CORT-induced reactivation was occurring through binding of corticosterone to GCR or MCR, quiescently infected neurons were treated with dexamethasone, a GCR agonist, or aldosterone, a mineralocorticoid receptor (MCR) agonist. Seventy-two hours after treatment, viral DNA was isolated and quantified by qPCR for the TK gene.

Dexamethasone, but not aldosterone, induced HSV-1 reactivation similar to CORT in quiescently infected sympathetic SCG neurons ($p < 0.001$) (Figure 4A). HBC, the water-soluble vehicle for CORT, and dimethyl sulfoxide (DMSO), the vehicle for the CORT agonists and antagonists, did not have any significant effects on reactivation (Figure 4A). To determine whether we could block CORT-induced reactivation of HSV-1 in sympathetic SCG neurons, quiescently infected neurons were treated with CORT and the GCR antagonist mifepristone, which effectively inhibited CORT-induced reactivation of HSV-1 in sympathetic SCG neurons (Figure 4B).

**Protein kinase and transcription factor inhibitors selectively block stress-induced reactivation.** Previously, the cAMP-response element binding protein (CREB) and the c-Jun N-terminal kinase (JNK) have been demonstrated to be important in stress-induced reactivation of

HSV [24-26]. The transcription factor β-catenin plays a role in bovine herpesvirus 1 latency and reactivation [27-29].

To determine whether epinephrine or corticosterone-induced reactivation requires the activation or repression of CREB, quiescently infected neurons were treated with EPI or CORT and the CREB inhibitor 666-15. This inhibitor prevents the phosphorylation of CREB at serine residue 133 by various cellular serine/threonine protein kinases, such as protein kinase A (PKA) or mitogen-activated protein kinases (MAPKs), which inhibits the CREB from interacting with CREB-binding protein (CBP) and p300 that are essential for CREB-mediated gene transcription [30]. Inhibition of CREB activity blocked both EPI and CORT-induced reactivation ($p < 0.05$ compared to EPI or CORT treatment) (Figure 5). This suggests that CREB is important for both EPI and CORT induction of HSV-1 reactivation.

To determine whether EPI or CORT-induced reactivation requires the activation or repression of JNK in primary adult sympathetic neurons, quiescently infected neurons were treated with EPI or CORT and the JNK inhibitor SP600125, which competitively inhibits ATP binding to JNK, thus reducing JNK's ability to phosphorylate c-Jun [31]. Inhibition of JNK decreased both EPI and CORT-induced reactivation ($p < 0.05$ compared to EPI or CORT treatment) (Figure 5), demonstrating that JNK is an important component of both adrenergic and corticosterone-induced reactivation.

To determine whether EPI or CORT-induced reactivation requires the activation or repression of β-catenin, quiescently infected neurons were treated with EPI or CORT and the β-catenin inhibitor iCRT14, which inhibits the interaction between nuclear β-catenin and N-terminal domain of T-cell factor (TCF) family members, specifically TCF4, to block the transcriptional targets of β-catenin signaling [32]. Inhibition of β-catenin reduced adrenergic reactivation, but β-

catenin with EPI was not significantly different from either EPI treatment or UNT (Figure 5A). β-catenin inhibition did not affect CORT-induced reactivation (Figure 5B). Therefore, β-catenin activity promotes adrenergic reactivation, but inhibition of β-catenin does not affect CORT-induced reactivation.

## DISCUSSION

In our studies, we demonstrate that the stress hormones EPI and CORT induce reactivation of HSV-1 selectively in sympathetic neurons, but not in sensory or parasympathetic primary adult neurons. Reactivation coincides with increases in ICP0 and VP16 gene expression, although the temporal kinetics differ for EPI and CORT. We also demonstrate that activation of more than one adrenergic receptor is necessary for epinephrine to induce HSV-1 reactivation, while only the glucocorticoid receptor is required for CORT-induced reactivation.

Previously, we demonstrated that EPI treatment during productive infection enhanced HSV-1 DNA replication and infectious virus titer in sympathetic, but not sensory, primary adult neurons [11]. Our current studies correlate with these findings, demonstrating that EPI also induces reactivation of HSV-1 during quiescence in the same neurons. . However, during productive infection, CORT decreased HSV-1 DNA replication and infectious virus titer [11], while our current studies show that CORT treatment during quiescence induces reactivation. This dichotomy could potentially be explained by differences in HSV gene expression between productive infection and reactivation [21, 33]. During productive infection, HSV carries VP16 into the neuron in its tegument, permitting the generation of the VP16/Oct1/HCF-1 initiation complex on immediate early genes, leading to the characteristic temporal cascade of gene expression to produce infectious virus. During reactivation from latency, however, first the genome undergoes

transient, disordered expression of viral lytic genes, referred to as Phase I of reactivation, which then progresses to a second phase more closely resembling the temporal cascade of productive infection [21]. Our gene expression results show that both EPI and CORT induce ICP0 and VP16 gene expression transiently 1-2 hrs post-treatment, coinciding with Phase I gene expression. Expression of ICP0 alone can induce reactivation of HSV-1 from latency [18, 34, 35]. VP16 has been shown to be required for the second phase of HSV-1 reactivation in a neonatal SCG quiescence model utilizing a PI3-K inhibitor as a reactivation stimulus [21]. Thus, by 1-2 hrs post-treatment, VP16 would be available to complex with host factors HCF-1 and Oct1 to initiate the lytic cycle.

EPI and CORT also significantly decreased ICP4 gene expression 24 hours post-reactivation. Previous studies have demonstrated that gene expression following a reactivation stimulus initially peaks 20 hpr and decreases at 25 hpr [21]. Since ICP4 inhibits its own expression [36], it is possible that EPI and CORT may have up-regulated ICP4 expression at a time point between 2 and 24 hpr, and therefore the significant decrease at 24 hpr is due to ICP4 inhibiting its own expression.

With the exception of ICP4, the immediate early and VP16 gene expression for sympathetic neurons undergoing adrenergic reactivation followed a similar pattern: an increase compared to untreated controls at 1 and 2 hpr and no difference at 24 hpr. Immediate early and VP16 gene expression following CORT treatment followed a similar pattern, which differed from the gene expression observed with adrenergic reactivation: no difference at 1 hpr compared to untreated controls, an increase at 2 hpr, and no difference at 24 hpr. Previous reports using PI3-K inhibition as a reactivation stimulus detected changes in ICP27 and VP16 expression at 15 and 20 hpr, but the earliest time point assayed was 10 hpr [21]. We found no significant differences in

ICP27 gene expression after EPI or CORT treatment. Taken together, this suggests that HSV-1 gene expression during the initial phase of reactivation may differ depending on the reactivation stimuli used.

We determined that the GCR, but not the MCR, was necessary for CORT-induced reactivation of HSV-1 in sympathetic neurons. GCR is expressed in both the sensory TG [10] and the sympathetic SCG [11]. Administration of the GCR agonist dexamethasone has been shown to reactivate HSV-1 in the rabbit and mouse models of ocular infection [6, 37, 38] and can stimulate expression of a reporter gene from the HSV-1 ICP0 promoter by activating several transcription factors [16]. CORT treatment of quiescently-infected SCGs increased VP16 expression, which has shown to be necessary for gene expression required during reactivation [21]. Both JNK and CREB activity was required for CORT-induced HSV-1 reactivation in sympathetic SCGs. GCR can activate CREB [39, 40], and CREB has been implicated in HSV-1 reactivation: There are multiple CRE binding sites in the LAT promoter that have been shown to bind CREB [25, 41, 42], and mutation of these CRE sites inhibit both epinephrine-induced reactivation and hyperthermia reactivation in the mouse ocular model [24, 43]. Activators of CREB, such as cAMP, have been demonstrated to induce HSV-1 reactivation *in vitro* [44]. Interestingly, JNK has been shown to phosphorylate GCR on serine 246 to inhibit GCR transcriptional activity and promote nuclear export [45, 46]. Therefore, it is unclear how JNK promotes CORT-induced reactivation through the GCR.

We have also demonstrated that EPI acts through specific adrenergic receptors to induce reactivation of HSV-1 from sympathetic neurons. Binding of EPI to any individual adrenergic receptor was not sufficient to induce reactivation; rather, the activation of two or more adrenergic

receptors was required. In addition, blocking the β-1 and β-2 ARs, but not α-1 or α-2 ARs, inhibited adrenergic reactivation.

We hypothesize that the ability of adrenergic receptors to couple to more than one G-protein is critical to understanding how EPI induces reactivation (Figure 6). Activation of the α-2 AR consistently induced HSV-1 reactivation when coupled with activation of another adrenergic receptor. Previously, use of two non-specific α-adrenergic receptor antagonists, thymoxamine and corynanthine, blocked HSV-1 reactivation in the murine and rabbit ocular models of infection [47]. The α-2 AR couples with the inhibitory G protein alpha ($G\alpha_i$) to inhibit adenylate cyclase and downstream cyclic AMP (cAMP) production, while also activating phospholipase C through the G protein alpha q ($G\alpha_q$) [48, 49], which through a protein kinase cascade activates the JNK pathway [50]. JNK inhibition blocked adrenergic reactivation, and JNK activity is required for HSV-1 reactivation in neonatal sympathetic SCGs using PI3-K inhibition as a reactivation stimulus [26]. Therefore, JNK activation by α-2 AR is a potential pathway by which EPI could induce HSV-1 reactivation.

The α-1 AR also couples with $G\alpha_q$ [50], and the α-1 AR agonist phenylephrine could only induce reactivation when coupled with the α-2 agonist, clonidine, but not when added with the β-1 or β-2 agonists. The effect of β-adrenergic receptor antagonist administration on HSV-1 reactivation in the ocular model of infection has been variable. Propranolol, a non-specific β-adrenergic receptor antagonist, decreased HSV-1 reactivation after hyperthermic stress or iontophoresis of epinephrine in rabbits [51, 52]. However, iontophoresis of timolol, another non-specific β-adrenergic receptor antagonist, did not decrease viral reactivation in rabbits, mice, or non-human primates [53-56]. In our studies, timolol effectively blocked EPI-induced reactivation.

The β-1 and β-2 ARs can couple to the stimulatory G protein alpha ($G\alpha_s$) to activate adenylate cyclase [57], which can activate mTORC and the transcription factor Akt that inhibits HSV-1 reactivation and promote latency through nerve growth factor signaling in the immature SCG neuronal culture model [58, 59]. However, cAMP production and protein kinase A (PKA) activation can release nuclear factor κ-B (NF-κB), which up-regulates the innate immune response and induces interferon production [60, 61]. Interferon-β and interferon-γ production blocks HSV-1 reactivation *in vitro* in neonatal sympathetic SCG neurons [62]. Consequently, activation of either β-1 or β-2 could effectively block HSV reactivation by inducing interferon expression.

However, combining the β-1 and β-2 agonists together did induce HSV-1 reactivation. Interestingly, the β-2 AR can couple with either the $G\alpha_s$ or the $G\alpha_i$ proteins [63], and when coupled to $G\alpha_i$ the β-2 AR can activats the Src pathway [64], which leads to activation of CREB and can thus compensate for lack of α-2 AR activation. CREB inhibition blocked adrenergic reactivation, and a cAMP response element in the LAT promoter and a 348 base pair sequence within LAT exon 1 were identified as essential for adrenergic reactivation of HSV-1 [65, 66], but follow-up studies were unable to further map specific functional sites within exon 1 [67]. Therefore, CREB activation could be an important step in how EPI induces HSV-1 reactivation in sympathetic neurons.

In addition, when three of the four adrenergic agonists were added together, all treatments containing the α-2 AR agonist stimulated HSV-1 reactivation levels above that of EPI. Therefore, induction of the α-2 AR to block cAMP production and downstream interferon gene expression is necessary, but not sufficient alone, to induce HSV-1 reactivation.

β-catenin promotes bovine herpesvirus 1 (BHV-1) productive infection and has been implicated in the latency-reactivation cycle of BHV-1 [27-29]. Induction of β-catenin inhibits

HSV-1 productive infection *in vitro* in fibroblasts [68]. Inhibition of β-catenin blocked adrenergic reactivation, but had no effect on corticosterone-induced reactivation. Activation of the glucocorticoid receptor by dexamethasone promotes β-catenin degradation [69], and β-catenin promotes BHV-1 latency in sensory neurons [27, 28]. Therefore, inhibition of β-catenin could promote CORT-induced HSV-1 reactivation. In contrast, activation of AR β-2 receptors by epinephrine promotes Wnt signaling and accumulation of activated β-catenin in airway bronchi and immune cells [70, 71]. However, the interactions between Wnt signaling and adrenergic receptors remains unclear.

Therefore, we propose two different mechanisms by which stress hormone-induced reactivation of HSV-1 occurs in sympathetic neurons. First, in EPI-induced reactivation, signaling through the AR α-2 is necessary but not sufficient alone to block adenylate cyclase and downstream interferon production and to activate phospholipase C and downstream targets CREB and JNK to stimulate HSV-1 reactivation (Figure 6). In CORT-induced reactivation, CORT binds to GCR which activates CREB transcriptional activity. Both of these pathways culminate in a transient increase in VP16 expression rapidly following hormone activation of cognate receptors, which is a critical determinant of HSV-1 reactivation [21].

## AUTHOR CONTRIBUTIONS

A. S. B. and A. M. I. designed the studies; A. M. I. performed the experiments; A. S. B., A. M. I., and R. P. D. analyzed and interpreted the data; A. S. B. and A. M. I. wrote the manuscript; and R. P. D. edited the manuscript.

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## FIGURES

**Figure 1: Epinephrine and corticosterone induce HSV-1 reactivation only in sympathetic neurons.** Primary adult murine neuronal cultures from trigeminal ganglia (TG), superior cervical ganglia (SCG), and ciliary ganglia (CG) were infected with HSV-1 for 5 days with 300 μM acyclovir. Media with acyclovir was removed, and cultures were treated with 1.2 μM trichostatin A (TSA; positive control), 10 μM epinephrine (EPI), or 10 μM corticosterone (CORT), or were untreated (UNT; ACV removed but no other treatment). Neurons and media were then collected for qPCR analysis at 72 hpr, reported as viral DNA copy number/200 ng total DNA (A) or plaque assays on Vero cells at 24 hpr (B). A) HSV-1 DNA load was significantly increased by both EPI and CORT treatment compared to HSV-1-infected untreated control neurons (UNT) ($p = 0.0002$ for EPI and $p = 0.0053$ for CORT) but not in TG or CG neurons ($n > 3$). 1.2 μM TSA significantly increased HSV-1 DNA load in TG and SCG, but not CG, neurons ($p = 0.04$ in TG and $p < 0.0001$ for SCG) ($n > 3$). B) Infectious virus titer results. ($n$ = number of cultures). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

**Figure 1.**

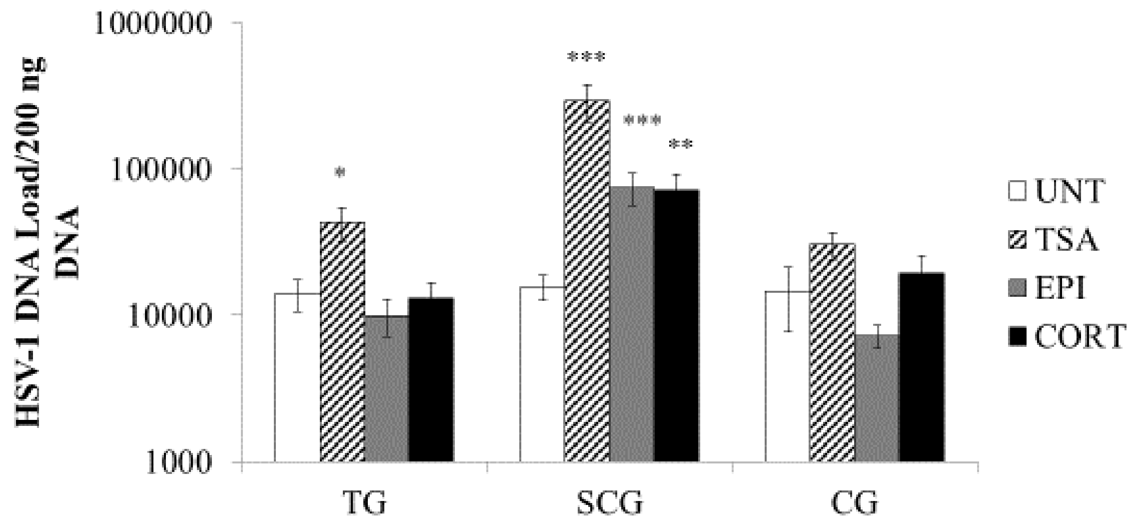


**Figure 2: Stress hormone treatment effects on HSV-1 gene expression in sympathetic neurons during reactivation.** Primary adult murine neuronal cultures from sympathetic SCG neurons were infected with HSV-1 for 5 days in the presence of 300 μM acyclovir. Media with acyclovir was removed, and cultures were treated with 10 μM epinephrine (EPI) or 10 μM corticosterone (CORT), or untreated (UNT). Neurons were collected at 1, 2, and 24 hpr for digital droplet PCR (ddPCR) analysis for viral immediate early genes ICP4, ICP0, and ICP27 and late gene VP16 expression. Gene expression is depicted as fold change compared to HSV-1-infected untreated SCG neurons (UNT). A) ICP4 gene expression was significantly decreased by EPI and CORT compared to UNT at 24 hpr ($p = 0.0073$ for EPI and $p = 0.0042$ for CORT) ($n > 3$). B) ICP0 gene expression was significantly increased at 1 hpr by EPI treatment ($p = 0.0024$) ($n > 3$). C) ICP27 gene expression was not significantly affected by either EPI or CORT treatment at the time points tested. D) VP16 gene expression was significantly increased at 1 hpr and 2 hpr by EPI treatment (p = 0.0006 and 0.0163, respectively) and significantly increased at 2 hpr by CORT treatment ($p = 0.04$) ($n > 3$). (*n* = number of samples analyzed). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

**Figure 2.**

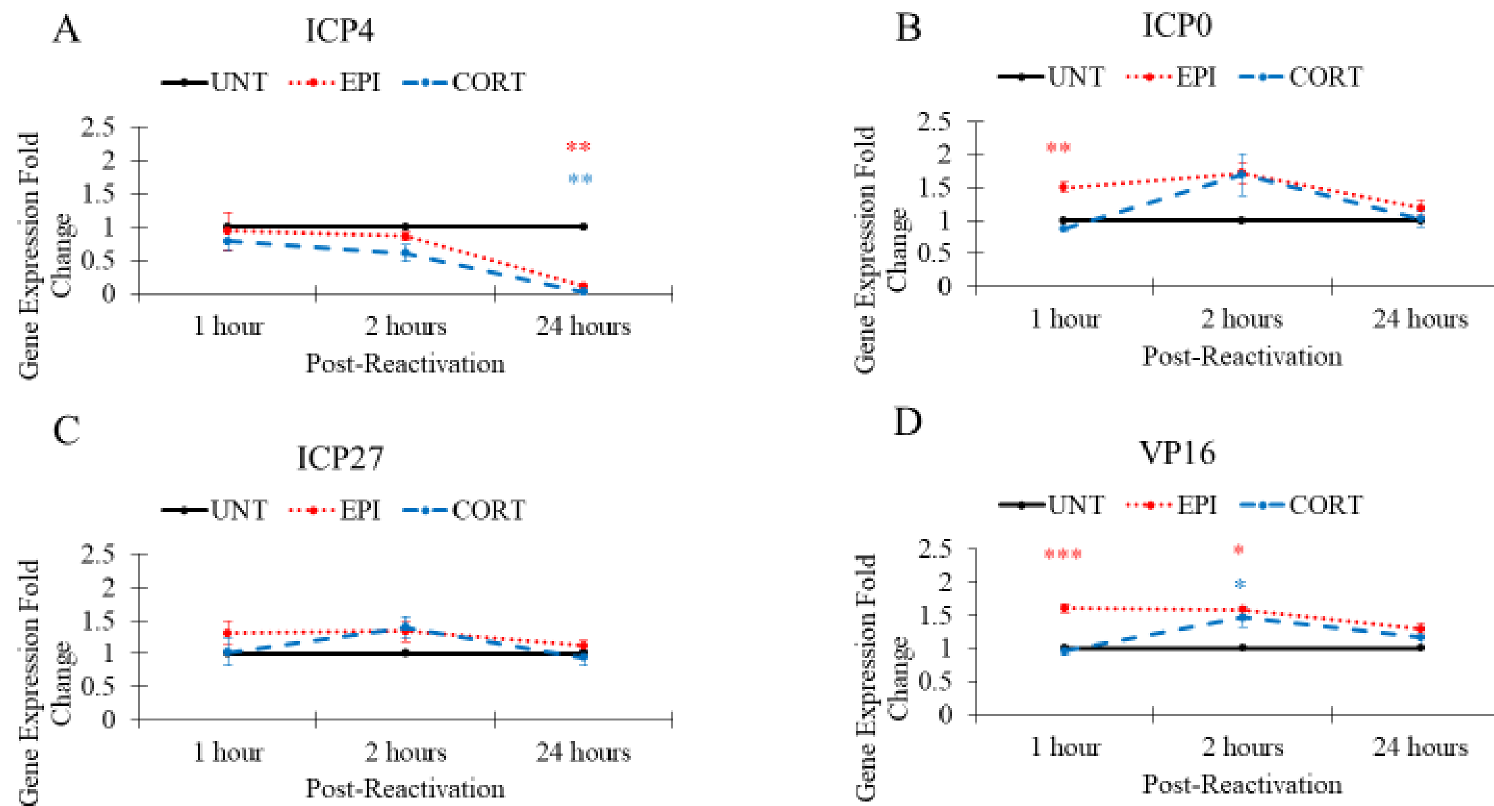


**Figure 3: Adrenergic receptor α-2 is sufficient, but not required, for epinephrine-induced reactivation of HSV-1 in sympathetic neurons.** Effect of adrenergic agonists and antagonists on HSV-1 DNA replication during quiescence. Primary adult murine neuronal cultures from superior cervical ganglia (SCG) were infected with HSV-1 for 5 days with 300 μM acyclovir. Media with acyclovir was removed, and cultures were treated with 10 μM adrenergic agonists or 10 μM epinephrine with 10 μM adrenergic antagonists. Adrenergic agonists include phenylephrine (α-1), clonidine (α-2), dobutamine (β-1), and terbutaline (β-2), while adrenergic antagonists include phentolamine (α-1 and α-2) and timolol (β-1 and β-2). Neurons and media were then collected for qPCR analysis at 72 hpr. A) No single adrenergic receptor agonist significantly affected HSV-1 DNA load compared to HSV-1-infected untreated controls (UNT). B) The non-specific α-AR antagonist did not inhibit adrenergic reactivation ($p = 0.0350$ compared to UNT) ($n > 3$). The non-specific β-AR antagonist blocked adrenergic reactivation ($p = 0.0032$ compared to epinephrine treatment) ($n > 3$). C) Administration of the α-1 and α-2 ($p = 0.0155$ compared to UNT), α-2 and β-1 ($p = 0.0433$ compared to UNT), and β-1 and β-2 agonists ($p = 0.0028$ compared to UNT) together significantly increased HSV-1 DNA load. D) All treatments with 3 adrenergic agonists significantly increased HSV-1 DNA load compared to UNT ($p < 0.0001$ for α-2 + β-1 + β-2; $p = 0.0144$ for α-1 + β-1 + β-2; $p = 0.0001$ for α-1 + α-2 + β-2; $p < 0.0001$ for α-1 + α-2 + β-1) ($n > 3$). (*n* = number of cultures). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

**Figure 3.**

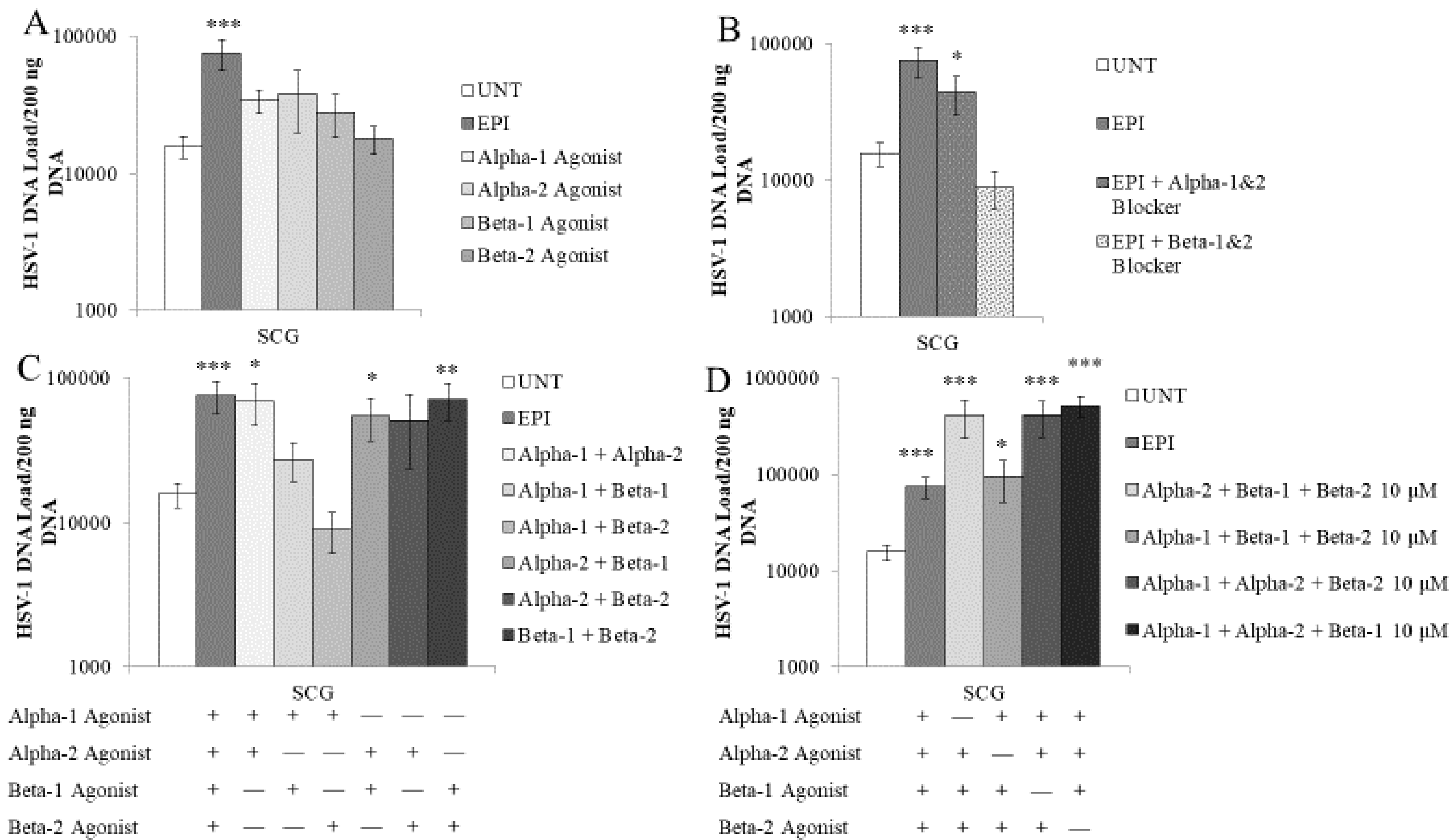


**Figure 4: Glucocorticoid receptor activation is necessary for corticosterone-induced HSV-1 reactivation.** Primary adult murine neuronal cultures from superior cervical ganglia (SCG) were infected with HSV-1 for 5 days with 300 μM acyclovir. Media with acyclovir was removed, and cultures were treated with either 10 μM steroid receptor agonists or 10 μM corticosterone with 10 μM steroid receptor antagonists. Neurons and media were collected for qPCR analysis at 72 hpr. A) The GCR agonist (dexamethasone) significantly increased HSV-1 reactivation compared to HSV-1-infected untreated SCG neurons (UNT) ($p = 0.0002$) ($n > 3$). The MCR agonist (aldosterone), HBC (2-Hydroxypropyl)-β-cyclodextrin, the carrier molecule for corticosterone), and DMSO (dimethyl sulfoxide, solvent for the MCR agonist) had no significant effects on HSV-1 DNA load. B) Administration of the GCR antagonist (mifepristone) with corticosterone significantly decreased HSV-1 DNA load compared to CORT treatment ($p = 0.0333$) ($n > 3$). ($n$ = number of cultures). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

**Figure 4.**

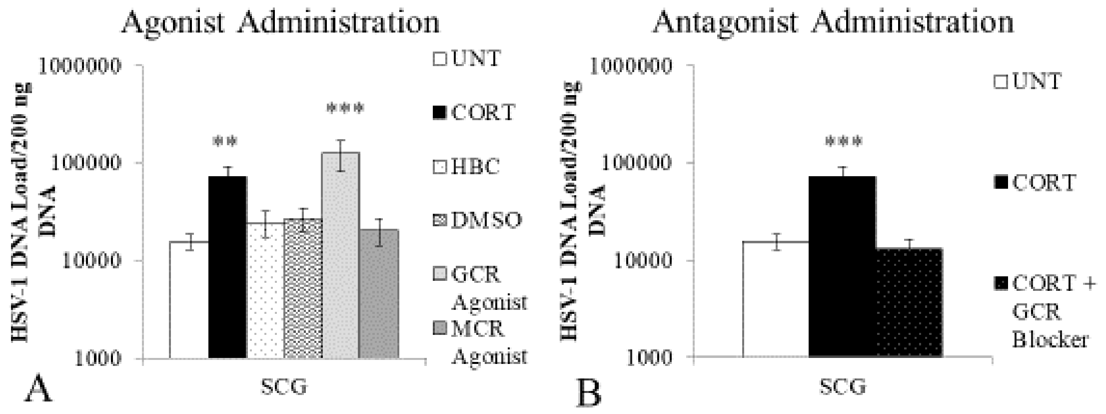


**Figure 5: Inhibition of specific transcription factors and protein kinases differentially affect adrenergic and corticosterone-induced reactivation.** A) Inhibitors of cAMP response element binding protein (CREB; 666-15) and c-Jun N-terminal kinase (JNK; SP600125) significantly decreased HSV-1 DNA load compared to neurons treated with EPI alone ($p = 0.0374$ and 0.0050, respectively) ($n > 3$). Administration of β-catenin inhibitor (iCRT14) did not significantly decrease HSV-1 DNA load compared to either EPI ($p = 0.1033$) or HSV-1-infected untreated control SCG neurons (UNT) ($p = 0.9092$). B) β-catenin inhibitor combined with CORT treatment significantly increased HSV-1 DNA load compared to UNT ($p < 0.0001$). CREB and JNK inhibitors combined with CORT treatment significantly decreased HSV-1 DNA load compared to EPI treatment ($p = 0.0180$ and 0.0131, respectively) ($n > 3$). (*n* = number of cultures). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

**Figure 5.**

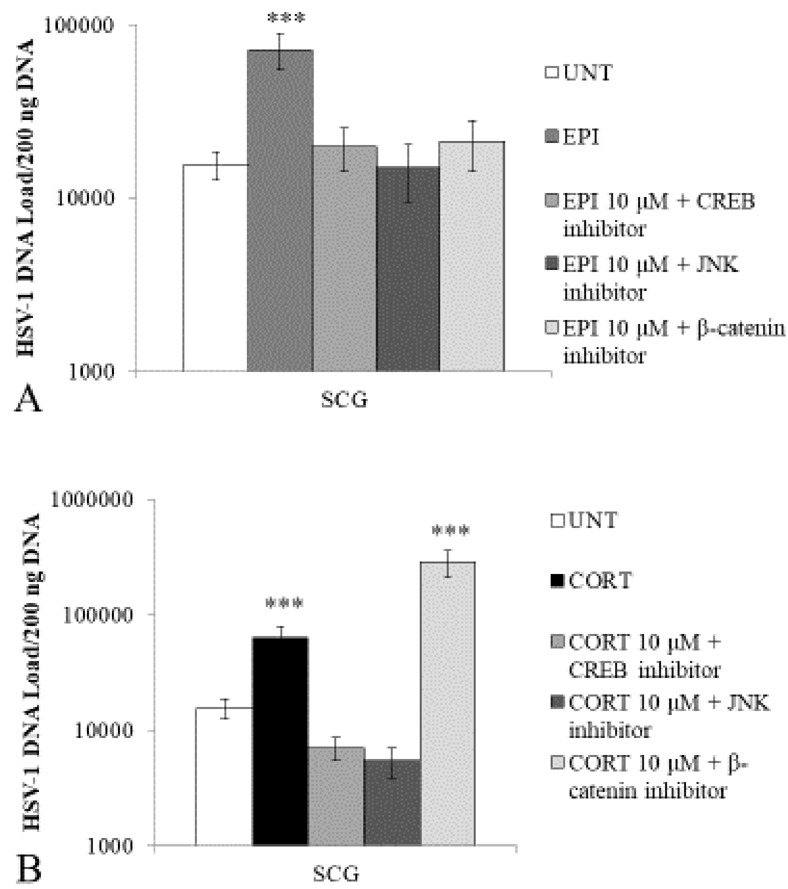
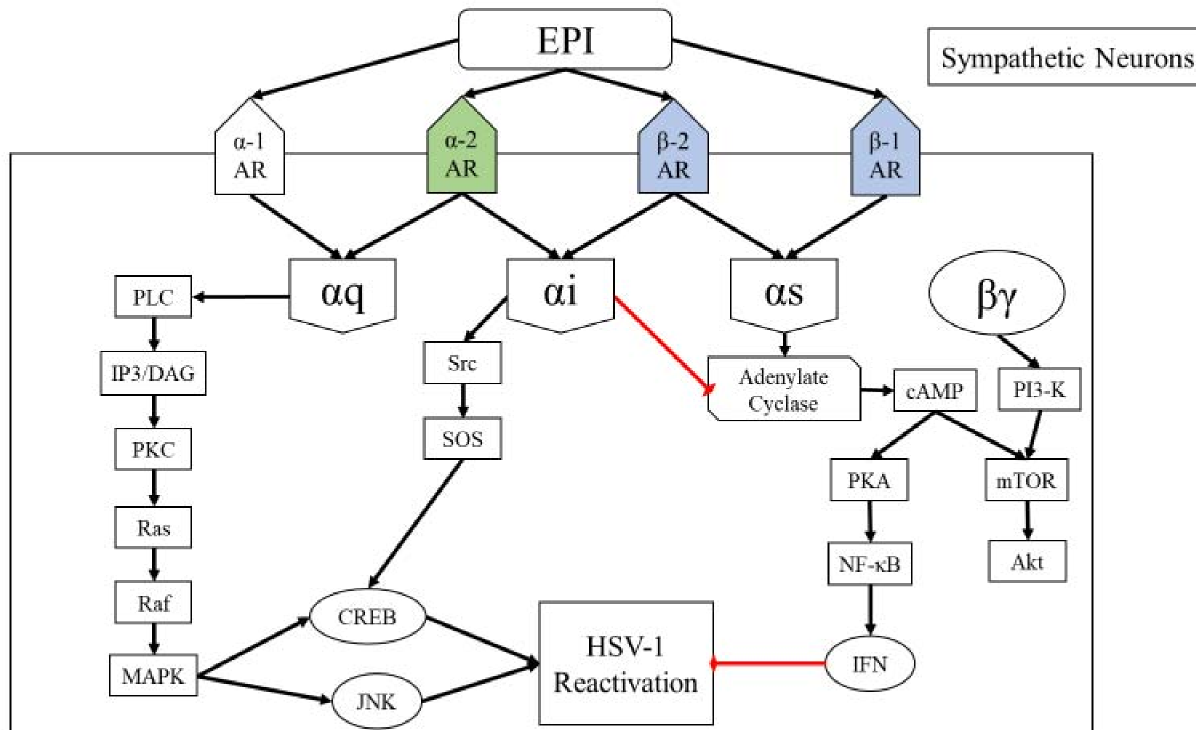


**Figure 6: Potential mechanism by which epinephrine could induce HSV-1 reactivation through multiple adrenergic receptors, the transcription factor CREB, and the cellular kinase JNK in sympathetic neurons.** Activation of either the α-1 or the α-2 adrenergic receptors (ARs) can stimulate the G protein alpha q ($G\alpha_q$), which activates phospholipase C (PLC). PLC initiates a signal cascade involving the secondary messengers inositol triphosphate (IP3) and diacylglycerol (DAG), protein kinase C (PKC), the Ras family of small GTPases, the Raf kinase family, and the mitogen-activated protein kinases (MAPKs), which can ultimately activate the transcription factor cAMP response element-binding protein (CREB) and the c-Jun N-terminal kinase (JNK), both of which have been previously implicated in HSV-1 reactivation. In our studies, inhibition of both CREB and JNK resulted in blocking adrenergic reactivation. Activation of either the α-2 or the β-2 ARs can stimulate the inhibitory G protein alpha ($G\alpha_i$), which can activate the Src tyrosine kinases and activate the SOS DNA repair response which can lead to CREB activation. Gi also inhibits adenylate cyclase activity. In contrast, β-2 AR can also activate the stimulatory G protein alpha ($G\alpha_s$), as can the β-1 AR, which stimulates adenylate cyclase to produce the secondary messenger cyclic AMP (cAMP). cAMP can activate the mTOR and Akt pathway, but also activate protein kinase A (PKA) and the transcription factor nuclear factor κ-B (NF-κB), which is known for stimulating interferon expression (IFN). Interferon expression can inhibit HSV-1 reactivation. Black arrows indicate stimulation or activation, while red lines indicate inhibition.

**Figure 6.**



## TABLES

**Table 1. Key resources used in this study. Related to Materials & Methods.**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Bacterial and Virus Strains | | |
| HSV-1 strain 17syn+ | John Hay at SUNY Buffalo, NY | N/A |
| Chemicals, Peptides, and Recombinant Proteins | | |
| Epinephrine bitartrate | Sigma-Aldrich | E4375 |
| Corticosterone:(2-Hydroxypropyl)-β-cyclodextrin | Sigma-Aldrich | C174 |
| Phenylephrine hydrochloride | Sigma-Aldrich | P6126 |
| Clonidine hydrochloride | Sigma-Aldrich | C7897 |
| Dobutamine hydrochloride | Sigma-Aldrich | D0676 |
| Terbutaline hemisulfate | Sigma-Aldrich | T2528 |
| Phentolamine hydrochloride | Sigma-Aldrich | P7547 |
| Timolol maleate | Sigma-Aldrich | T6394 |
| Dexamethasone: (2-Hydroxypropyl)-β-cyclodextrin | Sigma-Aldrich | D2915 |
| Aldosterone | Sigma-Aldrich | A9477 |
| Mifepristone | Sigma-Aldrich | M8046 |
| Trichostatin A | Sigma-Aldrich | T8552 |
| Vehicle (DMSO; Dimethyl sulfoxide) | ThermoFisher | BP231-100 |
| Vehicle (HBC; (2-Hydroxypropyl)-β-cyclodextrin) | Sigma-Aldrich | H107 |
| 666-15 (CREB inhibitor) | EMD Millipore | 5.38341.0001 |
| iCRT14 (β-catenin inhibitor) | Sigma-Aldrich | SML0203 |
| SP600125 (JNK inhibitor) | Sigma-Aldrich | SP600125 |
| Acyclovir | Sigma-Aldrich | PHR1254 |
| TRIsure | Bioline | 38033 |
| Neurobasal A medium | Gibco | 10888-022 |
| NeuroCult SM1 Neuronal Supplement | StemCell Technologies | 05711 |
| Penicillin/Streptomycin | Corning | 30-002-Cl |
| Glutamax | ? | ? |
| Nerve Growth Factor | Peprotech | 450-34 |
| Glial-derived neurotrophic factor | R&D Systems | 512-GF |
| Neurturin | R&D Systems | 477-MN |
| Ciliary neurotrophic factor | Peprotech | 450-50 |
| 5-fluorodeoxyuridine | Sigma-Aldrich | F0503 |
| Matrigel | Corning | 354234 |
| Papain | Worthington | LK003176 |
| Collagenase | Worthington | LS004176 |
| Neural Protease (dispase) | Worthington | LS02109 |
| Optiprep | Sigma-Aldrich | D1556 |
| Critical Commercial Assays | | |
| iTaq Universal Probes Supermix | Biorad | 172-5131 |

| | | |
|---|---|---|
| Eukaryotic 18S rRNA Assay (20X) | Applied Biosystems | 4319413E |
| iScript cDNA synthesis kit | Biorad | 17C-6891 |
| QX200 EvaGreen ddPCR Supermix | Biorad | 186-4034 |
| Experimental Models: Cell Lines | | |
| Vero 76 cells | ATCC | CRL-1587 |
| Experimental Models: Organisms/Strains | | |
| Swiss Webster ND4 mice | Harlan | ND4 |
| Oligonucleotides | | |
| Primers used in this study are listed in Table S1 | | |
| Software and Algorithms | | |
| SAS JMP 12 | | |
| Other | | |
| Lab-Tek II chamber slides | ThermoFisher | 154534 |

**Table 2. Primer sets used in this study. Related to Materials & Methods.**

**PRIMERS & PROBES**

**qPCR**

| **Target** | | ***Sequence*** |
|---|---|---|
| HSV-1 TK | Forward | TAATGACCAGCGCCCAGAT |
| | Reverse | CGATATGAGGAGCCAAAACG |
| | Probe | FAM-TGGGTTCGC-ZEN-GCGACGATATCG-3IABkFQ |

**ddPCR**

| **Target** | ***Sequence*** |
|---|---|
| HSV-1 ICP4 | TTTCCCACCCAAGCATCGACC |
| | TGCTTGTTCTCCGACGCCAT |
| HSV-1 ICP0 | GGATGCAATTGCGCAACAC |
| | GCGTCACGCCCACTATCAG |
| HSV-1 ICP27 | GTGCAAGATGTGCATCCACCACAA |
| | TGCAATGTCCTTAATGTCCGCCAG |
| HSV-1 VP16 | ACCTGTTTGACTGCCTCTG |
| | TGACGAACATGAAGGGCTG |
| Mouse 18S rRNA | GGAGAACTCACGGAGGATGAG |
| | CGCAGCTTGTTGTCTAGACCG |

# Chapter 4: Corticosterone induces HSV-2 reactivation by activating either the glucocorticoid or mineralocorticoid receptors in sensory and sympathetic neurons

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*Under preparation*

## ABSTRACT

Herpes simplex virus 2 (HSV-2) infects and establishes latency in sensory and autonomic neurons, from which it can reactivate to cause recurrent disease throughout the life of the host. Stress has been correlated with HSV-2 recurrences in both humans and in animal models. To determine whether stress-related hormones reactivate quiescent HSV-2 infections within sensory and autonomic neurons, we analyzed viral DNA and the production of viral progeny after treatment with the stress hormones epinephrine and corticosterone. Epinephrine had no effects on HSV-2 reactivation in any neuronal type. Corticosterone reactivated HSV-2 from both sympathetic superior cervical ganglia (SCG) neurons and sensory trigeminal ganglia (TG) neurons. CORT-induced reactivation occurred through activation of either the glucocorticoid receptor (GCR) or the mineralocorticoid receptor (MCR) for HSV-2 reactivation in both types of neurons. Inhibition of transcription factors β-catenin and cyclic AMP response element binding protein (CREB), protein kinases c-Jun N-terminal kinase and phosphatidylinositol 3-kinase (PI3-K), or mTOR did not block CORT-induced HSV-2 reactivation in either type of neuron. Therefore, corticosterone induces HSV-2 reactivation in sensory and sympathetic neurons through a mechanism that is independent of common second messenger signaling pathways downstream of glucocorticoid receptors.

## INTRODUCTION

Herpes simplex virus 2 (HSV-2) infects mucosal cells, then establishes a life-long latent infection in sensory and autonomic ganglia innervating the site of infection. The virus can reactivate to cause recurrent genital lesions throughout the life of the host. Symptomatic and asymptomatic recurrences can be associated with autonomic dysfunction, including urinary retention, constipation, and erectile dysfunction or impotence [1]. More rarely, HSV-2 causes recurrent sacral meningitis, typically unrecognized as HSV-2-related disease. Furthermore, 60-90% of those infected with HSV-2 experience recurrent disease. The mechanisms by which HSV-2 reactivates in primary adult neurons are not understood.

Stress is strongly correlated with recurrent HSV-2 disease in humans and with reactivation in animal models [2, 3]. The two major stress hormones, epinephrine and corticosterone, cause a "short-term flight or fight response" and a "long-term" stress response, respectively. Receptors for these stress hormones 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) [4-6].

Epinephrine, a catecholaminergic hormone secreted by the adrenal medulla, is regulated by the sympathetic nervous system. Epinephrine induces HSV-1 reactivation in an *in vivo* ocular model of infection in rabbits [7], although adrenergic reactivation is significantly reduced if the latency associated transcript (LAT) is deleted or mutated [8]. Iontophoresis of epinephrine during HSV-2 latent infection induces reactivation but not to the same extent as HSV-1 in the rabbit model, and replacement of the first ~1500 bp of the HSV-1 LAT with HSV-2 LAT significantly reduces epinephrine-induced reactivation [9]. A functional cAMP response element (CRE) has been identified in the LAT promoter of HSV-2 [10], and a CRE site in the LAT promoter and a

348 base pair sequence within LAT exon 1 were identified as essential for adrenergic reactivation of HSV-1 [11, 12], but follow-up studies were unable to further map any specific binding sites within exon 1 [13].

Corticosterone, a glucocorticoid secreted by the adrenal cortex, binds to the glucocorticoid (GCR) and mineralocorticoid receptors (MCR). Its major effects on humans and mammals include regulation of metabolism and suppression of the immune system. Corticosterone induces HSV-2 reactivation *in vivo,* presumably through immune system suppression [14-17]. However, several stress-induced cellular transcription factors expressed in TG neurons stimulate the HSV-1 ICP0 promoter [18] and ICP0 alone can initiate reactivation of HSV-1 and HSV-2 from latency [19]. In addition, we recently demonstrated that corticosterone decreases HSV-2 DNA replication and infectious virus titer during productive infection in sympathetic, but not sensory, neurons [6]. Thus, corticosterone could potentially induce HSV-2 reactivation by acting directly on neurons that harbor latent virus, rather than acting through immune system suppression to indirectly induce reactivation.

To determine if stress hormones differentially reactivate HSV-2 at a cellular level in different types of mature neurons, we administered either corticosterone (CORT) or epinephrine (EPI) to primary murine adult sensory and autonomic neurons quiescently infected with HSV-2. Quantification of viral replication, production of viral progeny, and gene expression showed that corticosterone, but not epinephrine, induces HSV-2 reactivation in sensory and sympathetic primary adult neurons. Furthermore, administration of stress hormone agonists and antagonists demonstrated that corticosterone can induce HSV-2 reactivation through activation of either glucocorticoid or mineralocorticoid receptors, although inhibition of downstream signaling molecules does not prevent corticosterone-induced reactivation.

## MATERIALS & METHODS

**Cell lines and Virus Strains.** HSV-2 strain 333 was originally transferred from Gary Hayward (Johns Hopkins, MD) to the Krause lab (FDA, Bethesda, MD). Virus was propagated in Vero cells (ATCC) and first passage stocks were transferred to the Margolis lab (UCSF, San Francisco, CA). Virus was then propagated in Vero cells and first passage stocks were transferred to the Bertke lab (Virginia Tech, Blacksburg, VA). Stocks were titrated in Vero cells in quadruplicate to determine concentration. Stock viruses were diluted in Neurobasal A medium (Gibco) for inoculation of primary adult murine neuronal cultures.

**Mice.** Female 6 week old Swiss Webster mice were purchased from Envigo. Mice were euthanized and tissues processed following delivery, without being housed in the animal facility. All animal care and handling was in accordance with the Virginia Tech Institutional Care and Use Committee (IACUC# 13-008-CVM and 15-237).

**Primary Adult Murine Neuronal Cultures.** Six-week old Swiss Webster female mice (Envigo) 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 removed, incubated at 37°C for 20 minutes in papain (≥100 units) (Worthington) reconstituted with 5 mL of Neurobasal A medium (Gibco), followed by 20 minutes in 4 mg/mL collagenase and 4.67 mg/mL dispase (Worthington) diluted in Hank's balanced salt solution (HBSS) (Gibco) on a rotator. Following mechanical trituration with a 1 mL pipette, the TG cell suspension was layered on a 4-step Optiprep (Sigma) gradient. Optiprep was first diluted with 0.8% sodium chloride (50.25:49.75 mL) to make a working solution (specific gravity 1.15). Then, cold Optiprep working solution was diluted with

37°C Neurobasal A medium to make each step of the gradient: first or bottom layer 550 μL Neurobasal A medium and 450 μL Optiprep working solution; second layer 650 μL Neurobasal A medium and 350 μL Optiprep working solution; third layer 750 μL Neurobasal A medium and 250 μL Optiprep working solution; and the fourth or top layer 850 μL Neurobasal A medium and 150 μL Optiprep working solution. The gradient with the TG cell suspension was centrifuged at 800 x g for 20 minutes, and the two middle layers containing neuronal cells (~ 2 mL) were collected. SCG and CG were triturated with a 200 μL pipette and did not go through a gradient step because they contain less ganglionic debris after dissociation. TG, SCG, and CG were washed twice with Neurobasal A medium supplemented with 2% SM-1 (StemCell Technologies) and 1% penicillin-streptomycin (Corning). Neurons were counted and plated on Matrigel-coated (Corning) Lab-Tek II chamber slides (ThermoFisher) at a density of 3000 neurons/50μL per well and incubated in 37°C/5% $CO_2$ incubator in Complete Neuro media (Neurobasal A media supplemented with 2% SM-1 (StemCell Technologies), 10 mg/mL penicillin-streptomycin (Corning), 10 μL/mL Glutamax (ThermoFisher), 10 μg/mL nerve growth factor (Peprotech), 10 μg/mL glial-derived neurotrophic factor (Peprotech), 10 μg/mL neurturin (Peprotech), 10 μg/mL ciliary neurotrophic factor (Peprotech; for CGs only) and 200 mM 5-fluorodeoxyuridine (Sigma) to deplete non-neuronal cells. After one hour, debris were removed by pipette and cells were maintained in 300 μL of fresh Complete Neuro media until infection.

**Establishment of Quiescent Infection.** Four days after plating, media was removed and neurons were inoculated with 30 multiplicity of infection (moi) of HSV-2 (strain 333). 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) for five days to establish and maintain a quiescent infection *in vitro*.

**Reactivation with Stress Hormones.** Five days after establishment of a quiescent 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. 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.

**Reactivation with Receptor Agonists & Antagonists.** For testing corticosterone receptor specificity, five days after establishment of a quiescent infection media containing acyclovir was removed and Complete Neuro media with no acyclovir and with either the corticosterone agonists dexamethasone (GCR agonist) or aldosterone (MCR agonist) (Sigma) were added to Complete Neuro media at 10 μM. The corticosterone antagonists mifepristone (GCR antagonist), eplerenone (MCR 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, five days after establishment of quiescent 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), or 100 nM mTOR inhibitor rapamycin (EMD Millipore) with or without 10 μM epinephrine or corticosterone-HBC were added to primary adult neuronal cultures quiescently infected with HSV-2.

**Quantitation of HSV viral load.** Viral DNA was extracted from neuronal cultures 72 hours post-reactivation (hpr) with TRIsure reagent (Biorad), according to the manufacturer's instructions. Viral DNA load was determined by quantifying viral DNA by qPCR using HSV-2 thymidine kinase (TK) gene-specific primers and probes (Table 2). All assays were normalized to 18s rRNA (Applied Biosystems) and reported as viral copy number in 200 ng of total DNA.

**Quantitation of HSV gene expression.** Viral RNA was extracted from neuronal cultures at 1 hour, 2 hours, and 24 hours hpr with TRIsure reagent (Biorad), according to the manufacturer's instructions, and converted to cDNA with iScript (Biorad). Gene expression was determined by quantifying the HSV immediate early genes ICP0, ICP4, ICP27, and VP16 by digital droplet PCR using HSV-2 gene-specific primers (Table 2) and EvaGreen QX200 Master mix (Biorad). All assays were normalized to 18s rRNA (Table 2) and reported as fold change of epinephrine or corticosterone treated samples compared to untreated.

**Quantification and Statistical Analysis.** Data are presented as means ± standard errors of the mean. Statistics were performed using parametric analyses with JMP Pro version 12, including analysis of variance with Tukey high standard deviation contrast tests. N represents the number of cultures. Where indicated, asterisks denote statistical significance as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

## RESULTS

**Stress hormones induce HSV reactivation *in vitro*.** To determine if stress hormones induce HSV-2 reactivation from quiescent infection, primary adult murine sensory trigeminal (TG), sympathetic superior cervical (SCG) and parasympathetic ciliary ganglia (CG) neurons were quiescently infected with HSV-2 (strain 333) for five days in acyclovir (ACV; 300 μM). Media

containing ACV was removed and epinephrine (EPI) or corticosterone (CORT, conjugated to (2-Hydroxypropyl)-β-cyclodextrin in the water-soluble form) were added to the quiescently infected neuronal cultures. Viral DNA was isolated 72 hours post-reactivation (hpr) and quantified by qPCR for the thymidine kinase (TK) gene.

CORT induced reactivation of HSV-2 in both sensory TG and sympathetic SCG neurons ($p < 0.0001$ for both) (Figure 1), but EPI had no effect. Neither EPI nor CORT had a significant effect on reactivation in parasympathetic CG neurons. Trichostatin A (TSA), a histone deacetylase inhibitor that has been shown to reactivate HSV-1 and HSV-2 reliably from quiescence in sensory and sympathetic neurons [20-22], was used as a positive control. Therefore, EPI and CORT have differential effects on HSV-2 reactivation in primary adult neurons.

**Stress hormones alter HSV immediate early gene expression during reactivation.** To determine which immediate early (IE) HSV genes respond to CORT treatment during reactivation, viral RNA was isolated 1 hour, 2 hours, and 24 hours following CORT-induced reactivation. IE ICP4, ICP0, and ICP27 transcript expression was quantified by digital droplet PCR. Viral protein 16 (VP16) transcript expression was also quantified, since previous reports have demonstrated its involvement in stress-induced reactivation [23, 24].

In HSV-2 quiescently-infected sympathetic SCG neurons, CORT significantly increased ICP4, ICP0, and VP16 gene expression by 1 hour post treatment ($p < 0.05$), compared to untreated control neurons (UNT), which had ACV removed but received no other treatment. By 2 hrs post treatment, ICP0 and ICP27 gene expression was significantly decreased, compared to untreated sympathetic control neurons ($p < 0.01$). By 24 hrs post treatment, gene expression of ICP4, ICP0,

ICP27, and VP16 were above untreated control neurons, although only ICP4 expression reached statistical significance ($p < 0.05$) (Figure 2).

In HSV-2 quiescently-infected sensory TG neurons, CORT treatment significantly decreased ICP4, ICP27, and VP16 gene expression by 1 hour post-reactivation ($p < 0.05$), which returned to untreated control levels by 2 hrs post treatment, with the exception of ICP27, which remained suppressed ($p < 0.01$). By 24 hrs post treatment, ICP0 and VP16 gene expression was increased compared to untreated controls ($p < 0.05$) (Figure 2). Therefore, CORT administration differentially affects HSV-2 gene expression, depending on the type of neuron being treated.

**Steroid receptor specificity of corticosterone-induced reactivation.** Previously we demonstrated that glucocorticoid receptors (GCR) are expressed in cultured primary adult murine sensory TG and sympathetic SCG neurons and HSV antigens generated during productive infection co-localize with receptor expression [6]. CORT can bind to and activate both glucocorticoid (GCR) and mineralocorticoid (MCR) receptors [25].

To determine whether CORT-induced reactivation was occurring through binding of CORT to GCR or MCR, quiescently infected neurons were treated with dexamethasone, a GCR agonist, or aldosterone, a MCR agonist. Seventy-two hours after treatment, viral DNA was isolated and quantified by qPCR for the TK gene.

In both sympathetic SCG and sensory TG neurons, dexamethasone (GCR agonist) and aldosterone (MCR agonist) individually induced HSV-2 reactivation similar to CORT treatment, which were statistically significant compared to untreated control neurons ($p < 0.001$) (Figure 3A and 3B). To determine whether we could block CORT-induced reactivation of HSV-2, SCG and TG neurons quiescently infected with HSV-2 were treated with CORT and the GCR antagonist

mifepristone or the MCR antagonist eplerenone. However, neither mifepristone nor eplerenone alone were able to block CORT-induced HSV-2 reactivation. To determine whether CORT is capable of inducing HSV-2 reactivation through both receptors, we administered both mifepristone and eplerenone together, which effectively blocked CORT-induced reactivation in both sensory TG and sympathetic SCG neurons (Figure 3C and 3D). Therefore, CORT induces HSV-2 reactivation in sympathetic SCG and sensory TG neurons through activation of either the glucocorticoid or mineralocorticoid receptors.

**Protein kinase and transcription factor inhibitors selectively block stress-induced reactivation.** Upon activation, the GCR and MCR transport into the nucleus to directly bind DNA of target genes, recruiting the p300/CBP coactivator complex to activate gene transcription through histone acetylation [26]. However, the GCR also activates other signaling pathways that include cAMP-response element binding protein (CREB) and c-Jun N-terminal kinase (JNK), and the Wnt pathway also becomes activated to inhibit β-catenin degradation and activation of Wnt responsive genes. Previously, CREB and JNK have been demonstrated to be important in stress-induced reactivation of HSV-1 [27-29]. The transcription factor β-catenin plays a role in bovine herpesvirus 1 latency and reactivation [30-32] and induction of β-catenin expression decreases HSV-1 replication *in vitro* [33]. Continuous activation of PI3-K and its downstream effector mTORC promote latency, and their inhibition has been implicated in inducing reactivation by nerve growth factor-deprivation in primary embryonic sympathetic neuronal cultures [34-37].

To determine whether corticosterone-induced reactivation requires activation or repression of CREB, JNK, β-catenin, PI3-K, or mTORC, quiescently infected SCG and TG neurons were treated with CORT and individual inhibitors for each of these factors. The CREB inhibitor 666-15

blocks CREB-mediated gene transcription [38-40], the JNK inhibitor SP600125 reduces c-Jun phosphorylation activity by JNK [29], the β-catenin inhibitor iCRT14 inhibits the interaction between β-catenin and T-cell factor (TCF) family members [32], the phosphatidylinositol 3-kinase (PI3-K) inhibitor LY294002 [29, 34], and the mTOR inhibitor rapamycin [35-37]. CREB, JNK, β-catenin, PI3-K, and mTOR inhibition did not block CORT-induced reactivation in either sympathetic SCGs or sensory TG neurons (Figure 4).

## DISCUSSION

In our studies, we demonstrate that epinephrine (EPI) had no effects on HSV-2 quiescent infection, regardless of neuron type. However, the stress hormone corticosterone (CORT) induces reactivation of HSV-2 in sympathetic and sensory neurons, but not in parasympathetic neurons. This is in contrast to CORT's effect on HSV-2 productive infection, in which CORT decreased HSV-2 DNA replication and infectious virus progeny production in sympathetic SCG neurons [6]. This difference may be due to differences in epigenetic regulation of the HSV genome between productive infection and latency [41]. Activation of the p300/CBP complex is involved in HSV-1 reactivation [42], and the transactivation domain of VP16 recruits p300 and CBP to viral immediate early genes [43]. Administration of CORT resulted in a significant increase in VP16 at 1 hour post-reactivation in sympathetic neurons and 24 hours post-reactivation in sensory neurons. In addition, we determined that either the GCR or MCR could be stimulated by CORT to induce HSV-2 reactivation, and these steroid receptors can interact with the p300/CBP complex [44]. Therefore, CORT could be potentially inducing HSV-2 reactivation and suppressing HSV-2 productive infection through the chromatin modulatory properties of these two receptors and the differences in chromatin conformation between productive infection and latency [45].

Trichostatin A (TSA), a histone deacetylase inhibitor, has previously been used to induce HSV-1 reactivation in quiescently infected rat pheochromocytoma cells (PC-12) and primary adult sensory and sympathetic neurons [20-22]. In our studies, TSA induced HSV-2 reactivation in sensory neurons, but not in sympathetic or parasympathetic neurons. We previously showed that following ocular infection in guinea pigs, HSV-2 was unable to fully establish latency or was not reactivation competent in parasympathetic ciliary ganglia, either *in vivo* or *in vitro*, and instead maintained a persistent lytic infection [46]. Taken together, these results suggest that differences in chromatin regulation of the viral genome may exist during latency in parasympathetic neurons, compared to sensory and sympathetic neurons. As chromatin modulation has previously been shown to be involved in HSV latency and reactivation, our findings support the hypothesis that different types of neurons maintain the viral genome in different histone configurations.

CORT treatment also significantly altered HSV-2 gene expression in both sympathetic and sensory neurons compared to untreated controls, although the patterns of gene expression differed substantially in the different types of neurons. CORT treatment in sympathetic SCG neurons increased ICP4, ICP0, and VP16 expression by 1 hr post-treatment compared to untreated controls. Expression of either ICP0 or ICP4 alone can induce reactivation of HSV-1 from latency [47-49]. Previous reports using PI3-K inhibition as a reactivation stimulus in quiescently infected embryonic sympathetic neuronal cultures detected changes in ICP27 and VP16 expression at 15 and 20 hpr, but the earliest time point assayed was 10 hpr [23]. In these embryonic sympathetic neurons, VP16 is required for the second phase of HSV-1 reactivation following a PI3-K inhibitor as a reactivation stimulus [23]. Thus, any of the three gene products may have been responsible for initiating the more ordered pattern of gene expression during Phase II of reactivation. In contrast, CORT treatment in sensory TG neurons significantly decreased ICP4, ICP27, and VP16

expression at 1 hr post-treatment and increased ICP0 and VP16 expression by 24 hrs. Taken together, this demonstrates that HSV-2 gene expression during the initial phase of reactivation differs between different types of neurons in response to the same stimulus.

We determined that the GCR and the MCR are redundant pathways for CORT-induced reactivation, with activation of either receptor being sufficient to induce HSV-2 reactivation. Administration of the GCR agonist dexamethasone has been shown to reactivate HSV-1 in the rabbit and mouse models of ocular infection [50-52] and can stimulate expression of a reporter gene from the HSV-1 ICP0 promoter by activating several transcription factors [18]. Activated GCR transports into the nucleus to directly bind DNA of target gene, recruiting p300/CBP initiation complex that acetylates histones and initiates gene transcription [26]. The MCR activates a similar initiation complex. However, the GCR can also activate additional signaling pathways, culminating in activation of CREB, Oct1, and JNK. Corticosteroids also activate the Wnt pathway, stabilizing β-catenin, which enters the nucleus and binds to T cell factor/lymphoid enhancer factor (TCF/LEF) family proteins to function as a transcription factor for Wnt target genes. Inhibition of the potential downstream effectors of GCR and MCR that we tested, including CREB, β-catenin, and JNK, did not block HSV-2 reactivation in either sympathetic SCG or sensory TG neurons. This suggests that the mechanism by which CORT induces HSV-2 reactivation involves direct binding of the GCR to the HSV-2 latent episome. Both GCR and MCR can remodel chromatin [44, 53], and a histone methyl/phosphorylation switch on HSV lytic promoters is important in stress-mediated HSV reactivation [29, 54].

Therefore, we propose that the mechanism by which CORT induces HSV-2 reactivation differs between sympathetic and sensory neurons due to differences in gene expression, and it most likely involves the chromatin remodeling properties of GCR and MCR.

## AUTHOR CONTRIBUTIONS

A. S. B. and A. M. I. designed the studies; A. M. I. performed the experiments; A. S. B., A. M. I., and R. P. D. analyzed and interpreted the data; A. S. B. and A. M. I. wrote the manuscript; and R. P. D. edited the manuscript.

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## FIGURES

**Figure 1: Corticosterone induces HSV-2 reactivation in sympathetic and sensory neurons.** Primary adult murine neuronal cultures from trigeminal ganglia (TG), superior cervical ganglia (SCG), and ciliary ganglia (CG) were infected with HSV-2 for 5 days with 300 μM acyclovir. Media with acyclovir was removed, and cultures were treated with 1.2 μM trichostatin A (TSA; positive reactivation control), 10 μM epinephrine (EPI), or 10 μM corticosterone (CORT). Neurons and media were collected for qPCR analysis at 72 hpr. HSV-2 DNA load was significantly increased by CORT treatment compared to HSV-2 quiescently-infected untreated control neurons (UNT) in both SCG ($p < 0.0001$) and TG neurons ($p < 0.0001$), but not in CG neurons ($n > 3$). ($n$ = number of cultures). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

**Figure 1.**

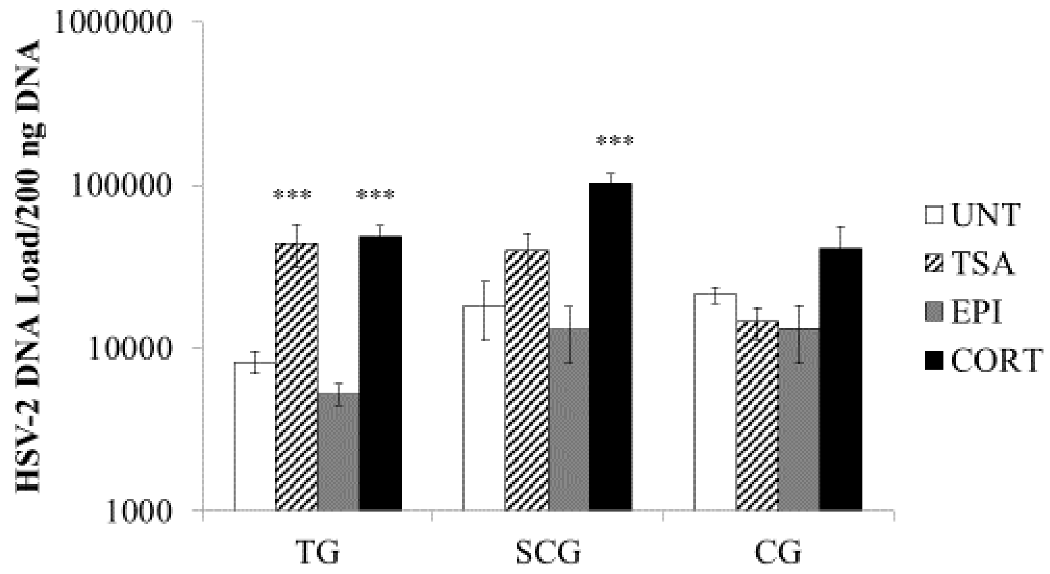
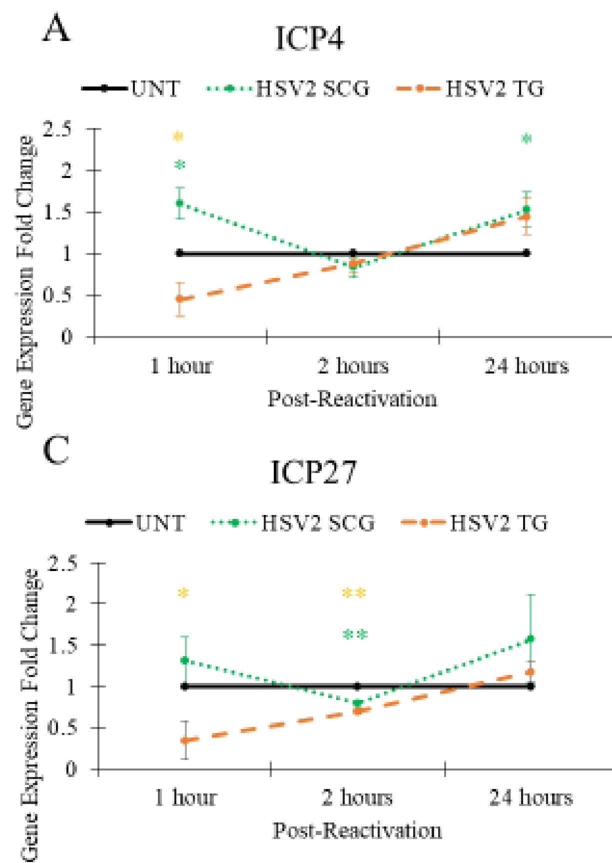


**Figure 2: Stress hormone treatment alters HSV-2 gene expression in sympathetic and sensory neurons.** Primary adult murine neuronal cultures from sympathetic SCG and sensory TG neurons were infected with HSV-2 for 5 days in the presence of 300 µM acyclovir. Media with acyclovir was removed, and cultures were treated with 10 µM corticosterone (CORT). Neurons were collected at 1, 2, or 24 hpr for ddPCR analysis for viral ICP4, ICP0, ICP27 and VP16 gene expression. Gene expression is depicted as fold change of treatment compared to HSV-2 quiescently-infected untreated SCG or TG neurons (UNT), respectively. A) ICP4 gene expression was significantly increased by CORT administration at 1 hpr ($p = 0.0201$) and 24 hpr ($p = 0.0478$) in SCG neurons ($n > 3$). B) ICP0 gene expression was significantly increased by CORT administration at 1 hpr in SCG neurons ($p = 0.0395$) and at 24 hpr in TG neurons ($p = 0.0255$), and significantly decreased at 2 hpr ($p < 0.0001$) in SCG neurons ($n > 3$). C) ICP27 gene expression was significantly decreased at 2 hpr in SCG neurons ($p = 0.0019$), and at 1 hpr ($p = 0.0097$) and 2 hpr ($p = 0.0096$) in TG neurons ($n > 3$). D) VP16 gene expression was significantly increased at 1 hpr in SCG neurons ($p = 0.0008$) and at 24 hpr in TG neurons ($p = 0.0042$), and significantly decreased at 1 hpr in TG neurons ($p = 0.0165$) ($n > 3$). (*n* = number of samples analyzed). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

**Figure 2.**



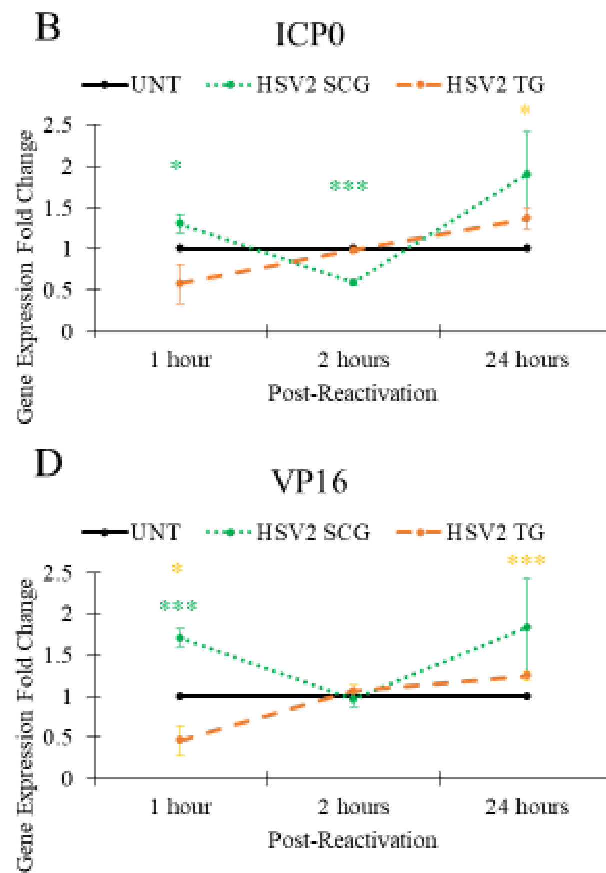



**Figure 3: Steroid receptors involved in corticosterone-induced HSV-2 reactivation in sympathetic and sensory neurons.** Primary adult murine neuronal cultures from sympathetic superior cervical ganglia (SCG) and sensory trigeminal ganglia (TG) were infected with HSV-2 for 5 days with 300 μM acyclovir. Media with acyclovir was removed, and cultures were treated with 10 μM GCR receptor agonist (dexamethaxone), 10 μM MCR agonist (aldosterone) or 10 μM corticosterone with 10 μM steroid receptor antagonists (GCR antagonist mifepristone or MCR antagonist eplerenone). Neurons and media were collected for qPCR analysis at 72 hpr. A) Both the GCR and MCR agonists significantly increased HSV-2 DNA load in sympathetic SCG neurons compared to HSV-2 quiescently-infected untreated control SCG neurons (UNT) ($p < 0.0001$ for dexamethasone and $p = 0.0009$ for aldosterone) ($n > 3$). B) Both the GCR and MCR agonists significantly increased HSV-2 DNA load in sensory TG neurons compared to UNT ($p < 0.0001$ for dexamethasone and aldosterone) ($n > 3$). C) The GCR antagonist and MCR antagonist alone had no effect on CORT-induced reactivation in SCG neurons ($p < 0.0001$ for both compared to UNT). Administration of CORT with both the GCR and MCR antagonist significantly decreased HSV-2 DNA load compared to CORT treatment ($p = 0.0035$) ($n > 3$). D) The GCR antagonist and MCR antagonist alone had no effect on CORT-induced reactivation in SCG neurons ($p < 0.0001$ for both compared to UNT). Administration of CORT with both the GCR and MCR antagonist significantly decreased HSV-2 DNA load compared to CORT treatment ($p = 0.0001$) ($n > 3$). Neither dimethyl sulfoxide (DMSO, solvent for aldosterone, mifepristone, and eplerenone) nor (2-Hydroxypropyl)-β-cyclodextrin (HBC, the carrier molecule for corticosterone) had

significant effects on HSV-2 DNA load. (*n* = number of cultures). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

**Figure 3.**

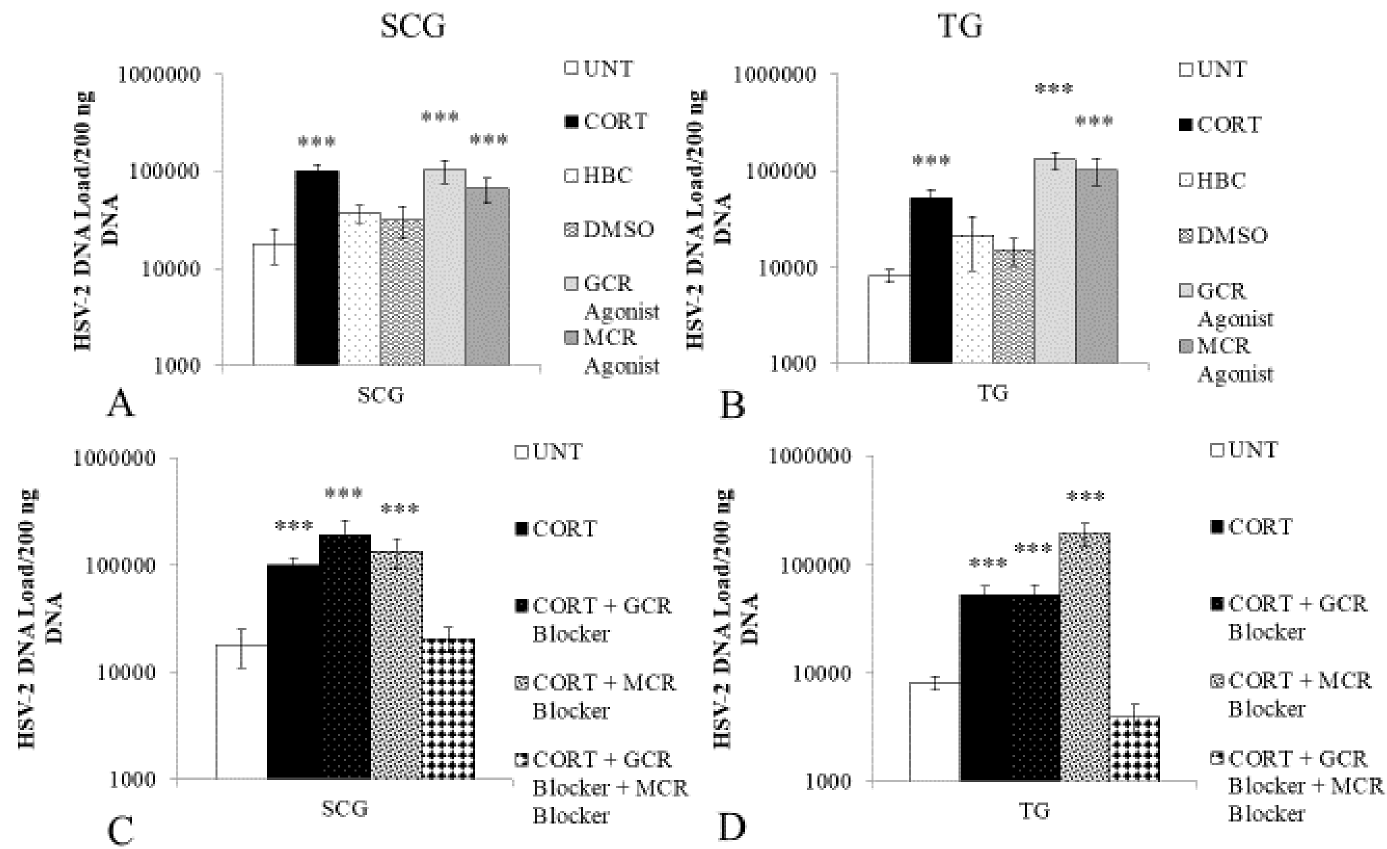
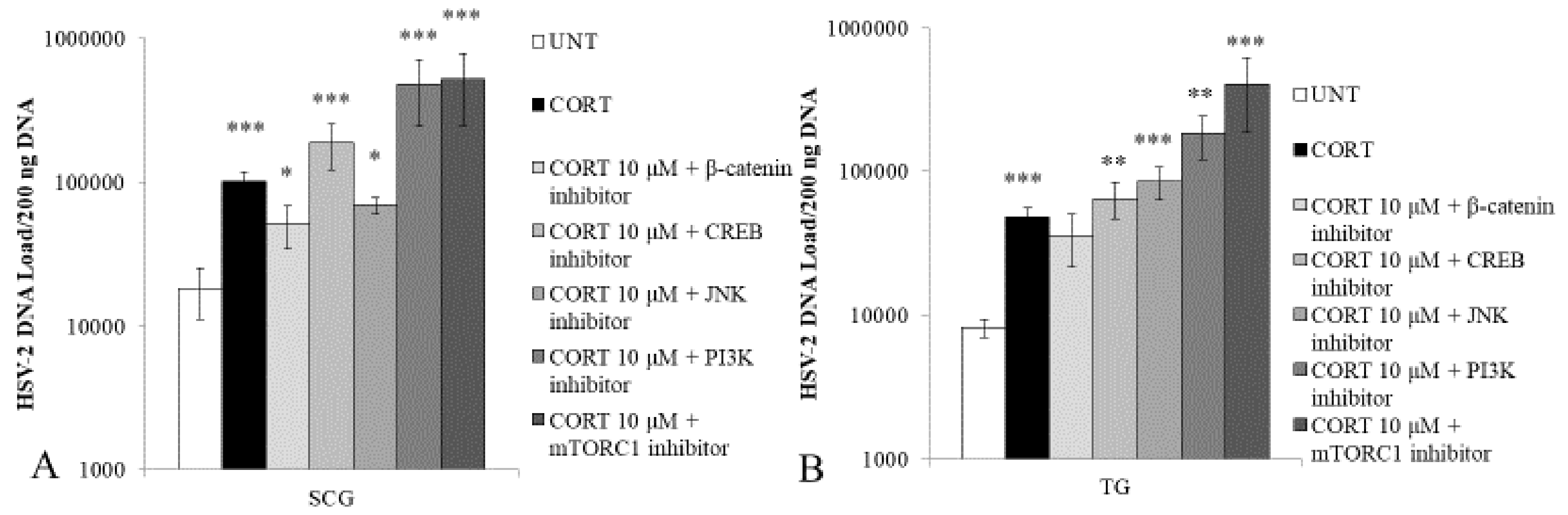


**Figure 4: Inhibition of specific transcription factors and protein kinases has no effect on corticosterone-induced reactivation.** Effect of transcription factor and protein kinase inhibitors on HSV-2 reactivation. A) β-catenin, cAMP response element binding protein (CREB), c-Jun N-terminal kinase (JNK), phosphatidylinositol 3-kinase (PI3K) and mTORC1 inhibitors did not inhibit CORT-induced HSV-2 reactivation in sympathetic SCG neurons ($n > 3$). B) β-catenin, CREB, JNK, (PI3-K), and mTOR inhibitors did not inhibit CORT-induced HSV-2 reactivation in TG neurons ($p > 0.05$ compared to untreated) ($n > 3$). (*n* =number of cultures). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

**Figure 4.**



**TABLES**

**Table 1. Key resources used in this study. Related to Materials & Methods.**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Bacterial and Virus Strains | | |
| HSV-2 strain 333 | Gary Howard at Johns Hopkins, MD | N/A |
| Chemicals, Peptides, and Recombinant Proteins | | |
| Epinephrine bitartrate | Sigma-Aldrich | E4375 |
| Corticosterone:(2-Hydroxypropyl)-β-cyclodextrin | Sigma-Aldrich | C174 |
| Dexamethasone: (2-Hydroxypropyl)-β-cyclodextrin | Sigma-Aldrich | D2915 |
| Aldosterone | Sigma-Aldrich | A9477 |
| Mifepristone | Sigma-Aldrich | M8046 |
| Eplerenone | Sigma-Aldrich | E6657 |
| Trichostatin A | Sigma-Aldrich | T8552 |
| Vehicle (DMSO; Dimethyl sulfoxide) | ThermoFisher | BP231-100 |
| Vehicle (HBC; (2-Hydroxypropyl)-β-cyclodextrin) | Sigma-Aldrich | H107 |
| 666-15 (CREB inhibitor) | EMD Millipore | 5.38341.0001 |
| iCRT14 (β-catenin inhibitor) | Sigma-Aldrich | SML0203 |
| SP600125 (JNK inhibitor) | Sigma-Aldrich | SP600125 |
| LY294002 (PI3-K inhibitor) | Sigma-Aldrich | L9908 |
| Rapamycin (mTOR inhibitor) | EMD Millipore | CAS 53123-88-9 |
| Acyclovir | Sigma-Aldrich | PHR1254 |
| TRIsure | Bioline | 38033 |
| Neurobasal A medium | Gibco | 10888-022 |
| NeuroCult SM1 Neuronal Supplement | StemCell Technologies | 05711 |
| Penicillin/Streptomycin | Corning | 30-002-Cl |
| Glutamax | ThermoFisher | 35050061 |
| Nerve Growth Factor | Peprotech | 450-34 |
| Glial-derived neurotrophic factor | R&D Systems | 512-GF |
| Neurturin | R&D Systems | 477-MN |
| Ciliary neurotrophic factor | Peprotech | 450-50 |
| 5-fluorodeoxyuridine | Sigma-Aldrich | F0503 |
| Matrigel | Corning | 354234 |
| Papain | Worthington | LK003176 |
| Collagenase | Worthington | LS004176 |
| Neural Protease (dispase) | Worthington | LS02109 |
| Optiprep gradient | Sigma-Aldrich | D1556 |
| Critical Commercial Assays | | |
| iTaq Universal Probes Supermix | Biorad | 172-5131 |
| Eukaryotic 18S rRNA Assay (20X) | Applied Biosystems | 4319413E |
| iScript cDNA synthesis kit | Biorad | 17C-6891 |
| QX200 EvaGreen ddPCR Supermix | Biorad | 186-4034 |

| Experimental Models: Cell Lines | | |
|---|---|---|
| Vero cells | ATCC | CRL-1587 |
| Experimental Models: Organisms/Strains | | |
| Swiss Webster ND4 mice | Harlan | ND4 |
| Oligonucleotides | | |
| Primers used in this study are listed in Table S1 | | |
| Software and Algorithms | | |
| SAS JMP 12 | | |
| Other | | |
| Lab-Tek II chamber slides | ThermoFisher | 154534 |

**Table 2. Primer sets used in this study. Related to Materials & Methods.**

**PRIMERS & PROBES**

**qPCR**

| **Target** | | ***Sequence*** |
|---|---|---|
| HSV-2 TK | Forward | AAAACCACCACCACGCAACT |
| | Reverse | TCATCGGCTCGGGTACGTA |
| | Probe | FAM-ACAATGAGC-ZEN-ACGCCTTATGCGGC-3IABkFQ |

**ddPCR**

| **Target** | ***Sequence*** |
|---|---|
| HSV-2 ICP4 | GTCGTCGTCGTCGTCAG |
| | CCGCCTCTGACTCATCAAA |
| HSV-2 ICP0 | GGTCACGCCCACTATCAGGTA |
| | CCTGCACCCCTTCTGCAT |
| HSV-2 ICP27 | CTTTCTGCAGTGCTACCTGAA |
| | CGACGCCTGTCGGACATTAAGGAT |
| HSV-2 VP16 | CATGCTAGATACCTGGAACGAG |
| | TCGACAGAAACTTGCACTCC |
| Mouse 18S rRNA | GGAGAACTCACGGAGGATGAG |
| | CGCAGCTTGTTGTCTAGACCG |

## Chapter 5: General Discussion

Previous studies have shown that epinephrine (EPI) and corticosterone (CORT), the two main stress hormones, can induce reactivation of HSV-1 in animal models of infection, as well as in humans [1-7]. In humans, stress is strongly correlated with the appearance of fever blisters, which are recurrent lesions caused by reactivating HSV-1, as well as anecdotally correlated with recurrences of genital herpes. Although anecdotal evidence correlates stress with HSV-2 reactivation as well, minimal experimental evidence exists. The neurons in which HSV establishes latency express receptors for EPI and CORT [8-10], suggesting that these endocrine factors may be capable of modulating HSV infection in the neurons directly, rather than indirectly through suppressive effects on the immune system.

In this dissertation research, I provide evidence to support the hypothesis that stress hormones affect HSV productive infection in these neurons, which likely impacts clinical disease during initial infection, as well as the ability of the virus to establish latency and reactivate at a later time. Furthermore, the data also demonstrate that stress hormones induce HSV-1 and HSV-2 reactivation by acting directly on the sensory and autonomic neurons in which the viruses are latent. The sensory and autonomic neurons in which HSV-1 and HSV-2 undergo productive infection express adrenergic (AR) and glucocorticoid receptors (GCR). In addition, EPI and CORT have selective effects on sympathetic neurons compared to sensory neurons, on productive compared to quiescent infection, and on HSV-1 compared to HSV-2.

### *Sympathetic Compared to Sensory Neurons*

Similar quantities of latent viral DNA are detected in both sympathetic and sensory neurons *in vivo* and *in vitro* [11-13]. A greater percentage of sympathetic neurons express stress hormone receptors compared to sensory neurons [10], and sympathetic neurons are more sensitive to

endocrine factors than are sensory neurons. Overall, stress hormones had more significant effects on HSV infection and reactivation in sympathetic neurons compared to sensory or parasympathetic neurons. Therefore, the post-ganglionic sympathetic neurons in which HSV establishes latency likely represent an important reservoir of reactivating virus. Previous studies have shown that HSV-1 and HSV-2 establish latency in different types of autonomic neurons [14-19]. Our recent work has also shown that ablation of sympathetic nerve terminals reduces HSV-1-associated clinical disease severity and both HSV-1 and HSV-2 cumulative recurrences in the genital guinea pig model (Figures 1 and 2). However, the role of the autonomic nervous system in HSV pathogenesis has remained largely unexplored. Overall, the autonomic nervous system is an important source of reactivating neurons, particularly in response to stress.

***Productive Compared to Quiescent Infection***

The effects of corticosterone (CORT) administration differed between productive and quiescent infection. CORT decreased HSV-1 and HSV-2 DNA replication and infectious virus progeny during productive infection, while it reactivated both HSV-1 and HSV-2 from quiescence. These results could be explained by the differences in HSV gene expression and chromatin conformation between productive infection and reactivation from latency. During productive infection, HSV undergoes a temporal cascade of gene expression to produce infectious virus. During reactivation from latency, first the genome undergoes transient, disordered expression of many viral lytic genes, referred to as Phase I of reactivation, which then progresses to a second phase more closely resembling the temporal cascade of productive infection [20]. In addition, CORT-induced reactivation of HSV-1 and HSV-2 utilized the GCR, which can couple with the p300/CBP to alter chromatin conformation [21], and chromatin conformation of the HSV episome differs between productive infection and latency [22, 23]. Because the stress hormones were

administered one hour after the inoculation to test for effects on productive infection, it is possible that CORT could inhibit one of the downstream steps of productive infection while being capable of inducing the first phase of reactivation due to differences in chromatin conformation between different phases of infection.

However, epinephrine has similar effects on both HSV-1 productive infection and quiescent infection. Therefore, EPI promotes productive infection and reactivation, at least for HSV-1, but CORT has differential effects on primary infection compared to recurrent infection.

***Differences between HSV-1 and HSV-2***

The effects of stress hormones had differential effects on HSV-1 and HSV-2. EPI increased HSV-1 productive infection and reactivation, but not HSV-2, suggesting that the short-term "fight or flight" response is capable of exacerbating clinical and recurrent HSV-1 disease but not HSV-2. CORT reactivated HSV-1 from quiescence, but only in sympathetic neurons. CORT also reactivated HSV-2, but from both sensory and sympathetic neurons. Therefore, the long-term stress response, characterized by increased levels of CORT in the host, can induce recurrent disease of both HSV-1 and HSV-2, although the sensory neurons harboring latent HSV-2 appear to be more responsive to this type of stress compared to those harboring HSV-1.

CORT-induced HSV-1 reactivation required the glucocorticoid receptor (GCR) only, not the mineralocorticoid receptor (MCR). In contrast, CORT could utilize either the GCR or the MCR to induce HSV-2 reactivation in both sensory and sympathetic neurons; inhibition of both receptors was required to block HSV-2 CORT-induced reactivation. Furthermore, CORT-induced HSV-1 reactivation required the activation of cAMP response element binding protein (CREB) and the inhibition of β-catenin, while CORT-induced HSV-2 reactivation was not affected by inhibition of either CREB or β-catenin. These results suggest that CORT reactivates HSV-1 by activating

specific cell signaling pathways. In contrast, reactivation of HSV-2 may occur primarily through GCR and MCR's ability to remodel chromatin [21, 24]. These studies demonstrate that the mechanisms behind stress hormone-induced reactivation differ between HSV-1 and HSV-2. However, further studies are needed to determine the precise pathways and mechanisms that differentially regulate CORT-mediated reactivation of HSV-1 and HSV-2.

***Conclusions and Future Studies***

Collectively, these studies demonstrate that stress hormones have a significant effect on HSV-1 and HSV-2 productive and latent infection, selectively inducing viral reactivation in a neuron type-specific manner. Although my results show that the mechanisms of reactivation differ for HSV-1 and HSV-2, further experiments are required to fully characterize the pathways by which EPI and CORT affect HSV latent infection in neurons. Studies to determine differences in protein binding to immediate early and VP16 gene promoters, as well as the latency associated transcript regulatory region, between stress hormone-treated and untreated controls are needed. The use of additional pharmacological inhibitors and small interfering-RNAs will aid in determining which second messengers and cellular proteins are required to mediate stress-induced reactivation in primary adult neurons. Animal studies are also needed to assess whether blockage of specific stress hormone receptors and cellular proteins can decrease cumulative recurrences in vivo.

In conclusion, the studies presented here establish that stress hormones are capable of acting directly on neurons productively or latently infected with HSV-1 and HSV-2, rather than just indirectly through suppression of the immune response. Alternative therapies that target neuronal responses to stress can be considered and tested to augment antiviral treatment, potentially improving the outcome of clinical disease. These studies also establish a substantial role for the

autonomic nervous system in HSV-related disease, challenging the paradigm that HSV only reactivates from sensory neurons to cause recurrent disease. Although additional studies are needed, this work provides a foundation for identifying alternative therapeutic targets to improve clinical outcomes of acute HSV-related disease and reduce and reduce recurrent disease in millions of people affected by HSV-1 and HSV-2.

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# FIGURES

**Figure 1. Sympathetic nerve terminal ablation with 6-hydroxydopamine (6-OHDA) in acute clinical disease.** Acute clinical disease was reduced in guinea pigs treated with 6-OHDA before HSV-1 infection. (Lee, Ives, Yanez, Bertke, unpublished)

**Figure 1.**

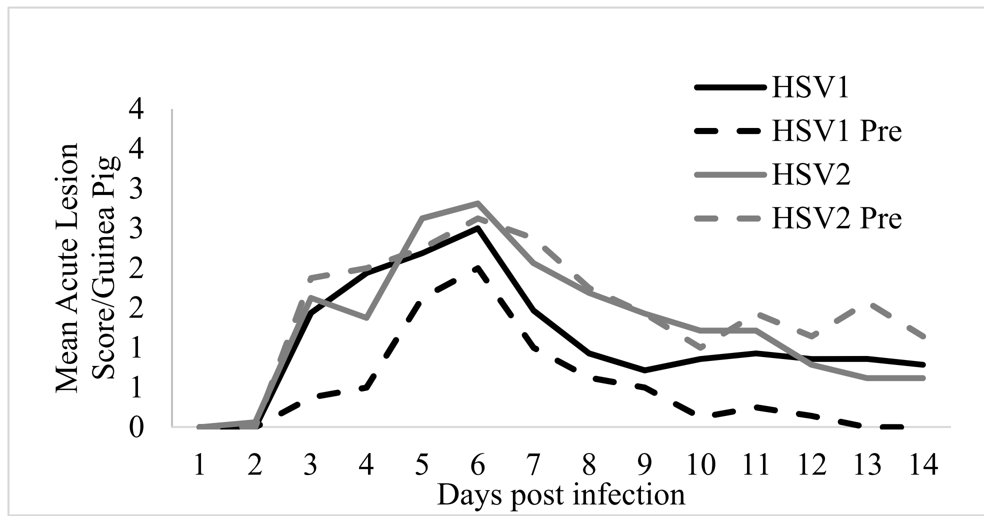

**Figure 2. Sympathetic nerve terminal ablation with 6-hydroxydopamine (6-OHDA) in cumulative recurrences.** Recurrences were reduced in guinea pig treated with 6-OHDA before infection with HSV-1 or HSV-2. Thus, sympathetic pathways are important for HSV-1 acute infection and recurrences of both HSV-1 and HSV-2. (Lee, Ives, Yanez, Bertke, unpublished).

**Figure 2.**