The Role of Autonomic Neurons in the Pathogenesis of Herpes Simplex Virus Type 1 and 2

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy In
Biomedical and Veterinary Sciences

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Dec 1st 2015
Blacksburg, VA

Keywords: Herpes Simplex Virus Type 1 and 2, Establishment of latency, Reactivation, Autonomic neurons

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ABSTRACT

Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) are major human pathogens. HSV establishes latency in the nervous system and reactivates to cause recurrent disease, resulting in transmission of progeny virions to naïve individuals. Though HSV-1 and HSV-2 share similar structure and genes, they have distinctive recurrence profiles. Generally, HSV-1 reactivation is associated with disease "above the waist" and HSV-2 reactivation is associated with disease "below the waist". This phenomenon was described decades ago but still remains unexplained.

The mechanism of HSV latent infection in the peripheral nervous system (PNS) has been extensively investigated, especially with in sensory neurons. Another component of the peripheral nervous system (PNS), autonomic neurons, were also known to be infected with HSV productively and latently, but largely ignored because of the assumption that there is no difference in the pathogenesis of HSV in the neurons and that both HSV-1 and HSV-2 behave in the same way in different types of neurons.

However, autonomic neurons differ in physiological function compared to sensory neurons. Activation factors of autonomic neurons, such as emotional stress, trauma and hormonal fluctuation, are also known HSV reactivation triggering factors. Therefore, I hypothesized that autonomic neurons innervating the site of HSV infection are responsible the different reactivation frequencies of HSV-1 and HSV-2 after peripheral invasion.

In this report, the role of autonomic neurons in HSV pathogenesis were examined using the female guinea pig reactivation model. Major findings of this report are that 1) parasympathetic ganglia innervating the ocular region support latent infection of HSV-1 selectively, thus contributing the more frequent HSV-1 reactivation, 2) mixed autonomic ganglia in the genital area support HSV-2 latent infection selectively, and 3) sympathetic neurons in the genital region supported productive and latent infection of HSV-1 and HSV-2 differently.

All of the results in this report indicate that autonomic neurons play a distinctive role in HSV pathogenesis compared to the sensory neurons and are responsible for the different reactivation frequencies of HSV-1 and HSV-2. This report raises the importance of autonomic neurons in HSV pathogenesis and challenges the paradigm of HSV pathogenesis.

Acknowledgement

I dedicate this dissertation to entire my family.

My wife, Soyoung Lee and my adorable sons, June and Sean.

Dr.Bertke, There is no word I can express my appreciation to you.

Angela Ives, my lab mate, her help for my research enabled me to finish PhD.

Kathleen, I greatly appreciated with your help from the beginning in this lab.

All the guinea pigs and mice I used for this research, I wish your contributions will be valuable for further researches.

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Introduction to Herpes Simplex Virus

Herpes simplex virus (HSV) is a double stranded DNA virus belonging to the *Herpesviridae* family. Among the three subfamilies, *alpha-, beta-* and *gammaherpesvirinae* in the *Herpesviridae* family, HSV is classified in the *alphaherpesvirinae* subfamily. There are two species of simplexvirus, HSV type 1 (HSV-1) and HSV type 2 (HSV-2) (1). Of the approximately 90 herpesviruses, HSV-1 and HSV-2 are the only species that naturally infect humans. Both HSV-1 and HSV-2 have a similar genome structure, share 40% of their nucleotide sequence homology and 83% sequence homology in the protein coding regions (2). However, HSV-1 and HSV-2 can be distinguished by neutralization assays, showing that these viruses have distinct antigenicity (3).

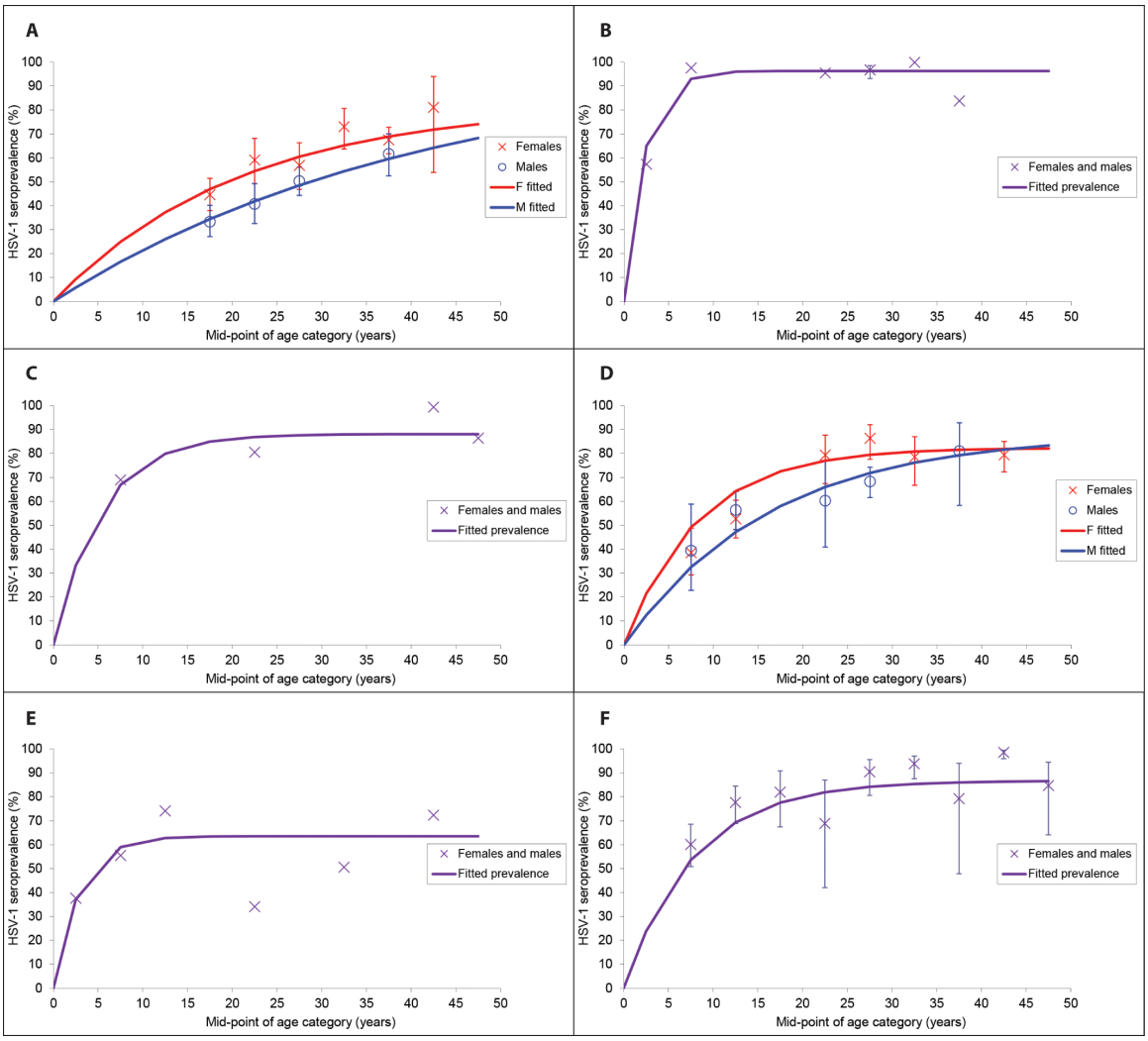
Epidemiology of HSV

HSV-1 infection is common throughout the world. HSV-1 infection is usually acquired during childhood and rates of infection increase with age. In the United states, 54% of the total population is infected with HSV-1; 30% of individuals between the ages of 14 and 19 are seropositive and this percentage increases to 64% by age 40 (Fig. 1) (4). The seroprevalence of HSV-1 is the highest in developing countries, reaching 97% in some countries (5-10). The prevalence of HSV-1 varies by geographical location and specific populations.

HSV-2 infection is also widespread in the population. However, the rate of HSV-2 infection is lower compared to HSV-1 prevalence (Fig. 2). Approximately 16% of the total

population is infected with HSV-2, but there is a significant difference in the prevalence of HSV-2 infection between males and females. HSV-2 infection is more common in females, with a seropositive rate of 18% in North America, while the seropositive rate of HSV-2 infection in males is 12%. In eastern Europe and Asia regions, HSV-2 infection rate is 12.3% in males and 29% in females (11, 12). Similar to HSV-1 infection, HSV-2 prevalence also increases with age. Between 0 and 4 years of age, the seropositive rate is 1.4%, increasing to 10.5% for individuals in their twenties and 26% by age 40 (13). However the rate of infection varies by country (14, 15). In developing countries, such as Africa, HSV-2 infection is more prevalent, ranging from 30% to 80% of adult females and up to 50% of adult males. In addition, HSV-2 infection is correlated with increased Human Immunodeficiency Virus (HIV) infection in Africa (13, 16, 17).

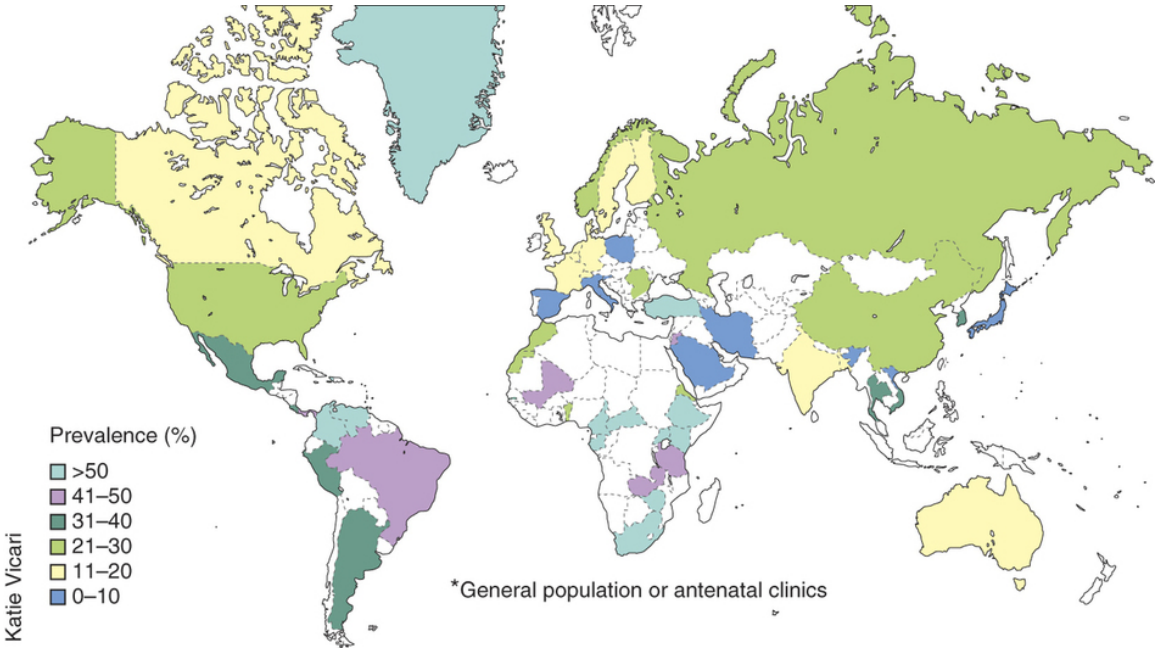
Figure 1. Global seroprevalence of HSV-1 by age in various geographical locations.



A) The Americas, B) Africa, C) Eastern Mediterranean, D) Europe, E) South-East Asia and F) Western Pacific.

Adapted by permission from Looker KJ et al.,: [PLoS ONE] (Global and Regional Estimates of Prevalent and Incident Herpes Simplex Virus Type 1 Infections in 2012), copyright (2012)

Figure 2. The seroprevalence of HSV-2 in various geographical regions.



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Clinical presentation of Herpes Simplex Virus infection

HSV infects the host through mucosal membranes or skin abrasion, replicates at the site of entry and often causes primary vesicular lesions. Two distinct serotypes of HSV, HSV-1 and HSV-2, can produce primary lesions without any remarkable differences at the site of infection. However, there are differences in the clinical disease associated with HSV-1 or HSV-2. Typical clinical signs associated with HSV-1 infection include fever, labialis (fever blister), tonsillitis, keratoconjunctivitis and gingivostomatitis. These clinical signs persist about a week, then lesions are usually resolved. The most common clinical signs of HSV-2 infection are fever, itching and formation of vesicular lesions at the genital area. The lesions usually last for two weeks, then resolve.

The transmission of HSV occurs via direct contact of mucosal membranes and secretions of infected individuals. Virus can transmit through contact with virus-containing saliva, tears, semen, vaginal secretions, sexual contact and contact with active lesions on the mucosal surfaces (18). As stated above, both HSV-1 and HSV-2 can infect and produce typical clinical signs at any mucosal area, including oropharyngeal or genital regions.

Most viral pathogens infect the host, produce various clinical signs, and sometimes lead to the death of the host. Infectious pathogens have developed their own strategies to promote viral spread, evading the host immune response and maintaining their existence in the population. Some pathogens, such as human immunodeficiency virus (HIV), can insert its viral genome into the host genome to establish persistent infection and produce progeny virions throughout the life of host. HSV has also developed a strategy for efficient viral spread and survival. Unlike most other infectious pathogens, HSV is not cleared completely from the host after healing of the primary lesions. After invasion of mucosal

epithelium, HSV is transported into the peripheral nervous system, where both HSV-1 and HSV-2 establish latent infection for the life of the host. The viruses reactivate in response to various stimuli, such as stress, ultraviolet light, skin damage, or hormonal fluctuations, intermittently producing progeny virions and recurrent clinical symptoms at or near the original site of infection.

Reactivation of latent HSV often leads to the formation of recurrent lesions in the orofacial or genital regions, but not all reactivations result in the formation of recurrent lesions. In some cases, asymptomatic or unrecognized reactivation occurs, but asymptomatic reactivations are still capable of producing infectious virions and transmitting viruses to naive individuals (19). Clinically however, HSV-1 and HSV-2 differ in their preferred anatomical location of recurrent symptoms. Even though both HSV-1 and HSV-2 can infect orafacial and genital regions and cause primary lesions in both areas, the majority of HSV-1 recurrent lesions tend to develop around the lips and nose, and occasionally other regions of the face. HSV-1 also causes recurrent stromal keratitis in the cornea, eventually leading to damage of the cornea and blindness (20). In contrast, HSV-2 rarely, if ever, reactivates to cause orofacial or ocular lesions, even if primary infection occurs in the mouth or eyes.

HSV-2 typically produces recurrent genital lesions (21, 22). Unrecognized or asymptomatic recurrence of HSV-2 with associated production of infectious viral progeny is common, however. HSV-2 can be shed during minimal clinical recurrences, leading to transmission of the virus to sexual partners and to neonates during vaginal delivery. HSV-2 infection in neonates can result in encephalitis, pneumonitis and disseminated infection, the most severe form of HSV-2 infection, which is usually fatal although aggressive

treatment is performed (23).

Primary genital HSV-1 infection is becoming more common due to changing sexual practices (24) but the genital recurrence rate of HSV-1 is relatively lower than HSV-2 recurrent genital disease. The genital recurrence rate of HSV-1 infection is 1.3 times per year and HSV-2 recurrence rate is 4.08 times per year (25). Only 14% of individuals with genital HSV-1 experience recurrent episodes, while 60% of those infected with HSV-2 experience recurrent genital lesions (22). Recurrences from genital HSV-1 decline rapidly (about 50% reduction) after one or two years but the HSV-2 recurrence rate exhibits slower reduction, declining three to five years after primary infection (26).

The differences in the reactivation efficiencies of HSV-1 and HSV-2 in the specific anatomical areas contribute to different patterns of HSV type-specific clinical illness. The mechanisms underlying these clinical observations are not yet fully understood.

Pathogenesis of Herpes Simplex Virus

HSV initially replicates at the site of entry (primary replication). Five surface glycoproteins of HSV (of a total of twelve glycoproteins) are involved with binding and fusion to host cells. First, surface glycoprotein D (gD) binds to one of three cellular receptors: the nectin family, herpes virus entry mediator (HVEM), or modified heparin sulfate proteoglycan (HSPG). Nectin family is a cell adhesion molecule in tight junctions and neuronal synapses, serving as the main entry receptor for HSV into epithelium and neurons (27). HVEM is a member of the tumor necrosis factor receptor family and expressed on lymphocytes. Both HSV-1 and HSV-2 can utilize nectin1 and HVEM to enter the target cells. Interaction of gD and the receptor triggers conformational changes and

activation of the fusogenic protein, gB. However, gB alone is not sufficient to induce membrane fusion or endocytosis. The gH/gL heterodimer complex is also required. This complex interacts with gB and leads to the transition of gB into an active fusion state (28).

These three surface glycoproteins, gB, gH and gL, form the core heterodimer and function for the fusion of HSV and the cell plasma membrane. Glycoprotein D (gD) is also required for efficient membrane fusion (29). Another glycoprotein, gC, binds to heparan sulfate on the cell surface to facilitate efficient membrane attachment. This gC is a dispensable protein for cell culture but recruitment of gC enhances membrane fusion 10-fold (30). A study using a soluble form of gB found that soluble gB competed for HSV entry, suggesting that there might be an additional specific receptor for gB (31). This viral fusion protein fuses the cell plasma membrane and viral envelope membrane by undergoing conformational changes. The rod shape viral protein brings one membrane near the other membrane so that fusion ensues.

Once membrane fusion has occurred, HSV is transported to the cellular nucleus to replicate. HSV replication depends on the host cellular transcription machinery, as with most other DNA viruses. HSV encodes several genes to manipulate host transcription machinery. Replication proceeds through an organized sequential order of gene expression in epithelial cells, beginning with immediate early genes, followed by early and late genes, to promote HSV replication in the host cells (32).

Immediate Early (IE) genes are the first genes expressed after HSV infection in the host epithelial cells. VP16, an HSV tegument protein carried into the host cells, forms a complex with host cellular protein Oct1 and Host Cell Factor 1 (HCF1) to activate IE genes. There are five IE genes, infected cellular protein (ICP) 4, 0, 22, 27 and 47. Synthesis of IE

genes is required for the transcription activity of E and L (late) genes in epithelial cells (32). For example, ICP4 is expressed to repress IE gene expression by interacting with host RNA polymerase II transcription factors, thus promoting progression from IE gene expression to the E and L gene expression, leading to completion of the lytic cycle. ICP27 also interacts with host RNA polymerase II. ICP27 contributes to host cell shutoff, downregulates the interferon response, and promotes viral transcription and translation (33). ICP27 stimulates E and L gene expression by recruiting RNA polymerase II to viral replication sites. ICP0 expression is required for efficient productive infection and reactivation from latency (34). ICP22 functions with UL13 to enhance L gene expression in a cell-specific manner (35). The peak of IE gene expression is present between 2 and 4 hours post-infection, after which the IE genes promote Early (E) gene expression. E genes encode the machinery for viral DNA synthesis and evasion of the immune response. The peak of E gene expression is at approximately 4-6 hours post-infection, after which Late (L) gene expression begins. L genes, which encode proteins for the viral structure, are transcribed approximately 6-8 hours post-infection, and then progeny virions are produced.

The sequential cascade of HSV gene transcription, determined in epithelial cell culture, has been a standard model for HSV replication.. In cultured embryonic superior cervical ganglion neurons, IE, E, and L viral gene expression occurs simultaneously instead of progressing in the characteristic temporal cascade (36). A recombinant HSV that lacks IE gene transcription showed more transcriptionally active expression of E and L genes in neurons compared to non-neuronal cells, suggesting that neuronal factors promote neuronal specific transcription (37, 38). ICP34.5, which is encoded by the flanking region of the unique long (UL) region of the genome, is not required for HSV-1 replication in

Vero cells but recombinant HSV-1 that has a mutation in ICP34.5 was limited in replication ability in neuronal cells and some primary human cells. The ICP34.5 recombinant HSV-1 also showed attenuation *in vivo* (39). The differences in the gene expression profiles between non-neuronal cells and neurons, particularly adult neurons, are not fully understood and the significance of the difference in the HSV pathogenesis was not determined.

After primary replication in mucosal epithelial cells, the virus spreads to the terminals of nerves that innervate the site of inoculation, such as Trigeminal ganglia (TG) or Dorsal root ganglia (DRG). However, HSV does not encode viral proteins for transport in neurons. Due to the relatively long distance between the axonal terminal and neuronal cell body, passive diffusion is not an efficient method for the delivery of HSV to neuronal nuclei. Hence, HSV utilizes and rearranges the cellular microtubule system for transport of the viral capsid to neuronal nuclei. The HSV viral capsid and tegument proteins must utilize host neuronal transportation machinery and manipulate the direction of transport, retrograde transport for neuronal infection or establishment of latency and anterograde for recurrent lesions following viral reactivation. Microtubules serve as the basic structure of transport in the axon. In the axon, all of the microtubules are oriented in the same direction (minus end toward neuronal body and plus end toward the axon terminal). The microtubules are not only one molecule, but are staggered and overlapped. This overlapped microtubule inside the axon serves as a highway for transportation of protein and vesicles. Two molecular motor systems were identified as a vehicle for HSV transport. The anterograde directional motor molecule (from neuronal cell body to axon) is kinesin and retrograde directional motor molecule is dynein (40).

Establishment of latency in the nervous system

After transport into the neuronal cell bodies, HSV encounters two potential fates: productive infection or latent infection. During productive infection, HSV undergoes replication in the neurons and can infect nearby neurons and glial cells. During latency, HSV genomes are present in the host nuclei in a nucleosome-associated episomal state and HSV gene expression is repressed, with the exception of the Latency Associated Transcript (LAT) (38). Since the first discovery of LAT in latent infection of HSV, the function and underlying mechanism of LAT has been the focus of much research. The exact mechanism of how LAT enhances establishment of latency in the neurons is not fully understood. The expression of LAT is limited during the acute phase of infection. Previous studies have shown that during latency, the expression of lytic genes are suppressed in LAT(+) HSV-1 virus compared to the LAT(-) virus (41). Since there is no protein encoded in the LAT, it has been suggested that microRNAs (miRNA) expressed during latency from the LAT region inhibit ICP0 and ICP4 protein expression but not at the mRNA level, thus promoting latent infection in the neurons (42). In addition to the establishment of latency, the function of LAT includes involvement in reactivation, anti-apoptic activity and virulence of HSV. When LAT was deleted or the LAT promoter region was deleted from HSV-1 or HSV-2, the *in vivo* reactivation rate was significantly reduced, but the deletion did not influence replication of the mutant virus or virulence in the rabbit ocular model or guinea pig genital model of infection (43, 44).

One of the mechanisms by which LAT promotes the establishment of latency in neurons is the protection of neurons from apoptosis. When the LAT promoter region and

first half of the stable 2kb LAT were deleted from HSV-1, the replication capacity was conserved but neurons infected with the mutant virus exhibited a higher apoptotic rate compared to neurons infected with the wild type virus (45). There are also differences between HSV-1 and HSV-2 latent infection in the neuronal population. In latently-infected trigeminal ganglia of mice, HSV-1 LAT expression was observed in A5+ sensory neurons, whereas HSV-2 LAT was observed in KH10+ neurons, which are a different population of sensory neurons. This result suggested that host or viral factors regulate virus-specific preferential establishment of latent infections in specific subpopulations of neurons (46).

HSV LAT exon 1 region influences this preference, not the promoter region. When HSV-2 LAT exon 1 region was swapped by the corresponding region from HSV-1, the mutant virus was impaired for reactivation after genital inoculation, despite the similarity of viral load of mutant and rescuant virus. These altered phenotypes were suspected due to the difference in the region between TATA box and intron splice site of LAT. In that region, HSV-1 contains an 18bp long transcription factor binding site whereas HSV-2 LAT has a 38bp long region in which there were two transcription factor binding sites (47). Thus, LAT exon 1 is suspected to be responsible for the HSV-1 and HSV-2 virus-specific reactivation phenotype. However, the molecular mechanism underlying regulation of latent infection of HSV is not fully understood yet. There is some evidence that LAT (-) mutant HSV-1 can reactivate as efficiently as wild type HSV-1 (48). There is also evidence showing that latent HSV load is related to increased recurrence rate (49, 50). Reactivation of HSV is a common occurrence but to date, the mechanisms that regulate reactivation are not understood.

Reactivation of HSV

After transport into neurons, HSV can enter into the latent phase. VP16 and host cellular factor 1 (HCF1) are the essential elements for the induction of IE gene expression and the absence of those elements in the neuronal nuclei represses transcription of IE genes, thus preventing E and L gene expression, with the exceptions of LAT and microRNAs (51). The absence of these essential elements for productive infection in the neurons creates a latent infection friendly environment for HSV.

During latency, reactivation is triggered by various stimuli, such as use of adrenergic agents (52), UV radiation (53), psychological stress (54) and fever (55). Reactivation of HSV can be clinically asymptomatic or symptomatic. Rarely, a viremic state of individuals with reactivation was able to be detected by sensitive PCR assay so it is possible that reactivated HSV could be disseminated (56), but most clinical manifestations of HSV infection or reactivation are generally restricted to mucocutaneous regions. It has not been determined how these various triggering factors can commonly induce reactivation of latent HSV. Most of these triggering factors, physical and psychological stresses, are related to elevated levels of the stress hormone glucocorticoids, such as cortisol and corticosterone. The influence of these stress hormones on HSV-1 reactivation was tested previously in cultured neonatal sensory neurons from (TG) (57). The results showed that dexamethasone, not epinephrine, could induce reactivation in a dose-dependent manner and enhance reactivation in response to heat in TG. It is not clear how the stress hormone exert its function in the reactivation process. Heat stress induced reactivation of HSV-1 in cultured TG model, suggesting that reactivation of HSV is not simply derived from the hormonal influences. Reactivation of HSV from the different

anatomical sites are correlated with the HSV serotype. It is still remained unknown that HSV-1 and HSV-2 reactivate in response to the specific stimuli (such as hormone) or anatomical location specific factors are regulating HSV reactivation.

Herpes Simplex Virus infection in the nervous system

The nervous system is composed of the central nervous system (CNS) and peripheral nervous system (PNS). While HSV is capable of infecting the CNS, often resulting in a fatal outcome even with aggressive treatments, the PNS is the main target of HSV productive and latent infection. The PNS can be further classified into motor, sensory and autonomic nervous systems, according to function and anatomical location. The function of sensory neurons is the reception of information such as heat, pain, and pressure from the periphery, then transfer of the stimuli to the brain. Sensory neurons, such as those found within the trigeminal (TG) and lumbosacral dorsal root ganglia (DRG), have been researched extensively for HSV pathogenesis and establishment of latent infection. Another component of the PNS, the autonomic nervous system (ANS), controls the host systems to maintain homeostasis in response to external or internal stimuli. The ANS can be further divided into the sympathetic nervous system, which controls "fight or flight" response, and parasympathetic nervous system, which controls rest and digest functions. Many factors can induce ANS activation, including well known HSV reactivation triggers. For example, hormonal changes and stress are strong ANS activators and are also known HSV reactivation triggering factors. Evidence of autonomic neuron infection by either HSV-1 or HSV-2 have been confirmed in human and experimental animal species (58-64). Despite growing evidence of involvement of autonomic neurons in HSV pathogenesis, it

is not clear if latent HSV-1 or HSV-2 is directly transported to autonomic ganglia from the primary site of infection or transported from infected sensory ganglia. However, the recovery of infectious HSV from autonomic ganglia implies that HSV infection in autonomic ganglia has the potential to induce clinical HSV recurrences (65). HSV can reactivate in response to various stimuli such as trauma, surgery, fever, use of immunosuppressant, hormonal fluctuation and the exposure to the UV light, all of which impact autonomic neurons. HSV reactivation triggering factors, such as hormonal fluctuation and stress hormones, elicit activation of the autonomic nervous system to a greater degree than the sensory nervous system. Despite of the growing evidences, correlation between HSV infection in the autonomic neurons and recurrence from the autonomic neurons has not been determined yet.

Both HSV-1 and HSV-2 can infect neurons, however specific neuronal populations in the sensory ganglia support either latent infection or productive infection of HSV-1 and HSV-2 differently (66). This result implies that particular cellular and/or viral factors determine the fate of HSV productive or latent infection. In human infection cases, most primary HSV-1 infection is derived from exposure to virus at oropharyngeal regions, but recurrent HSV-1 herpetic lesions are relatively common in the ocular regions, in addition to the oral mucosa. This implies that HSV-1 infection of oral mucosa is able to spread to the eye. Trigeminal ganglia innervate both lip and eye via maxillary and ophthalmic branches, which might contribute to recurrent ocular lesions. Autonomic neurons also connect the lip and ocular regions. The superior cervical ganglion (SCG) innervates iris and mucosal glands around the lip, so reactivation of HSV from autonomic neurons could potentially contribute to recurrent ocular infection. Human herpetic anterior uveitis cases

without concurrent or past keratitis history indicate involvement of autonomic neurons, independent of TG sensory neurons (60).

The genital area is also extensively innervated with both sensory fibers extending from DRG and autonomic fibers from the major pelvic ganglia (MPG). Experimentally, it has been suggested that autonomic neurons play a role in the transmission of HSV to the spinal cord and there is a fundamental difference between HSV-1 and HSV-2. In an experimental HSV-2 infection study using the female guinea pig model, the pathway of HSV-2 from the peripheral site of inoculation to the spinal cord or DRG was determined by comparing inoculation of the genitals and the footpad, which are innervated by sensory and autonomic pathways differently. HSV-2 inoculated vaginally successfully reached the spinal cord, while inoculation into the footpad, which lacks parasympathetic innervation, produced more efficiently spread to sensory DRG with minimal virus reaching the spinal cord. It was determined that when HSV-1 and 2 were inoculated at different sites, the dynamics of viral DNA detection exhibited different patterns. In particular, HSV-2 infection of the genital area, which is more extensively innervated by autonomic nerves, resulted in increased HSV-2 DNA load in the spinal cord, independent of sensory DRG. HSV-1 and 2 infection at the footpad, which lacks parasympathetic innervation, resulted in significantly lower HSV DNA detected in the spinal cord. These results implied that spinal cord and autonomic ganglia are also sites of HSV replication and reactivation for HSV pathogenesis after genital infection (58, 67).

It is not clear whether sole infection of autonomic neurons could be sufficient to induce recurrent herpetic lesions, since sensory and autonomic neurons are connected at a higher level (e.g spinal cord). However, the latency associated transcript (LAT) is

expressed more abundantly in sensory neurons than autonomic neurons, suggesting that sensory neurons plays a major role in HSV reactivation. Since autonomic neurons also express LAT to some degree, autonomic neurons may also be responsible for recurrent herpetic lesions after infection (68).

Selection of the laboratory animal species

Humans are the natural host for HSV. However, HSV-1 and HSV-2 can infect a variety of mammals so several laboratory animal species can be used for HSV research. Mice, rabbits, guinea pigs, and non-human primates have all been used to study HSV pathogenesis. Each animal model has advantages and disadvantages and investigators select an appropriate animal model based on the question they are trying to answer.

Mice in HSV research. Mice are the most widely used laboratory species for laboratory research of any kind. The small size of mice is an advantage for reducing the amount of drugs or viruses used for studies, but a disadvantage is the small amount of tissue harvested. Mice have been used to investigate ocular infection, as well as genital and foot pad infection with HSV (which accounts for human genital infection). HSV can establish latency in mouse neurons after ocular or genital infection, thus mice can be a good candidate for HSV research when investigating latency establishment. However, severity of acute infection of HSV is virus strain dependent and can be lethal to mice. HSV-1 strain 17+ is highly pathogenic and lethal in mice, so additional treatment, such as injection of acyclovir (ACV) or HSV-specific immunoglobulin, must be added to protect mice from death and establish viral latency. Another disadvantage of mice in HSV research is that

once HSV establishes latency, spontaneous reactivation in mice is not as efficient as other species (69). Spontaneous reactivation is extremely low and recurrent lesions are not found in immunocompetent mice. Nervous tissue, such as the dorsal root and trigeminal ganglia, collected from HSV latently infected mice and explanted into culture dishes has been used for HSV reactivation research. However, the limitation of this method is that reactivation in humans is characterized by recurrent lesions around organs innervated by neurons infected with HSV, so the explant reactivation model is not biologically relevant for naturally occurring reactivation. In addition, the tissue explant method has focused primarily of sensory ganglia, causing investigators to overlook the possibility of other factors involved in viral reactivation. Results obtained from sensory ganglia explants can provide insight into HSV pathogenesis but in a normal infection in a human, it is possible that HSV infection in autonomic neurons could have a role in clinical presentation and/or recurrences. Therefore, a complete picture could not be gained using the mouse model. Lastly, reactivation can be mice strain specific, depending on the methods used to induce reactivation and possibly attributed to differences in their immune response.

Rabbits in HSV research. The rabbit provides a more relevant and reliable model for HSV pathogenesis. Early research showed that for HSV-1, the rabbit is an excellent model species. HSV-1 infection in the rabbit eye mimics human ocular infection. HSV-1 infects the rabbit cornea after topical inoculation, followed by establishment of latency in the trigeminal ganglion. The latent virus can be recovered after a period of latency. Reactivation in the rabbit eye model occurs spontaneously at low frequency but can be experimentally induced by use of adrenergic agents. Since most of the clinical

complications of HSV infection are due to recurrent herpetic disease, the finding of recurrent lesions from the rabbit eye made this species a valuable tool for HSV research. The eye of the rabbit is big so it is easily accessible. However, there are also disadvantages in these species. HSV-2 does not reactivate efficiently in the rabbit eye model and since the rabbit is not a natural host of HSV, mortality often occurs after infection. Economic issues are also a problem, as inbred strains of rabbit are extremely expensive to obtain, and feeding and housing are costly.

Nonhuman primates in HSV research. Nonhuman primates could be the best model species, since nonhuman primates are genetically similar to humans and HSV can establish latency in nonhuman primate neurons just like in humans. Since anatomical and physiological characteristics of nonhuman primates are similar to humans, HSV infection of nonhuman primates could provide similar results as actual human infection. However, research using nonhuman primates is limited due to the extremely high cost compared to other laboratory animal species and the complexity of caring for nonhuman primates, such as emotional status.

Guinea pigs in HSV research. Guinea pigs are a reliable and biologically relevant animal model, and currently the best available for investigation of the HSV pathogenesis and reactivation. Genital inoculation of HSV-1 or HSV-2 produces a primary symptomatic infection with characteristic lesions, and the viruses reactivate spontaneously to cause recurrent lesions around the genitalia. Similar to human infections, HSV-2 infection produces a higher genital recurrence rate compared to HSV-1 infection, making guinea

pigs the ideal tool for HSV research on reactivation. Disadvantages of guinea pigs in HSV research, especially regarding the autonomic neurons, are that there might be different host factors correlated with HSV infection and there are anatomical differences between human and guinea pigs. For example, guinea pigs do not have a discrete ciliary ganglion. They have a loose aggregation of neuronal cells in a ciliary plexus instead.

Research hypothesis

Recurrences of HSV from neuronal system contributed to the world wide prevalence of HSV. The molecular mechanism of reactivation from latency has been extensively investigated. However, still several questions remained undetermined. One of the remaining question is that HSV-1 and HSV-2 have type specific preferences of reactivation in the different anatomical site. Neurons in the nervous system have been identified as a source of recurrent HSV. Among the neuronal populations, sensory neurons have been extensively investigated for the HSV pathogenesis however, autonomic neurons were proved to be a site of latent HSV infection. But the role of autonomic neurons in the HSV pathogenesis was not of focus since HSV infection in the sensory and autonomic neurons has been regarded as the same. Recently researchers found distinctive HSV pathogenesis in the autonomic neurons compared to the sensory neurons. Still, several aspects of HSV infection in the autonomic neurons need to be determined before defining the role of autonomic neurons in the HSV pathogenesis, including latent HSV in the autonomic neurons is reactivation-competent or not.

I hypothesized that HSV infection in the autonomic neurons contributed to the HSV recurrences and anatomical preferences of reactivation derived from the autonomic neurons, further investigating a different mechanism which regulates reactivation of HSV-1 and 2 selectively.

Specific Aims

The goal of this research is to elucidating the role of autonomic nervous system after HSV infection in the female guinea pig model. More precisely, determining the contributions of ANS to the HSV acute and recurrent disease after peripheral infection. The specific aims of this research includes:

1. Investigation of HSV productive and latent infection after ocular infection
    a. To compare the acute HSV-1 and HSV-2 infection between sensory and autonomic ganglia after ocular inoculation.
    b. To compare the latent HSV-1 and HSV-2 infection between sensory and autonomic ganglia after ocular inoculation.
    c. To compare the HSV-1 and HSV-2 infection and gene expression between sympathetic and parasympathetic ganglia in acute and latent phase.
2. Investigation of HSV productive and latent infection after genital infection
    a. To compare the acute HSV-1 and HSV-2 infection between sensory and autonomic ganglia after genital inoculation.
    b. To compare the latent HSV-1 and HSV-2 infection between sensory and autonomic ganglia after genital inoculation.
3. Determination of the effect of sympathetic nervous system ablation in HSV reactivation
    a. To assess the effect of chemical sympathetic fiber ablation in the guinea pigs
    b. To determine the effect of chemical sympathetic fiber ablation in HSV-1 and HSV-2 latent infection and reactivation.

Chapter 2. Materials and Methods

Stock virus production

HSV-1 strain 17+, originally obtained from Dr. John Hay (SUNY Buffalo, NY), and HSV-2 strain 333, originally obtained from Gary Hayward (Johns Hopkins, MD), and GFP tagged HSV-1 and 2 (HSV1-VP26-GFP or HSV2-VP26-GFP) were propagated in Vero cells to produce high titer stocks of viruses that were used for both *in vivo* and *in vitro* studies. Vero cells were purchased from American Type Culture Collection (ATCC) and grown as a monolayer in uncoated culture flasks with minimum essential medium supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin. To propagate virus stocks, Vero cells were seeded into T175 flasks to achieve an 80-90% monolayer, media was aspirated and 0.5 moi (multiplicity of infection) of HSV-1 or HSV-2 were inoculated. After one hour of adsorption period, media was replaced and Vero cells were incubated at 37°C for 48 hours.

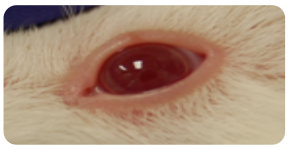
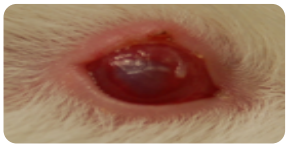
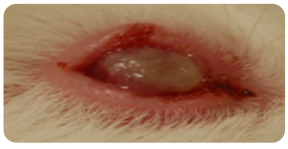
Stock viruses were titrated using plaque assays. Virus stocks were thawed on ice and serial 10-fold dilutions were performed. Vero cells, seeded into 12 well culture plates to achieve a 90% monolayer, were inoculated with the serially diluted virus. After one hour of adsorption period, media was replaced, then Vero cells were incubated at 37°C for 48 hours. After incubation period, media was aspirated and crystal violet dye with 10% paraformaldehyde (PFA) was added to fix and stain live cells. The number of plaques was counted to determine the amount of virus. Virus stock aliquots were stored in a -80°C freezer and thawed as needed for studies.

Guinea pig ocular infection

Female 3 week old Hartley guinea pigs (HillTop Laboratories) were infected with $2x10^5$ PFU of HSV-1 or HSV-2. For the ocular infection, guinea pigs were sedated with use of Xylazine and Ketamine. HSV-1 or HSV-2 was inoculated by pipette to the surface of the eye after scarification of the cornea with 27 gauge needle. Guinea pigs were observed daily for 60 days post-infection (dpi). Weight of individual guinea pigs was recorded and acute infection severity was assessed by counting the number of lesions. Severity of acute infection was scored for each guinea pig through Day 14, based on a scale of 0 - 4 (0 = no symptoms, 1 = inflammation or redness, 2 = one or two lesions, 3 = 3 - 5 lesions, 4 = more than five or coalescence of lesions, Table 1). The rate of recurrences was determined by daily observation of recurrent lesions around eye and nose area from Days 15-60 pi. All procedures were performed in a way to minimize unnecessary stress or pain to the guinea pigs.

At various time points (Days 1, 2, 3, 4, 7, 10, 14, 30, and 60), guinea pigs were sacrificed and sympathetic superior cervical ganglia (SCG), parasympathetic ciliary ganglia (CG), and sensory trigeminal ganglia (TG) were collected. All samples were placed into RLT buffer (Qiagen) after collection, homogenized, and frozen until further processed for quantitative PCR or RT-PCR. All procedures were carried out according to the institutional animal care and use committee (IACUC) guidelines and were approved by and conducted in accordance with the Virginia Tech Institutional Care and Use Committee (IACUC# 13-003-CVM).

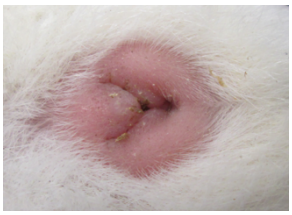
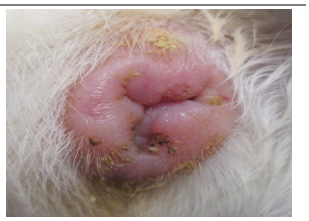
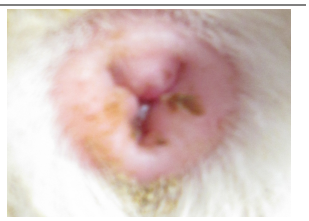
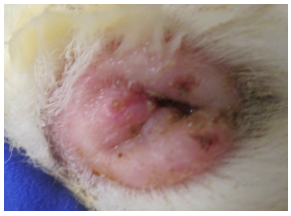
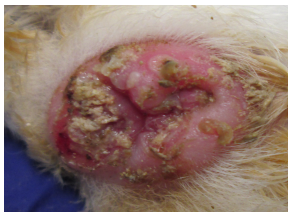
Table 1. Scoring system for the evaluation of HSV ocular infection.

Score	Number of lesion	Clinical presentation
0	No lesion	
1	Inflammation or conjunctivitis	
2	One or two lesions on or around eye	
3	Three to five lesions on or around eye	
4	More than five lesions on or around eye	

Guinea pig genital infection

Female 3 week old Hartley guinea pigs (HillTop Laboratories) were infected with $2x10^5$ PFU of HSV-1 or HSV-2. The guinea pigs were sedated with use of Xylazine and Ketamine, then HSV-1 or HSV-2 in 50 ul of media was inoculated into vagina directly with a pipette. After HSV infection, guinea pigs were monitored and weighed daily for 60 days post-infection (dpi) and scored for presence and severity of lesions (Table 2). Lesions that developed at the genital area or occasionally, the foot pad, were counted to determine the severity of acute disease (from Day 1 to 14) and rate of recurrences (from Day 14 to 60). All procedures were carried out as to not cause any unnecessary stress or pain to the guinea pigs. Each day, the guinea pigs' genital area was cleaned with baby wipes to prevent secondary bacterial infections and antibiotic ointment was applied to any open lesions, if necessary. Some guinea pigs developed urinary incontinence. The urinary bladder of those guinea pigs were manually massaged to empty. Hind limb paresis was present in some guinea pigs; they were closely monitored and euthanized if there was no improvement for greater than 5 days. At various time points (Days 1, 2, 3, 4, 6, 7, 8, 10, 14, 30, and 60), guinea pigs were sacrificed and mixed autonomic major pelvic ganglia (MPG) and sensory dorsal root ganglia (DRG) were collected. All samples were placed into RLT buffer (Qiagen) after collection, homogenized, and frozen until further processed for quantitative PCR or RT-PCR. All procedures were carried out according to the institutional animal care and use committee (IACUC) guidelines. All studies were approved by and conducted in accordance with the Virginia Tech Institutional Care and Use Committee (IACUC# 13-008-CVM).

Table 2. Scoring system for the evaluation of HSV genital infection.

Score	Number of lesion	Clinical presentation
0	No lesion	
1	Inflammation or redness	
2	One or two lesions	
3	Three to five lesions	
4	More than five lesions or coalescence of lesions	

Primary culture of adult mouse neurons

Trigeminal (TG), superior cervical (SCG), ciliary, (CG), dorsal root (DRG), and major pelvic ganglia (MPG) were removed from 6 week old Swiss Webster mice and cultured on Matrigel-coated 8-well Lab-Tek II chamber slides or in 6, 12, or 24 well cultures plates (ThermoScientific). Ganglia were digested in papain, collagenase and dispase (Worthington), followed by mechanical trituration with a pipette. TG and MPG were passed through an OptiPrep (BD Biosciences) gradient (45%, 35%, 25% and 15% of gradient from the bottom respectively) to enrich for neurons; DRG, SCG and CG were plated without the gradient step, since they contain minimal axonal debris in the cell suspension. Cells were washed and plated in Neurobasal A medium supplemented with 2% B27, 1% penicillin-streptomycin, L-glutamine, neurotrophic factors (2.5ug of Nervous growth factor, Glial cell line derived neurotrophic factor and Ciliary neurotrophic factor), and mitotic inhibitors (0.1ug of Floxuridine and 0.83ug of Aphidicolin, Life Technologies).

Acute and latent HSV infection of primary neuronal culture

Four days after plating at 3000 neurons per well, media was removed, neurons were inoculated with 30 multiplicity of infection (moi) of HSV-1 or HSV-2 for acute phase infection and 10 moi for latent infection. Viruses were allowed to adsorb for one hour, and complete Neuro media (Neurobasal A, B27, L-glutamine and neurotrophic factors, with no mitotic inhibitors) was added.

Viral load and gene expression profiles were analyzed at various time points after inoculation of HSV-1 or HSV-2. To analyze gene expression during lytic infection, neurons infected with HSV-1 or HSV-2 were collected at 0, 1, 2, 5, 8, 12, 16 and 24 hours

post-inoculation. For latent infection, neurons were incubated with 300 uM concentration of ACV to limit HSV replication but not HSV entry into the neurons. Neurons were incubated for one week and Neuro media containing ACV was removed to promote spontaneous reactivation in the neurons.

Determination of spontaneous *ex vivo* reactivation of Herpes Simplex Virus

Six week old Swiss-Webster mice (Harlan) were sedated with the mixture of Xylazine and Ketamine. Then mice were ocularly infected after corneal scarification with $2x10^7$ PFU of HSV1-VP26-GFP or HSV2-VP26-GFP. Mice were euthanized 21 days post-inoculation, and TG, SCG and CG were collected into Neurobasal A medium, supplemented with 2% B27 and 1% penicillin-streptomycin. Ganglia were dissociated, as described above for primary neuronal cultures, and plated onto 24-well plates coated with Matrigel (BD Biosciences). Reactivation was determined by observation of GFP expression in neurons twice per day for the first three days after plating. All studies were approved by and conducted in accordance with the Virginia Tech Institutional Care and Use Committee (IACUC# 13-003-CVM).

Quantitative PCR assays

Viral DNA and RNA were extracted from homogenized tissues with the Qiagen AllPrep DNA/RNA minikit (Qiagen) according to the instructions of the manufacturer. After RNA extraction, cDNA was synthesized using the iScript cDNA Synthesis kit (Bio-Rad). Quantitative PCR was performed on a Viia7 real-time PCR machine (Applied Biosystems), using the iTaq universal probe mix (Bio-Rad) and ZEN primer/probe sets (IDT) specific

for genes encoding HSV-1 or HSV-2 thymidine kinase (TK) and latency associated transcript (LAT) and immediate early (IE) genes encoding ICP0 (infected cell protein 0), ICP27, ICP4 and Late (L) gene VP16 (Table 1), Viral DNA load was determined by quantifying viral DNA by quantitative PCR (qPCR) using HSV-1 and HSV-2 thymidine kinase (TK) gene-specific primers and probes (47, 70). All assays were normalized to 18s rRNA (Applied Biosystems) and reported as quantity in 200 ng of DNA or RNA.

Table 3. Primers and Probe sets for the Quantitative PCR assays.

Primer/Probe	Gene bank accession number	Sequence (5” to 3” direction)	Concentration
HSV1 Thymidine Kinase			
TK1 Forward	JN555585.1	AAAACCACCACCACGCAACT	900nM
TK1 Reverse		TCATCGGCTCGGGTACGTA	900nM
TK1 Probe		FAM-TGGGTTCGCGCGACGATATCG-BHQ	250nM
HSV2 Thymidine Kinase			
TK2 Forward		TAATGACCAGCGCCCAGAT	900nM
TK2 Reverse	KP192856.1	CGATATGAGGAGCCAAAACG	900nM
TK2 Probe		FAM- ACAATG AGCACGCCTTATGCGGC -BHQ	250nM
HSV1 Latency Associated Transcript			
LAT1 Forward		ACCCACGTACTCCAAGAAGGC	900nM
LAT1 Reverse	JN555585.1	TAAGACCCAAGCATAGAGAGCCA	900nM
LAT1 Probe		FAM- TCCCACCC CGCCTGTGTTTTTGT -BHQ	250nM
HSV2 Latency Associated Transcript			
LAT2 Forward		TAATGACCAGCGCCCAGAT	900nM
LAT2 Reverse	KP192856.1	CGATATGAGGAGCCAAAACG	900nM
LAT2 Probe		FAM- ACAATG AGCACGCCTTATGCGGC -BHQ	250nM
HSV1 Infected Cell Protein (ICP) 0			
HSV1 ICP0 Forward		GATGCAATTGCGCAACAC	450nM
HSV1 ICP0 Reverse	JN555585.1	GCGTCACGCCCACTATCAG	450nM
HSV1 ICP0 Probe		FAM- GCTGTGCAACGCCAAGCTGGTGTA -BHQ	250nM
HSV2 Infected Cell Protein (ICP) 0			
HSV2 ICP0 Forward		GGTCACGCCCACTATCAGGTA	450nM
HSV2 ICP0 Reverse	KP192856.1	CCTGCACCCCTTCTGCAT	450nM
HSV2 ICP0 Probe		FAM-CAACGGAATCCAGGTCTTCAT-BHQ	250nM
HSV1 Infected Cell Protein (ICP) 4			
HSV1 ICP4 Forward		CATGGCGTAGCCCAGGT	450nM
HSV1 ICP4 Reverse	JN555585.1	GGCCTGCTTCCGGATCT	450nM
HSV1 ICP4 Probe		FAM- CCGGTGATGAAGGAGCTG -BHQ	250nM
HSV2 Infected Cell Protein (ICP) 4			
HSV2 ICP4 Forward		GTC GTCGTCGTCGTCAG	450nM
HSV2 ICP4 Reverse	KP192856.1	CCGCCTCTGACTCATCAAA	450nM
HSV2 ICP4 Probe		FAM- ATGCAGACGAGGAGGAGGAG -BHQ	250nM
HSV1 Infected Cell Protein (ICP) 27			
HSV1 ICP27 Forward		GCGGCTGTGCTGGATAA	450nM
HSV1 ICP27 Reverse		GCGAACACAGTTCGT CCA	450nM
HSV1 ICP27 Probe	JN555585.1	FAM-TTTCTCCAGTGCTACCTGAAGGCGCGA -BHQ	250nM
HSV2 Infected Cell Protein (ICP) 27			
HSV2 ICP27 Forward		GTC CTTTCTGCAGTGCTACCTGAA	450nM
HSV2 ICP27 Reverse	KP192856.1	CAGGATGACCAACACAAAGGA	450nM
HSV2 ICP27 Probe		FAM- C GACGCCTGTCGGACATTAAGGAT -BHQ	250nM
HSV1 VP16			
HSV1 VP16Forward	JN555585.1	ACCTGTTTGACTGCCTCTG	450nM
HSV1 VP16Reverse		TGACGAACATGAAGGGCTG	450nM
HSV1 VP16Probe		FAM- CGACCTGGAGAGCTGGCGT -BHQ	250nM
HSV2 VP16			
HSV2 VP16Forward		CATGCTAGATACCTGGAACGAG	450nM
HSV2 VP16Reverse	M60050.1	TCGACAGAAACTTGCACTCC	450nM
HSV2 VP16Probe		FAM- C TCCCGACCAACGCCGACAT -BHQ	250nM

6-Hydroxy dopamine (6-OHD) treatment

To further investigate the role of autonomic neurons, especially sympathetic neurons, and distinguish the effect of sympathetic and parasympathetic neurons in the primary and recurrent infection of HSV, female guinea pigs were treated with 6-hydroxy dopamine (6-OHD), which selectively ablates sympathetic neuronal axons, but does not destroy the neuronal cell body. Guinea pigs were treated 10 days before HSV infection (pre-infection group, n=8) or 15 days after infection (post-infection group, n=8) with 100mg/kg concentration of chemical 6-OHD via intra-peritoneal route. Guinea pigs were monitored to determine the acute clinical disease and recurrences for 60 days in same manner with the genitally inoculated guinea pigs.

Statistics

Acute severity was statistically analyzed by Mann-Whitney test (SPSS) using area under the curve per guinea pig. Cumulative recurrences were statistically analyzed by Mann-Whitney test (SPSS) using cumulative recurrences per individual animal and gene expression.

Chapter 3. Reactivation of Herpes Simplex Virus type 1 and 2 from autonomic neurons after ocular infection

Introduction

Herpes simplex virus 1 (HSV-1) and HSV-2 infect and establish latency in peripheral sensory ganglia, including the trigeminal ganglia (TG) after orofacial infection and the dorsal root ganglia (DRG) after genital infection. While both viruses can reactivate from latency to cause recurrent lesions throughout the life of the host, HSV-1 and HSV-2 demonstrate different patterns of recurrent disease. HSV-1 is more likely to produce recurrent lesions in the orofacial region, while HSV-2 rarely, if ever, recurs orofacially, even if primary infection occurs in the mouth, nose, or eyes. HSV-1 is becoming more common as a cause of genital herpes, but HSV-2 recurs much more efficiently after genital infection. Although 60 to 90% of individuals with genital HSV-2 demonstrate symptomatic recurrences, only 25% with genital HSV-1 infections experience symptomatic recurrences (21, 22), demonstrating that HSV-1 and HSV-2 characteristic recurrent disease patterns are not simply a result of differences in the site of initial infection.

Upon reaching the sensory ganglia after peripheral inoculation, the viruses replicate in some neurons, while establishing latent infection in others. The sensory neuronal populations that are permissive for productive infection differ between HSV-1 and HSV-2. Sensory neurons recognized by monoclonal antibody Fe-A5 (A5+) limit productive HSV-1 infection (66, 71). In contrast, sensory neurons bound by the monoclonal antibody KH10 or isolectin IB4 (IB4+) limit productive infection of HSV-2 (72). Similar

percentages of these nonoverlapping populations of sensory neurons are found in TG (10 to 12%) and DRG (13 to 15%). HSV-1 and HSV-2 demonstrate neuronal specificity for A5 and IB4 neurons, respectively, regardless of the route of infection. One-half of the LAT-positive latent HSV-1 reservoir is found in A5 neurons, and one-half of the LAT-positive latent HSV-2 reservoir is found in IB4 neurons, in the TG after ocular infection or in the DRG after genital infection (62-64, 73). Thus, different patterns of HSV-1 and HSV-2 recurrence cannot be adequately explained by preferential establishment of latency in different types of sensory neurons.

The orofacial and genital regions innervated by the sensory TG and DRG are also extensively innervated by autonomic nerve endings. Sympathetic neurons of the superior cervical ganglia (SCG) and parasympathetic neurons in the ciliary ganglia (CG) innervate the conjunctival epithelium and stroma (73), and mixed autonomic neurons in the major pelvic ganglia (MPG) innervate the genitourinary tract. In both humans and animal models, HSV-1 and HSV-2 latent viral DNA has been detected in autonomic ganglia, including the SCG, CG, pterygopalatine ganglia (PTG), and MPG (also referred to as the paracervical ganglia) (58, 74, 75). The autonomic pathways are intimately involved in physiological activities associated with symptomatic recurrences in humans, including the stress response, febrile response, and hormone regulation. Although sensory ganglia are considered to be the site of reactivating HSV, reactivation of latent virus residing in autonomic ganglia could contribute to recurrent symptoms.

To determine whether differences between HSV-1 and HSV-2 in autonomic ganglia may contribute to the viruses' different patterns of recurrent disease, we used a guinea pig ocular infection model to evaluate clinical signs and to analyze viral DNA load and gene

expression in sensory and autonomic ganglia at various time points postinoculation. After ocular infection, reactivation of HSV-1 occurred from autonomic ganglia to cause recurrent disease symptoms, but there was no evidence that HSV-2 could independently reactivate from autonomic ganglia to cause recurrences. The differences in viral gene expression in sympathetic and parasympathetic ganglia are likely responsible for differences between HSV-1 and HSV-2 virulence and reactivation.

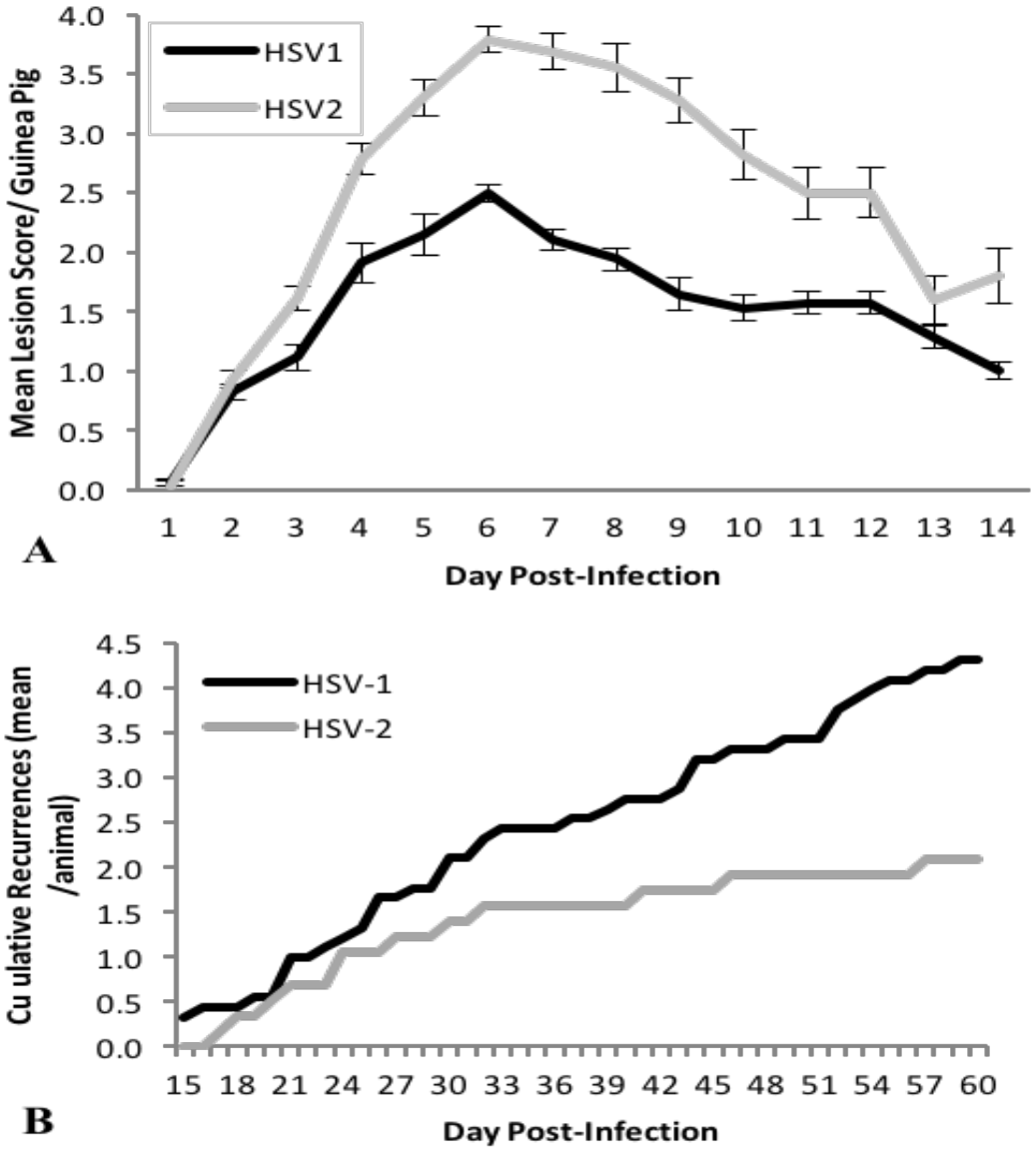
Results

Acute disease and cumulative recurrences in the ocular guinea pig model.

To characterize HSV-1 and HSV-2 ocular disease in a guinea pig model, female guinea pigs were inoculated by topical application of virus ($5x10^5$ PFU of virus) and observed for 60 days for clinical signs. During the acute phase of infection (1 to 14 dpi), HSV-2 produced significantly more severe ocular disease than did HSV-1 infection (Fig. 3A) (*P value*= 0.002 by Mann-Whitney test). Both viruses produced corneal and periorbital lesions, corneal clouding, conjunctivitis, and blepharitis. However, HSV-2 produced deep stromal ulcerations in 18 of 42 guinea pigs (42.9%), of which 12 were bilateral, while similar deep lesions were observed in only 1 of 40 (2.5%) guinea pigs infected with HSV-1. Between days 5 and 9 p.i., head tilting, rotational head movement, and postural instability consistent with vertigo were observed in 13 guinea pigs infected with HSV-2; these symptoms resolved in 2 to 4 days, and similar signs were not observed in HSV-1-infected guinea pigs. HSV-1 produced recurrent corneal and periocular lesions at a

significantly higher frequency from days 15 to 36 p.i. than did HSV-2 (Fig. 3B) (*P value*= 0.020 by Mann-Whitney test).

Fig 3. Acute lesion severity and cumulative recurrences of HSV-1 and HSV-2 after ocular infection



A) Severity of acute infection from 1-14 dpi, graphed as mean lesion score for each group of animals on each day of observation, based on a scale of 0-4; HSV-1 (n=40); HSV-2 (n=42); p=0.002 by Mann-Whitney test. B) Cumulative recurrences per guinea pig for each group during latent infection from 15-60 dpi; HSV-1 (n=11); HSV-2 (n=9); p=0.020 by Mann-Whitney test.

HSV-1 produced asymptomatic latent infection with defined episodes of symptomatic recurrences, consisting of 1 or 2 corneal and/or periocular lesions that cleared in 2 to 4 days. However, HSV-2 produced a more persistent form of symptomatic disease, characterized by continuous eruption of lesions and corneal clouding over a period of 5 to 18 days with minimal clearance between episodes. In addition, all nine of the HSV-2-infected guinea pigs observed throughout the 60-day period developed vesicular lesions on the nose, while none of the animals infected with HSV-1 developed nose lesions, suggesting a more extensive zosteriform spread with HSV-2 than HSV-1.

Viral DNA in sensory and autonomic ganglia.

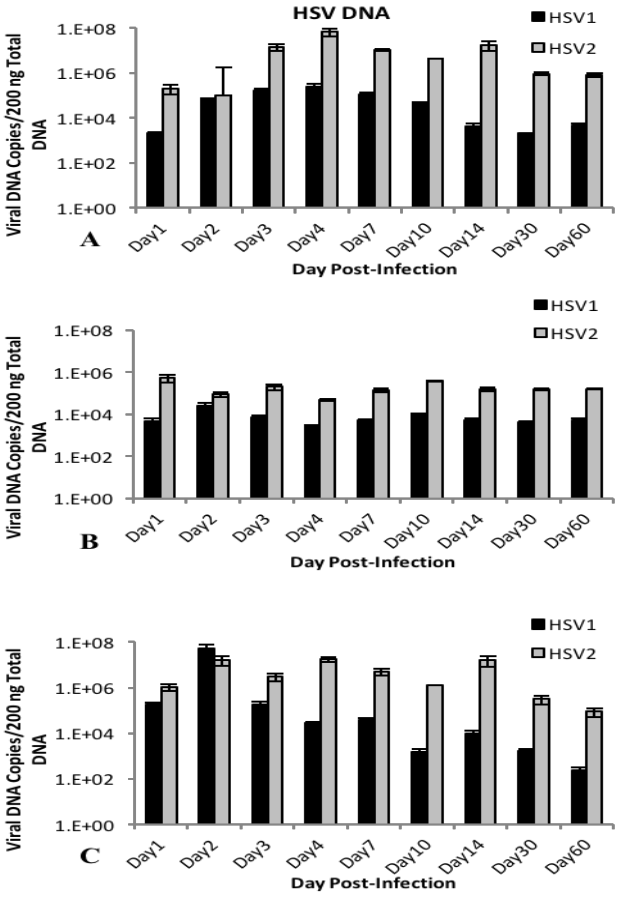
To determine if differences in viral DNA load were responsible for differences in lesion severity and recurrence frequency, viral DNA levels were evaluated at various time points in sensory and autonomic ganglia, including the sensory trigeminal ganglia (TG), sympathetic superior cervical ganglia (SCG), and parasympathetic ciliary ganglia (CG). Both HSV-1 and HSV-2 efficiently infected sensory neurons in the TG after ocular infection, as expected (Fig. 4A). Viral DNA increased during the first 4 days of infection, correlating with increasing clinical severity of the infections. By day 14 p.i., a latent viral DNA reservoir was established, and it was maintained throughout the 60-day experiment (Fig. 4A). Although the quantity of HSV-1 DNA was consistently lower than the quantity of HSV-2 (*P value*=0.0001), the viruses produced nearly identical patterns within the TG, reaching a peak on day 4 p.i. and decreasing thereafter. In the sympathetic SCG, HSV-1 viral DNA increased transiently on day 2 post-infection and then maintained a static quantity of viral DNA in the ganglia, suggesting that the virus replicated briefly within the ganglia early after infection and then established a latent reservoir in the SCG, which

remained stable throughout the 60-day observation period (Fig. 4B). HSV-2 viral DNA showed minor variability between time points but remained relatively stable throughout the infection period. While there was a significant difference in the quantities of viral DNA detected in the SCG (*P value*= 0.0001), the overall patterns were similar, with both viruses maintaining a similar quantity of viral DNA at all time points.

In the parasympathetic CG, viral DNA was detected 1 day after inoculation and both HSV-1 and HSV-2 viral DNA increased on day 2 p.i. However, HSV-2 DNA remained elevated from day 3 through day 14 p.i., while HSV-1 DNA began decreasing on day 3 p.i. (*P value*= 0.0001) (Fig. 4C).

A significantly greater quantity of HSV-2 viral DNA was detected in all three sensory and autonomic ganglia analyzed, implying that the viral load could have been responsible for the difference in severity of disease but not for the difference in recurrence frequency.

Fig 4. HSV viral load in sensory (A), sympathetic (B) and parasympathetic (C) ganglia after ocular infection



HSV-1 and HSV-2 viral DNA extracted from ganglia was quantified by qPCR in A) Sensory trigeminal ganglia (TG), B) Sympathetic superior cervical ganglia (SCG), and C) Parasympathetic ciliary ganglia (CG).

Thymidine kinase expression in sensory and autonomic ganglia.

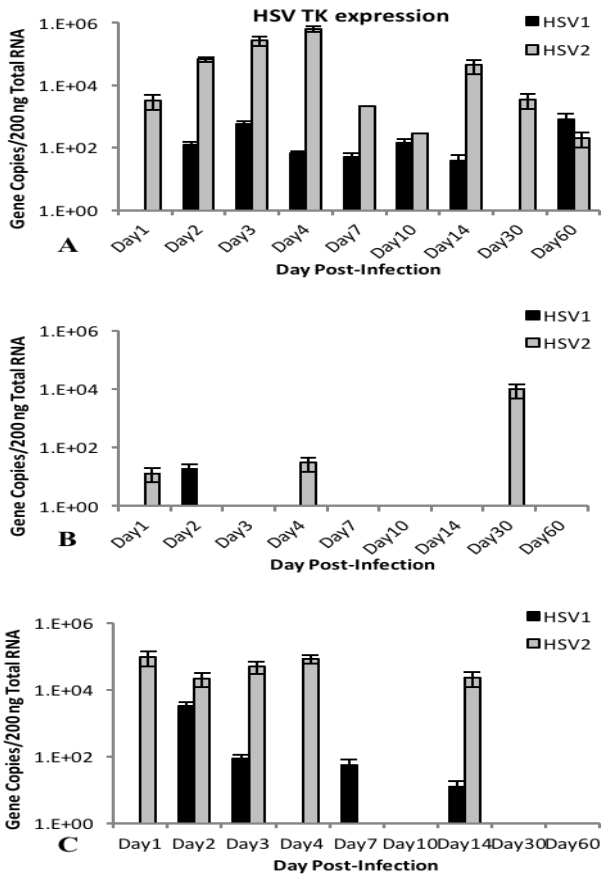
HSV encodes the enzyme TK, which is important for HSV viral replication in neurons (76). Expression of TK was analyzed to verify active viral replication in the ganglia, as opposed to just the presence of viral DNA.

During acute infection (1 to 14 dpi), HSV-2 expressed higher levels of TK in TG, SCG, and CG than did HSV-1, correlating with the more-severe acute disease symptoms observed in the guinea pigs. In the sensory TG, HSV-1 and HSV-2 expressed relatively similar patterns of TK expression (Fig. 5A), although HSV-2 TK expression was generally higher than HSV-1 expression (*P value*= 0.0001). In the sympathetic SCG, however, TK expression was detected from HSV-2-infected animals only on days 1 and 4 p.i. and from HSV-1-infected animals on day 2 (Fig. 5B). Considering the quantity of viral DNA detected in the SCG, these results indicate that adult sympathetic SCG limit both HSV-1 and HSV-2 replication during acute infection of guinea pigs after ocular in- fection. In the parasympathetic CG, HSV-2 TK expression was sustained at high levels for the first 4 days after infection (Fig. 5C), which was significantly different from HSV-1 TK expression during the same time period (*P value*= 0.025). HSV-1 TK expression was detected at a high level on day 2 and at decreased levels on days 3 and 7. Combined with the detected DNA quantities, these patterns of TK expression suggest that sensory TG and parasympathetic CG support both HSV-1 and HSV2 replication, resulting in large quantities of viral DNA in the ganglia at latent time points. However, CGs preferentially support HSV-2 replication rather than HSV-1 during the first 4 days of acute infection. Even though large quantities of HSV-1 and HSV-2 viral DNA are present in the SCG, neither virus replicates efficiently in the SCG after ocular infection.

During latent time periods, HSV-2 TK expression was detected in TG, SCG, and CG (Fig. 5A, B and C), coincident with observed recurrent lesions. On day 14, TK expression was detected in 2 of 4 TGs; one of these guinea pigs concurrently expressed TK in the CG. On day 30, TK was detected in a single guinea pig in both TG and SCG. On day 60, TK was detected in a single guinea pig in the TG only. HSV-2-infected guinea pigs that had lesions at the time of tissue analysis expressed TK in just TG or in both TG and autonomic ganglia; thus, the recurrent lesions could have originated from replicating virus in either the TG or the autonomic ganglia. HSV-1 TK was detected in TG and CG, but not SCG during latent time points. On day 14, one guinea pig with no lesions expressed TK in the TG only, demonstrating that HSV-1 can replicate in the TG without producing peripheral lesions. An- other guinea pig with lesions on day 14 p.i. expressed TK in CG only, demonstrating that HSV-1 can reactivate from the CG independently of the TG to cause recurrent ocular lesions. HSV-1 TK expression was also detected on day 60 in the TG of a single animal, which had lesions but no detectable TK expression in other ganglia. Thus, HSV-1 can reactivate from either TG or CG to produce recurrent ocular lesions.

Fig 5. HSV replication in sensory (A), sympathetic (B) and parasympathetic (C) ganglia

after ocular infection



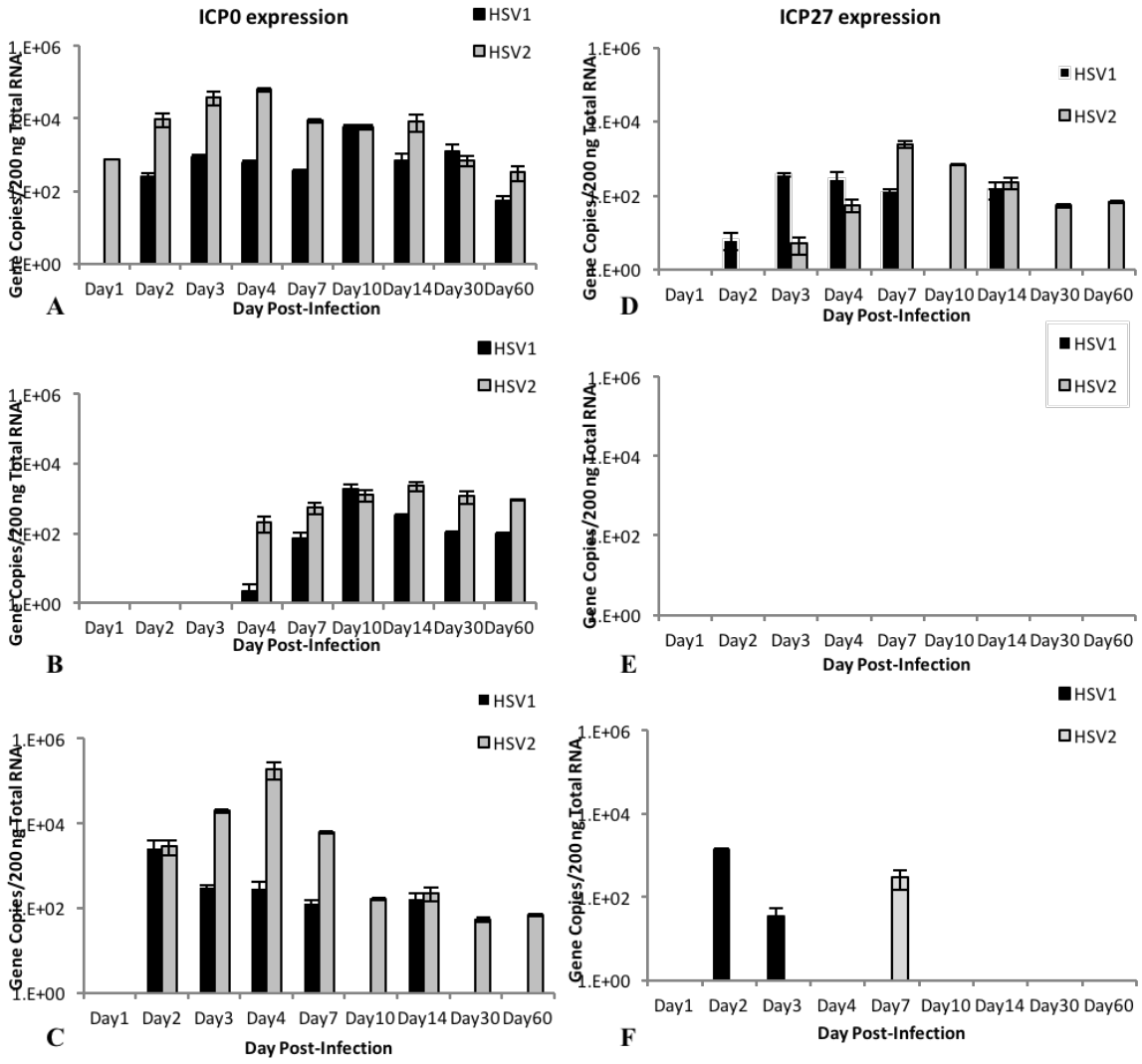
HSV-1 and HSV-2 viral gene thymidine kinase copy number was quantified by qRT-PCR in A) Sensory trigeminal ganglia, B) Sympathetic superior cervical ganglia, and C) Parasympathetic ciliary ganglia. n=2-3 samples per group per time point.

IE gene expression in sensory and autonomic ganglia.

HSV produces immediate early (IE) genes, which manipulate the host cell replication and antiviral mechanisms to promote viral early (E) and late (L) gene expression. Infected cell protein 0 (ICP0) is a ubiquitin ligase involved in both lytic and latent infections and has been implicated in reactivation from latency (77, 78). ICP0 expression was detected in guinea pig TGs very early after infection, on day 1 p.i. for HSV-2 and day 2 p.i. for HSV-1, but there was no significant difference in expression profiles (Fig. 6A). In sympathetic SCG, ICP0 expression was delayed compared to expression in TGs, but the expression profiles for HSV-1 and HSV-2 were similar (Fig. 6B). Expression profiles differed in CG, however; while both HSV-1 and HSV-2 ICP0 transcripts were detected on day 2 p.i., HSV-2 continued to express ICP0 at all time points but HSV-1 expressed ICP0 at detectable levels during acute infection only, and at lower levels than HSV-2 (Fig. 6C). These results suggest that the function of ICP0 may differ between HSV-1 and HSV-2 in parasympathetic neurons.

Another viral IE gene encodes a multifunctional protein, ICP27, that contributes to host cell shutoff, downregulates the interferon response, and promotes viral transcription and translation (33). In guinea pig TGs, HSV-1 and HSV-2 both expressed ICP27 during acute infection (days 1 to 14 p.i.), but expression was also detected in HSV-2-infected ganglia analyzed at latent time points, suggestive of an ongoing persistent infection (Fig. 6D). No ICP27 was detected in SCGs from either virus (Fig. 6E), and expression was sporadically detected in CGs (Fig. 6F), suggesting that ICP27 expression is not required for lytic infection in autonomic neurons.

Fig 6. Immediate-early gene expression in sensory and autonomic ganglia of guinea pig.



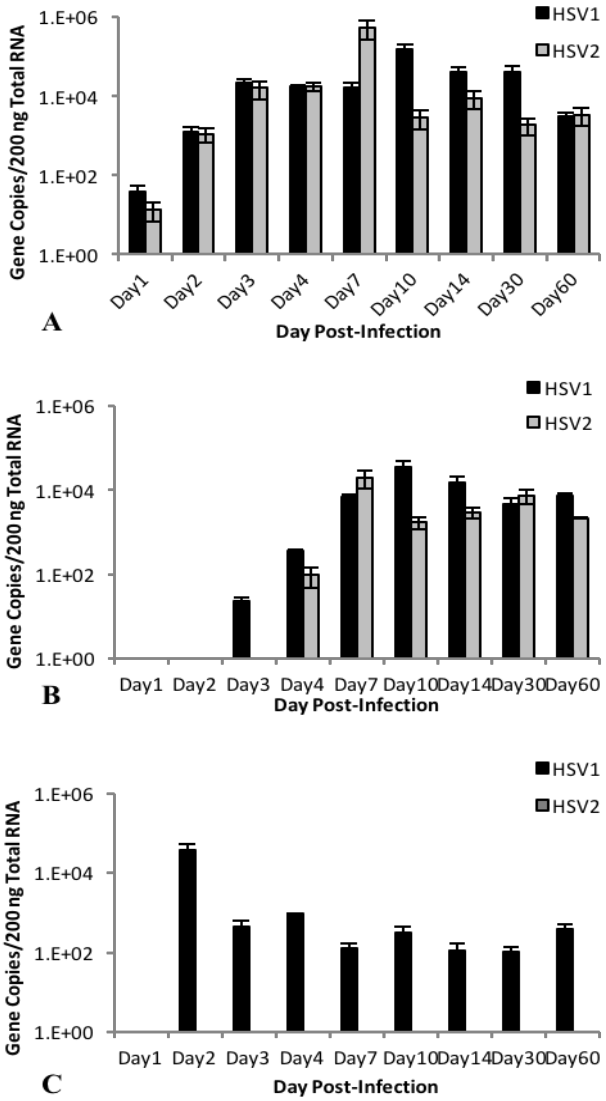
HSV-1 and HSV-2 viral immediate early (IE) genes ICP 0 and ICP27 copy number was quantified by qRT-PCR in A) ICP0 expression in sensory trigeminal ganglia, B) ICP0 expression in sympathetic superior cervical ganglia, and C) ICP0 expression in parasympathetic ciliary ganglia D) ICP27 expression in sensory trigeminal ganglia, E) ICP27 expression in sympathetic superior cervical ganglia, and F) ICP27 expression in parasympathetic ciliary ganglia. n=2-3 samples per group per time point.

LAT expression in sensory and autonomic ganglia.

The latency-associated transcript (LAT) is the most abundant gene expressed during HSV latency. While not required for establishment of latency, LAT exon 1 is necessary for HSV type-specific neuron specificity and characteristic patterns of reactivation (71, 79). To determine if differences in LAT expression play a role in differences between HSV-1 and HSV-2 recurrence frequency, LAT expression was analyzed in the sensory and autonomic ganglia.

In the TG, LAT expression was detected on day 1 p.i. and increased thereafter, as expected, demonstrating the establishment of latent infection in the TG (Fig. 7A). A similar pattern of LAT expression was detected in the sympathetic SCG, although LAT expression was first detected later after infection on day 3 for HSV-1 and day 4 for HSV-2 (Fig. 7B). The sustained presence of viral DNA and LAT expression in the SCG demonstrates that both HSV-1 and HSV-2 established latency in sympathetic ganglia as effectively as in sensory TG, and there were no significant differences between HSV-1 and HSV-2 LAT expression in the TG or SCG (*P value*= 0.231 and 0.200, respectively). A different pattern of LAT expression was identified in parasympathetic CG (Fig. 7C). HSV-1 LAT expression was detected at a high level on day 2 p.i. and persisted throughout the 60-day period of analyses. Since both viral DNA and LAT expression persisted, these data show that HSV-1 established a LAT-positive latent infection in the guinea pig CG after ocular infection. However, HSV-2 LAT expression was not detected in the CG at any time point (*P value*= 0.0001), suggesting either that HSV-2 is not capable of reactivation from ciliary ganglion neurons or that LAT is not required for reactivation from ciliary ganglia.

Fig 7. LAT gene expression in sensory and autonomic ganglia of guinea pig after ocular infection.



HSV-1 and HSV-2 viral gene LAT copy number was quantified by qRT-PCR in A) Sensory trigeminal ganglia, B) Sympathetic superior cervical ganglia, and C) Parasympathetic ciliary ganglia. n=2-3 samples per group per time point.

Viral infection and reactivation in primary cultured neurons.

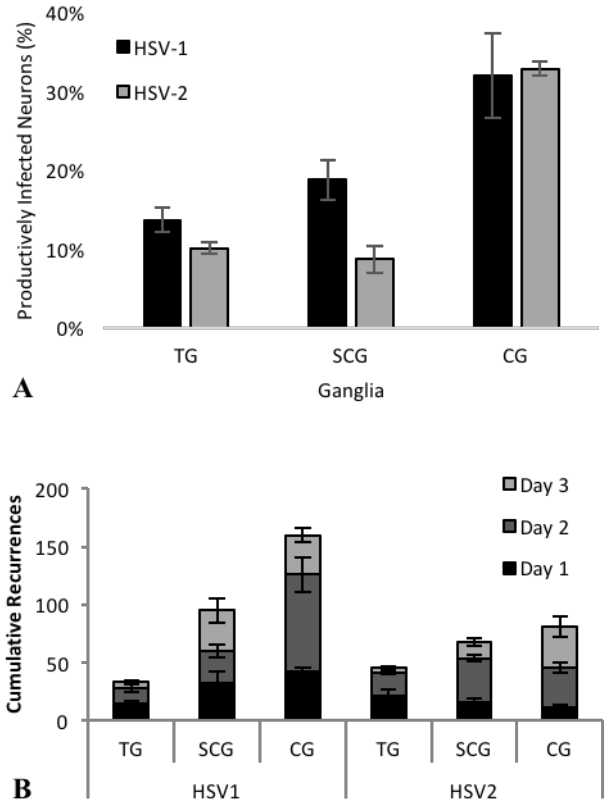
Animal models of HSV infection are necessary to identify differences in severity of disease and recurrence frequencies of HSV-1 and HSV-2. In an animal model, however, hormone levels and the immune response are involved in regulating disease severity and also contribute to viral reactivation to either limit or promote symptomatic recurrences. To identify any differences in the abilities of HSV-1 and HSV-2 to productively infect sensory and autonomic neurons without the influence of the adaptive immune response or exogenous hormone induction, cultured primary adult murine ganglionic neurons from TG, SCG, and CG were infected. Nine hours after infection, the percentages of cultured neurons that were productively infected with HSV-1 or HSV-2 were determined, using polyclonal antisera to visualize HSV productive cycle antigens. HSV-1 and HSV-2 were detected in similar percentages of TG and CG cultured neurons (Fig. 8A). In cultured SCG, however, HSV-1 productively infected a significantly greater percentage of cultured neurons (18.8%) than did HSV-2 (8.7%).

In the guinea pigs, neither HSV-1 nor HSV-2 productively infected SCGs efficiently, suggesting that extracellular factors limit HSV-1 productive infection of SCG neurons *in vivo* in the guinea pig model. Additional studies are required to determine whether these extracellular factors are immune system- or hormone-related factors or a host-specific restriction mechanism.

The guinea pig ocular infection studies demonstrated that HSV-1 could reactivate from CG independently from TG to cause symptomatic recurrences. However, the guinea pig studies did not provide evidence that HSV-2 recurrences could be caused by viral

reactivation in autonomic ganglia independently from TG. To determine if HSV-1 and HSV-2 could reactivate from individual autonomic neurons, mice were infected with HSV-1 or HSV-2 viruses that express a VP26-GFP fusion protein during replication. VP26, expressed as a late gene, is a small capsid protein that decorates the outer surface of the mature capsids, bound to VP5 (80) (81) (82) ; previous studies have demonstrated that the HSV-VP26- GFP reporter viruses effectively represent productive infection (66, 72). Twenty-one days post infection, when the viruses had established latency, TG, SCG, and CG were removed from infected mice, cultured, and observed for viral reactivation, visualized by expression of GFP in individual neurons. Cumulatively over 3 days after culture, HSV-1 and HSV-2 both reactivated in a greater number of autonomic neurons than of TG neurons (Fig. 8B), demonstrating that autonomic neurons effectively support viral reactivation. HSV-1 also reactivated much more efficiently from CG neurons, with an average of 160 cumulative reactivations, than did HSV-2, with an average of 61 cumulative reactivations in individual neurons, demonstrating the viral type selectivity of HSV reactivation.

Fig 8. HSV-1 and HSV-2 infection in the mice primary cultured sensory, sympathetic and parasympathetic neurons



A) Percentage of cultured primary adult neurons productively infected with HSV-1 or HSV-2 at 10 moi for 10 hrs, immunostained for HSV antigens with polyclonal antisera, in trigeminal ganglia (TG, p=0.045), superior cervical ganglia (SCG, p=0.015), and ciliary ganglia (CG, p=880). B) Cumulative number of reactivating neurons over 3 days *ex vivo* from ganglia harvested and cultured from adult mice latently infected with GFP-expressing HSV-1 or HSV-2.

Discussion

Although HSV-1 and HSV-2 infect the same tissues and produce lesions with similar characteristics, there are significant differences in recurrent disease patterns between HSV-1 and HSV-2. HSV-1 is most often associated with recurrent orolabial lesions and keratitis, while HSV-2 is more commonly associated with recurrent genital lesions. Ocular infections caused by herpesviruses present a serious clinical problem throughout the world, and an estimated 500,000 people have recurrent ocular HSV infections in the United States alone. While both HSV-1 and HSV-2 can cause ocular disease, HSV-1 is far more likely to spread to the eyes and cause recurrent disease, causing herpes simplex keratitis characterized by dendritic lesions and inflammation of the cornea, eventually leading to irreversible blindness. While HSV-1 orolabial lesions and herpes keratitis are commonly seen in healthy adults, reports of recurrent HSV-2 oral or ocular disease are rare and typically associated with immunocompromised status (83) (84). HSV-2 ocular disease most often manifests as acute retinal necrosis, rather than the recurrent keratitis characteristic of HSV-1 (85). While the incidence of HSV-1 genital disease is increasing due to changes in sexual behavior, HSV-2 is more likely to cause recurrent genital lesions, and the anatomical specificity of recurrent disease is not simply due to the site of infection; 60 to 90% of individuals with genital HSV-2 experience recurrences, while only about 25% of people with genital HSV-1 develop recurrent lesions (21, 22). It is not clear why these similar viruses reactivate preferentially in an anatomical site-specific manner to cause different patterns of recurrent disease.

In our guinea pig model, HSV-1 produced acute disease symptoms, which cleared in most animals by day 14, and then produced periodic recurrences in the form of corneal

and/or periocular lesions. The cornea became hazy during these episodic recurrences, suggestive of inflammation. Thus, guinea pig ocular disease caused by HSV-1 was clinically similar to human disease, which is characterized by recurrent corneal and/or periocular lesions along with inflammation and corneal clouding. After HSV-2 ocular infection, the guinea pigs experienced a more persistent form of symptomatic infection with deep stromal involvement, rather than defined episodic recurrences. The rare occurrences of human HSV-2 ocular disease typically take the form of persistent retinal necrosis. Although we did not evaluate the retinas of our guinea pigs, the persistent, necrotic disease characteristics observed in our guinea pigs were consistent with human HSV-2 ocular dis- ease. HSV-1 reactivated in the guinea pigs spontaneously to cause episodic symptomatic recurrences, while HSV-2 rarely reactivated after day 30 p.i., also consistent with recurrent disease frequencies in humans. Therefore, the guinea pig ocular model is a valuable model for investigating differences between HSV-1 and HSV-2 ocular disease.

Previous studies have demonstrated that HSV-1 and HSV-2 viral DNA is regularly found in the autonomic ganglia of humans and also in animal models after ocular or genital infection (58-62, 75, 86-88). Although many investigators have reported findings related to HSV activity in the sympathetic and parasympathetic autonomic ganglia, it is still not clear whether virus in the autonomic ganglia contributes to the pathogenesis of herpetic disease, either for severity of acute disease symptoms or for recurrent disease episodes. Since autonomic innervation and response patterns differ significantly between the face and the genitalia, it is highly likely that differences in viral behavior within autonomic ganglia account for the anatomical differences in HSV-1 and HSV-2 recurrent disease.

Our results in the guinea pig model demonstrate that HSV-1 and HSV-2 infected and established latency in autonomic and sensory ganglia after ocular infection. HSV-1 and HSV-2 behaved similarly in sensory trigeminal ganglia after ocular infection, with respect to patterns of viral DNA loads and gene expression throughout the time of analyses. However, the viruses behaved very differently in autonomic ganglia, which likely contributed to differences in the pathogenesis of acute disease as well as differences in reactivation. Both viruses established latency in sympathetic SCG, with comparable viral DNA loads and expression of LAT. However, HSV-1 did not express TK in the SCG with the exception of a single animal on day 2 p.i., and HSV-2 TK expression was sporadic in only a few animals. In addition, neither HSV-1 nor HSV-2 expressed ICP27 in the SCG. Thus, neither virus replicated efficiently in adult SCG after ocular inoculation. Although some viral replication of HSV-2 did occur in the sympathetic ganglia of three guinea pigs during symptomatic recur- rent disease, there was no evidence that the recurrence originated in the SCG, since virus was simultaneously replicating in the TG. HSV-1 was, however, capable of productively infecting cultured SCG neurons *in vitro*, suggesting that extracellular factors may act on the SCG neurons to inhibit HSV-1 viral replication *in vivo* in the guinea pig model. Additional studies are needed to determine whether those factors are immunological or hormonal in nature or whether they represent a species-specific restriction.

In the ciliary ganglia, both HSV-1 and HSV-2 replicated during acute infection, shown by increases in viral DNA and expression of TK and ICP0. In several animals, HSV-1 and HSV-2 TK levels were higher in the CG than in the TG, particularly for HSV-2, suggesting that virus replicating in the CG during acute infection was contributing to acute

disease symptoms. However, HSV-2 failed to express LAT in the CG at latent time points, suggesting either that HSV-2 was incapable of reactivating from the CG or that LAT is not necessary for HSV-2 reactivation from the CG. During HSV-2 recurrences, TK expression was detected in TG and CG simultaneously in a single animal, providing no evidence that recurrences originated from the CG since virus was also replicating in the TG. In contrast, HSV-1 expressed LAT in the CG throughout the 60-day period of analyses. At latent time points, guinea pigs displaying recurrent HSV-1 lesions expressed TK in either TG *or* CG, but never both, demonstrating that HSV-1 symptomatic recurrences could originate from either the TG or the CG. To develop more effective antivirals that can inhibit re- activation and prevent recurrent herpetic disease and viral transmission to new hosts, it is imperative to fully understand the processes involved in viral reactivation from neurons. HSV-1 and HSV-2 demonstrate preferences for productively infecting and establishing latency in specific types of neurons, and not only sensory neurons but autonomic neurons as well. Different populations of neurons are dependent on, and responsive to, a broad range of neurotrophic factors and hormones, depending on the repertoire of receptors and host factors expressed. Physiological stimuli that are known to reactivate HSV-1 and/or HSV-2, *in vivo* or *in vitro*, have a greater effect on activation and signaling cascades in autonomic neurons than in sensory neurons, and autonomic neurons harbor latent virus. Our studies demonstrate that HSV-1 is capable of reactivation from autonomic ciliary ganglia, independently from sensory trigeminal ganglia, to cause recurrent lesions after ocular infection, while actively replicating HSV-2 was not detected in CG independent of TG. Although additional studies are necessary, the ability of HSV-1, but not HSV-2, to independently reactivate from ciliary ganglia to cause recurrent disease may explain the

greater orofacial recurrence frequency of HSV-1 than of HSV-2.

Since autonomic innervation and response patterns differ significantly between the face and the genitalia, it is highly likely that differences in viral behavior within autonomic ganglia account for the anatomical differences in HSV-1 and HSV-2 recurrent disease.

Chapter 4. Latency Establishment of Herpes Simplex Virus type 1 and 2 in Sensory and Autonomic Ganglia After Genital Inoculation

Introduction

Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) infect and establish latency in peripheral sensory dorsal root ganglia (DRG) after genital infection. Both viruses can reactivate to cause recurrent genital lesions throughout the life of the host. Although HSV-1 is estimated to be responsible for approximately 30% of new genital herpes infections, the majority of recurrent genital lesions are caused by HSV-2 (24). Only 14% of individuals with HSV-1 genital infections develop symptomatic recurrences (22) (89). In contrast, 60% of HSV-2 genital infections reactivate to cause recurrent genital lesions (22).

HSV-1 and HSV-2 also infect and establish latent infection in autonomic ganglia. However, the relevance of these neurons to the establishment of viral latency and their contribution to recurrent disease have not been well established, particularly after genital infection. Most animal studies of HSV autonomic infection have focused on HSV-1 ocular infection, where it is relatively easy to dissect the autonomic ganglia (64, 90) (87, 91). After ocular infection of guinea pigs, HSV-1 can reactivate from autonomic ganglia independently from sensory ganglia to cause recurrent ocular lesions, but there has been no evidence that HSV-2 is capable of independently reactivating from autonomic ganglia after ocular infection (92). Thus, the autonomic ganglia may contribute to HSV-1 and HSV-2 recurrence frequency differences. Both HSV-1 and HSV-2 have also been detected in human autonomic ganglia in the head (61, 62, 64).

HSV-1 and HSV-2 latent virus has also been detected in the autonomic major pelvic ganglia (MPG) after genital infection of mice (58, 86, 93). In guinea pigs, HSV-1 and HSV-2 spread from the vagina to the spinal cord through autonomic pathways instead of through the sensory dorsal root ganglia, demonstrating that the pelvic autonomic ganglia play a

substantial role in HSV pathogenesis after genital infection (67). Primary and recurrent genital infection in humans is associated with urinary retention attributed to autonomic dysfunction, providing evidence of HSV infection of genitourinary autonomic neurons in humans (89). However, it is not clear whether latent HSV in autonomic ganglia after genital infection is reactivation competent and capable of contributing to recurrent symptoms.

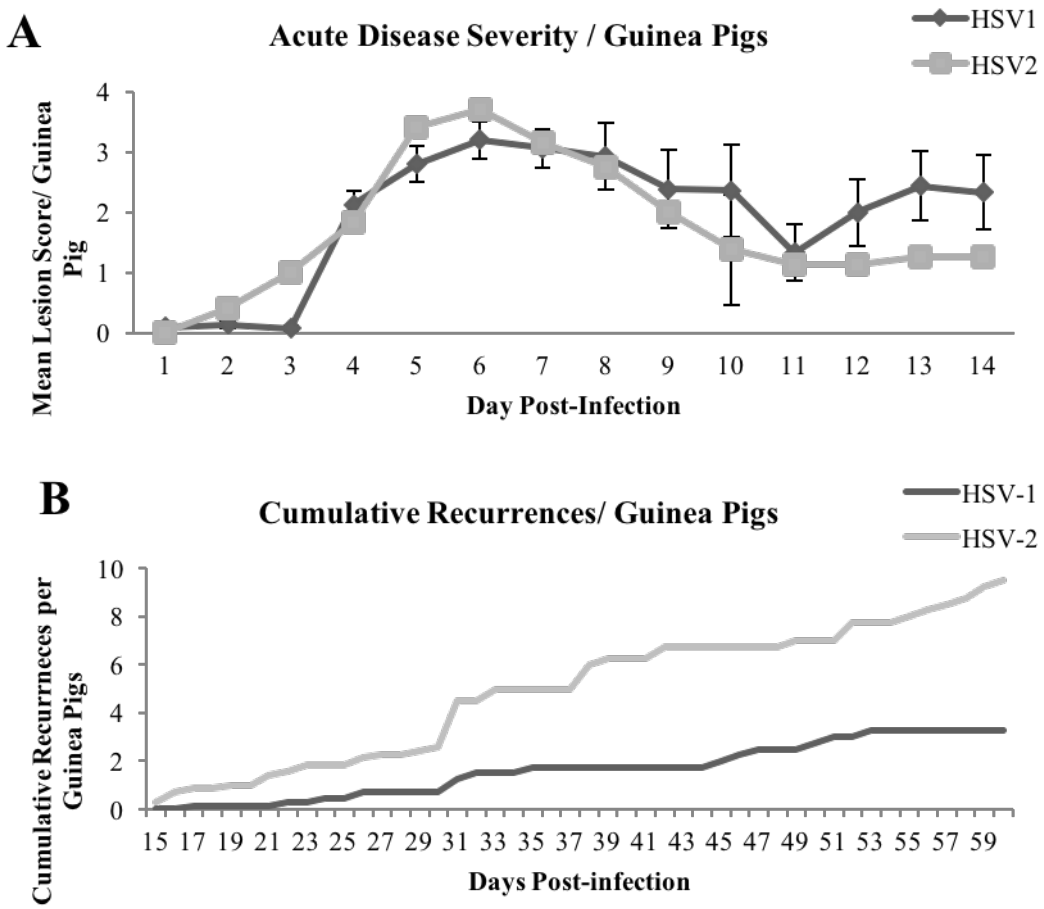
In the present study, we sought to characterize the role of the pelvic autonomic ganglia in the pathogenesis of recurrent genital herpes caused by HSV-1 and HSV-2, and determine whether autonomic pelvic ganglia may contribute to the higher HSV-2 genital recurrence frequency. After intravaginal infection of female guinea pigs, we analyzed viral DNA load and gene expression in the major pelvic ganglia (MPG) at various time points. We found that the clinically more frequent recurrences of HSV-2 result from more efficient lytic infection in the autonomic neurons. In addition, gene expression of HSV-1 and HSV-2 differed between sensory and autonomic neurons.

Results

Clinical lesion severity and recurrence frequencies after guinea pig genital infection.

To determine if autonomic major pelvic ganglia (MPG) innervating the genitals contributes to a higher frequency of HSV-2 genital recurrences, we infected adult guinea pigs intravaginally with HSV-1 or HSV-2. HSV-1 and HSV-2 produced herpetic lesions around the genital region during the acute phase (1 to 14 days post-infection (dpi)), although there were no significant differences between HSV-1 and HSV-2 (Fig 9A). After HSV-1 and HSV-2 established latent infection in the guinea pigs, recurrences were monitored through Day 60 pi and cumulative recurrences were graphed (Fig 9. B). Similar to observations with humans, HSV-2 genital infection produced significantly more frequent recurrent lesions in the guinea pigs compared to HSV-1 consistent with previously published studies (94).

Fig 9. Acute lesion severity and cumulative recurrences of HSV-1 and 2 after genital infection

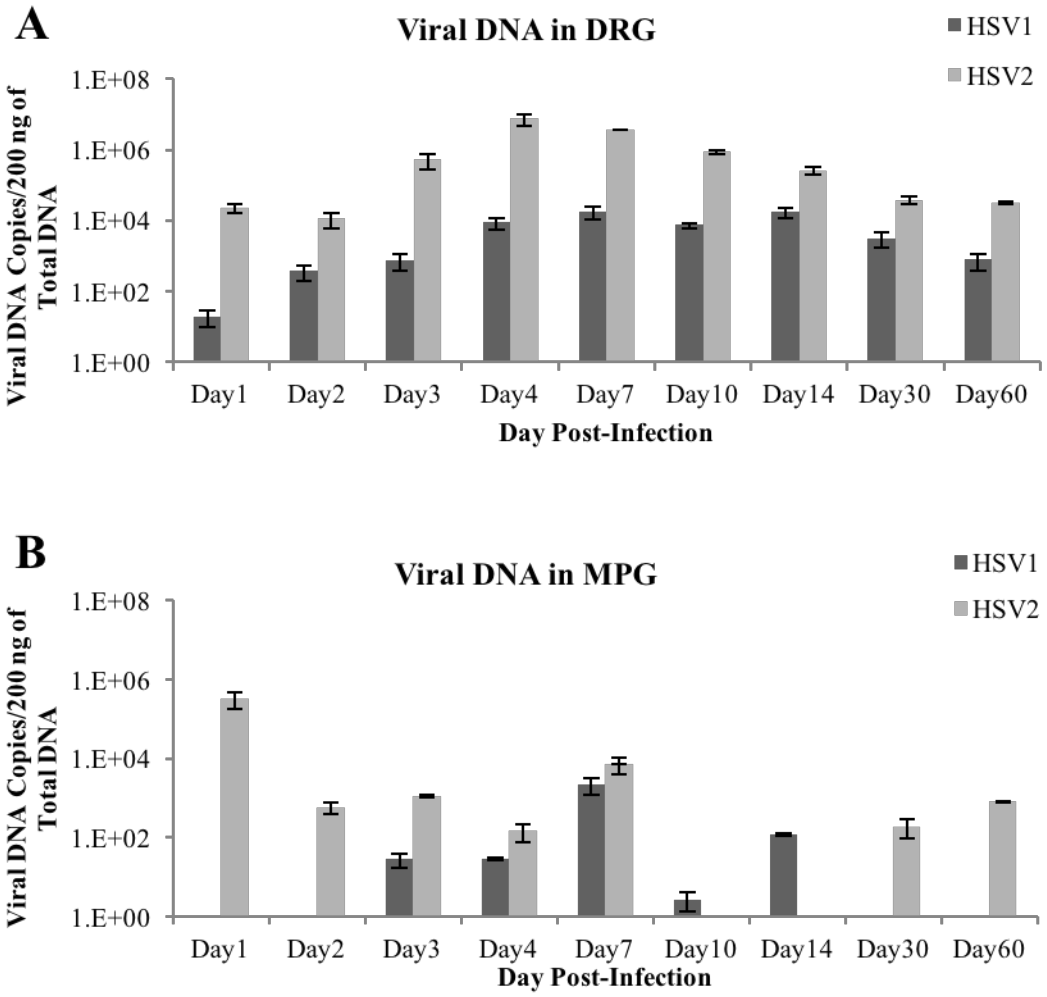


A) After genital HSV inoculation, female guinea pigs were daily monitored to evaluate clinical disease severity. There was no remarkable difference in acute lesion severity in acute phase (1 to 14 dpi). B) Cumulative recurrences of HSV was generated per guinea pigs. HSV-2 produced greater frequency of reactivation compared to HSV-1 after genital infection at latent time phase (15 to 60 dpi).

HSV viral DNA loads in sensory and autonomic ganglia.

HSV-1 and HSV-2 viral DNA loads in the sensory and autonomic ganglia were quantified to identify any differences between HSV-1 and HSV-2 after genital infection. In the sensory DRG, HSV-1 and HSV-2 viral DNA was detected from the first day of the study and persisted throughout the period of study, peaking on Days 4-7 pi and correlating with the severity of acute lesions (Fig 10. A). At all time points, HSV-2 DNA loads were higher than HSV-1 in DRG. In autonomic MPG, HSV-2 DNA was detected at high quantities on Day 1 pi, persisting throughout the study period (Fig 10. B), while HSV-1 DNA was only detected during acute infection from Days 2 through 14 pi. These results suggest that HSV-1 did not successfully establish latency in the autonomic MPG after genital infection.

Fig 10. HSV viral load in sensory dorsal root ganglia (DRG) and autonomic major pelvic ganglia (MPG)



HSV viral load was evaluated after genital inoculation. A) Both HSV-1 and HSV-2 viral DNA were detectable from the first day of study and remained stable through the end of study in sensory ganglia, although HSV-2 DNA load was higher than HSV-1 at all time points. B) In autonomic ganglia, HSV-1 DNA was first detectable after 3 dpi while HSV-2 DNA was persisted at acute phase (1 – 14 dpi). At latent phase (15 – 60 dpi), only HSV-2 DNA was present in autonomic ganglia.

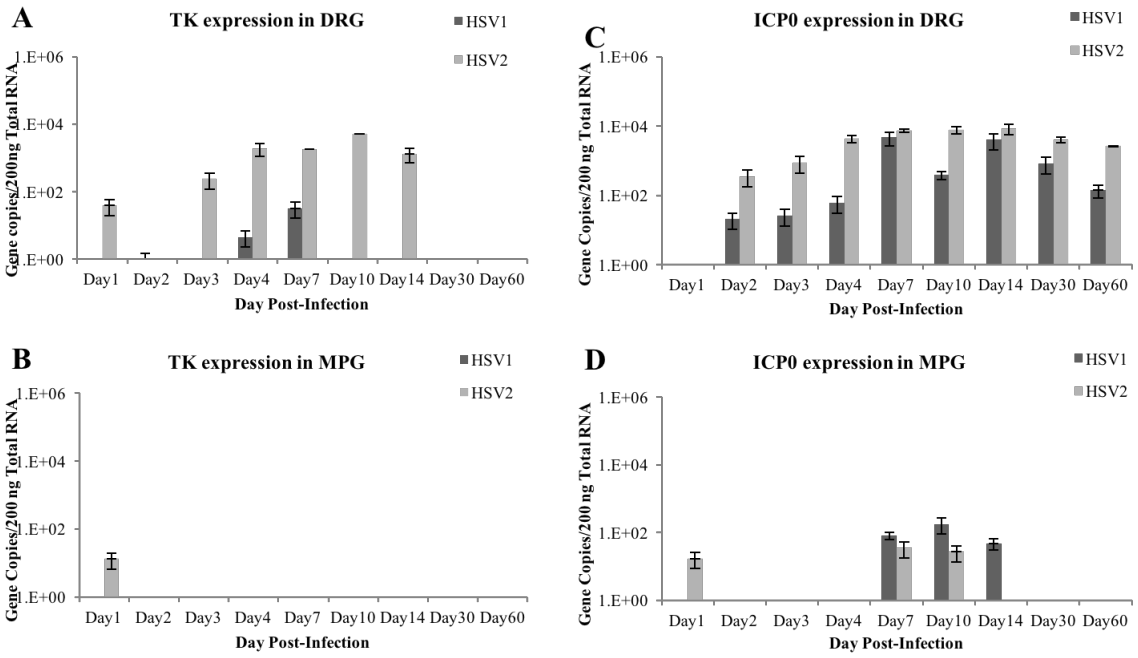
Viral gene expression in sensory and autonomic ganglia.

Thymidine kinase (TK) is an HSV-encoded enzyme that is important for HSV replication in neurons and the (95). In this study, TK expression was analyzed as a marker for HSV replication in ganglia (Fig 11. A and B). In sensory DRG, HSV-2 TK expression was detected at all time points through Day 14 pi, showing that HSV-2 replicated efficiently in the DRG during acute infection (Fig 11. A). HSV-1 TK expression was also detected during acute infection, although at lower quantities and for a shorter period of time post-infection. TK was not detected from either HSV-1 or HSV-2 during the latent time points (Days 30 and 60 pi), demonstrating that the viruses had established latent infections and were not reactivating at the time of euthanasia and analysis. In the autonomic MPG, TK was below the level of detection for both HSV-1 and HSV-2 at all time points, with the exception of a small quantity of HSV-2 TK on Day 1 pi, suggesting that HSV-2 replication occurs in the MPG after genital infection but is not sustained as it is in the DRG.

Immediate early (IE) gene expression in sensory and autonomic ganglia.

IE genes function to manipulate host cellular machinery and produce viral products required for progression of the lytic cycle. In this study, ICP0, ICP4 and ICP27 RNA transcripts were quantified by qPCR to identify differences between HSV1 and HSV2 in the sensory and autonomic ganglia. ICP0 gene expression is required for efficient productive infection and reactivation from latency (34). ICP0 expression from both HSV-1 and HSV-2 was first detected in sensory ganglia at 2 dpi, then continued to be expressed through 60 dpi without remarkable differences between HSV-1 and HSV-2 (Fig 11. C). In the autonomic MPG, however, ICP0 was transiently detected from HSV-2 24 hpi. ICP0 was also detected during the peak of infection from both HSV-1 and HSV-2, on Days 7-14 dpi for HSV-1 and Days 7-10 dpi for HSV-2 (Fig 11. D). ICP4 suppresses LAT and its own expression, but promotes early (E) lytic gene expression, thus preventing establishment of latency and driving productive infection forward. ICP4 and ICP27 expression was also analyzed by qRT-PCR assay. However, only HSV-2 ICP4 expression was detected in sensory ganglia, while no detectable ICP4 expression was present in autonomic ganglia. HSV-1 and HSV-2 ICP27 expression was detectable in the sensory ganglia but not detectable in autonomic ganglia (data not shown).

Fig 11. HSV viral replication in DRG and MPG and immediate early gene (IE) Infected cellular protein 0 (ICP0) expression in DRG and MPG

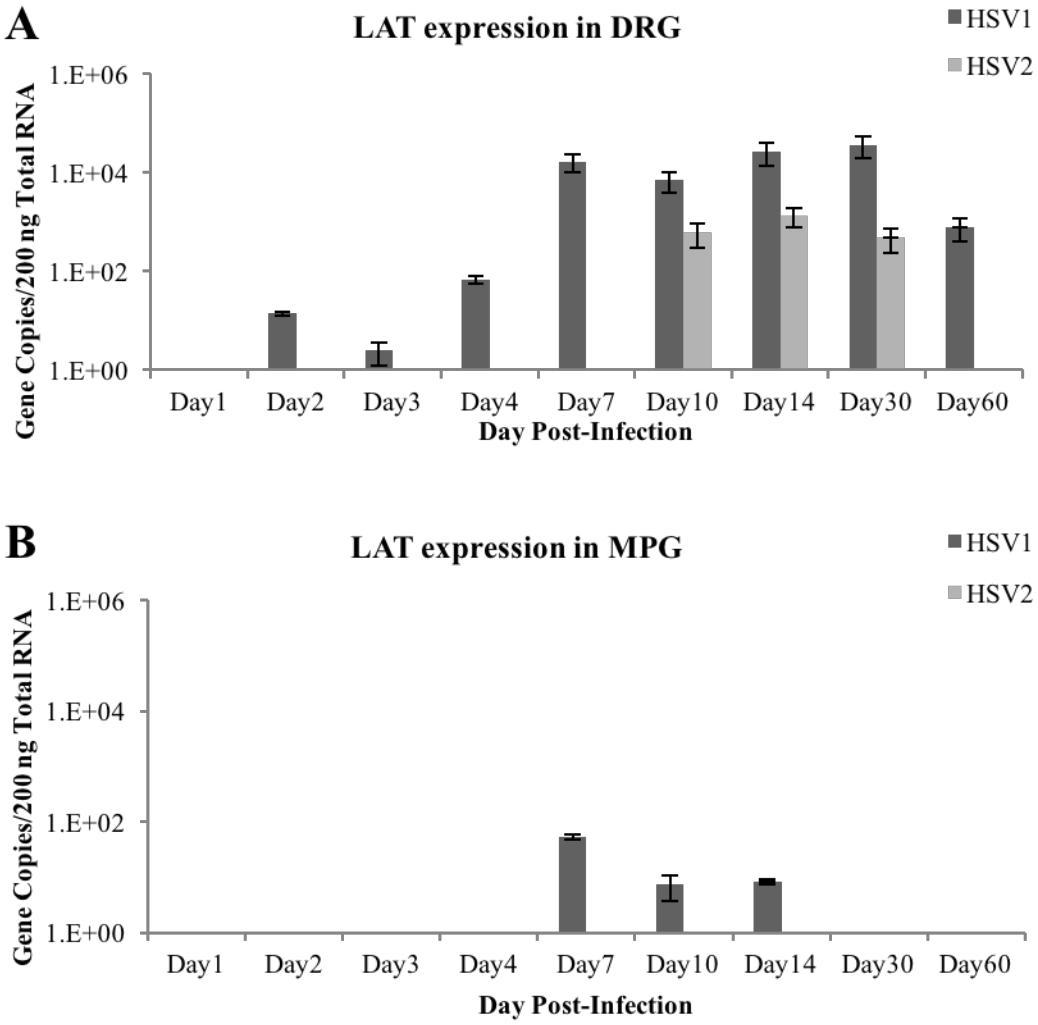


Productive infection of HSV in sensory and autonomic ganglia was evaluated. A) Thymidine kinase (TK) gene expression was measured as a marker for HSV replication in sensory ganglia. Both HSV-1 and HSV-2 replicated efficiently in sensory ganglia at acute phase. B) Only HSV-2 replication was observed in autonomic ganglia. C) ICP0 expression in sensory ganglia showed no remarkable differences between HSV-1 and HSV-2. D) Both HSV-1 and HSV-2 expressed ICP0 at second week of study and sporadic HSV-2 ICP0 expression was present at 1 dpi.

LAT expression in sensory and autonomic ganglia.

The latency-associated transcript (LAT) is the most abundant gene expressed during HSV latency (Fig 12.). In the DRG, HSV-1 LAT expression was detected beginning on Day 2 pi and continued to increase throughout acute infection. HSV-2 LAT was not detected in the DRG until Day 10 pi. In the autonomic MPG, only HSV-1 LAT was detected. Taken together, these results suggest that HSV-2 establishes latency in autonomic MPG, replicates during acute infection, but does not express LAT during latent infection.

Fig 12. Latency associated transcript (LAT) expression in DRG and MPG.



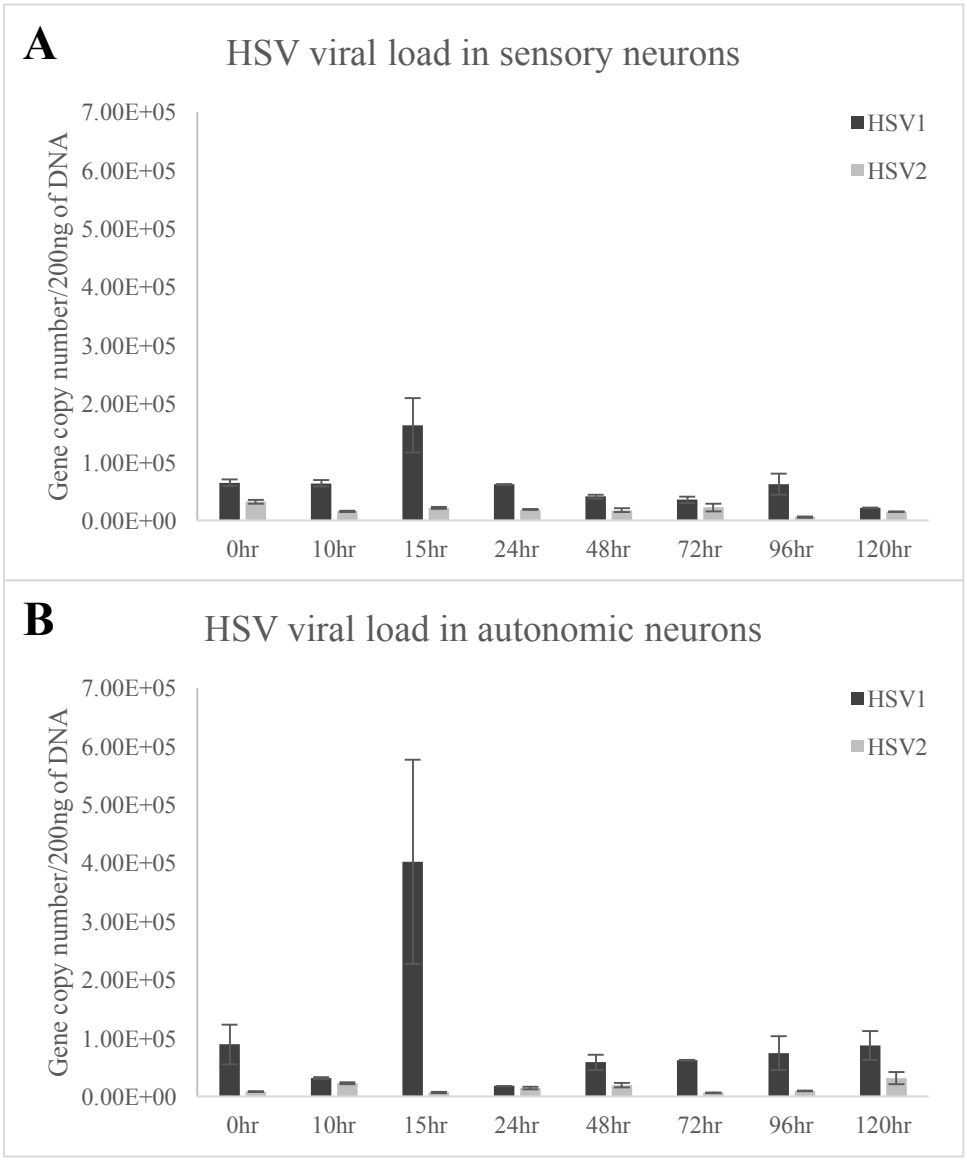
LAT expression was evaluated as a marker for HSV latency in sensory and autonomic ganglia. A) In the sensory ganglia, HSV-1 LAT expression was detectable from 2 dpi to 60 dpi and HSV-2 LAT expression was from 10 dpi to 30 dpi. Thus LAT expression from sensory ganglia alone was not sufficient to explain the clinical difference of HSV recurrences. B) In the autonomic ganglia, only HSV-1 LAT expression was detectable.

HSV viral replication in primary adult cultured DRG and MPG.

In an animal model, HSV reaches sensory and autonomic ganglia from the periphery. Few neurons become infected *in vivo.* To better understand HSV-1 and HSV-2 replication characteristics in the DRG and MPG, we cultured primary adult sensory DRG and autonomic MPG neurons and infected them *in vitro.* In cultured adult DRG neurons, HSV-1 and HSV-2 replicated similarly for the first 12 hours post-inoculation. At 24 hpi, HSV-2 DNA was significantly higher than HSV-1 in DRG neurons (Fig 13. A). In autonomic MPG, both HSV-1 and HSV-2 replicated very slowly for the first 12 hours, followed by a significant increase in HSV-2 DNA, while HSV-1 DNA remained very low (Fig 13. B). These results indicate that while HSV-1 and HSV-2 replicated similarly in sensory DRG neurons, while autonomic neurons in the MPG selectively supported HSV-2 replication.

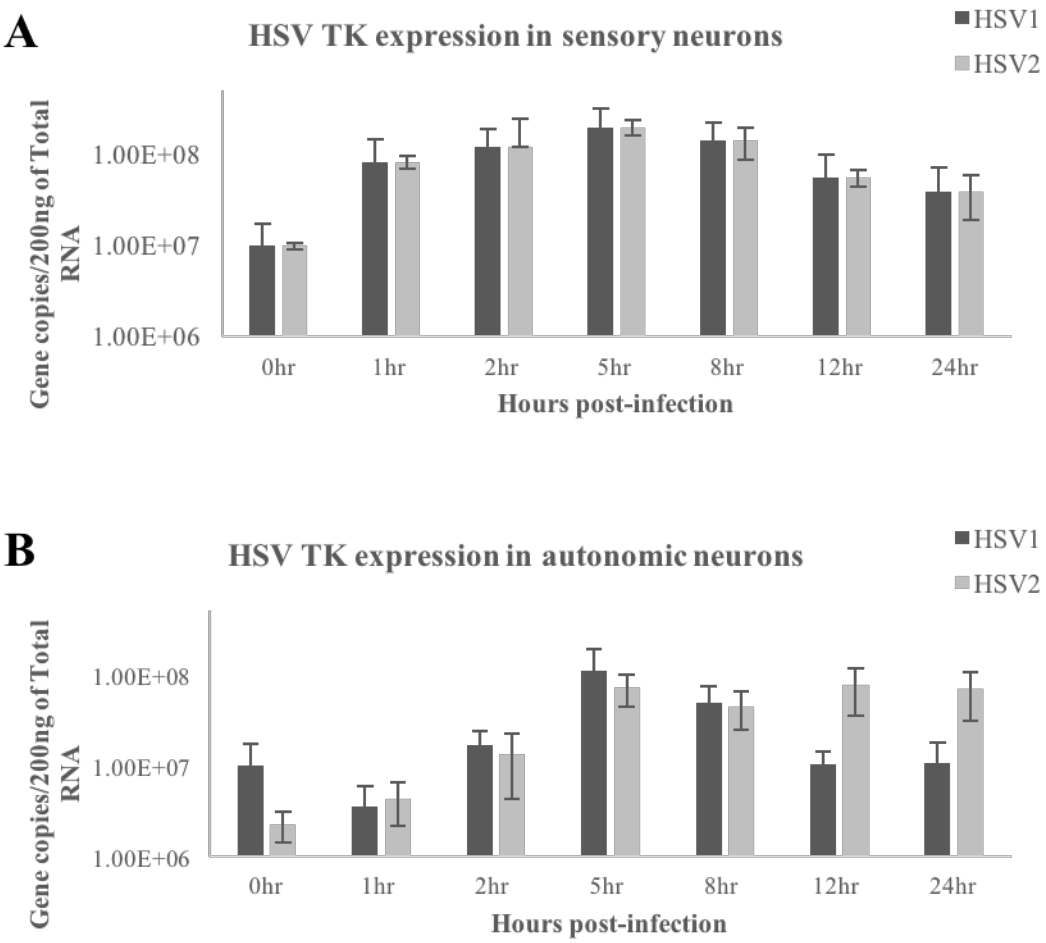
HSV TK expression was analyzed in adult neuronal cultures to further verify replication capabilities of HSV in sensory and autonomic neurons (Fig 14.). No significant differences between HSV-1 and 2 TK gene expression were present in sensory neurons. In autonomic neurons, HSV-1 and HSV-2 patterns of TK expression were similar only through the first 8 hpi. After 12 hpi, HSV-2 TK expression was significantly higher than HSV-1, suggesting that autonomic MPG neurons selectively maintain persistent HSV-2 replication during the first 24 hours.

Fig 13. *In vitro* HSV viral load change in mice primary cultured adult sensory (DRG) and autonomic (MPG) neurons



A) In vitro HSV infection showed similar pattern of fluctuation in sensory neurons. B) HSV infection of autonomic neurons showed stable viral load till 12 hours pi then HSV-2 viral load was increased selectively at 24 hour pi.

Fig 14. *In vitro* HSV replication in mice primary cultured adult sensory (DRG) and autonomic (MPG) neurons

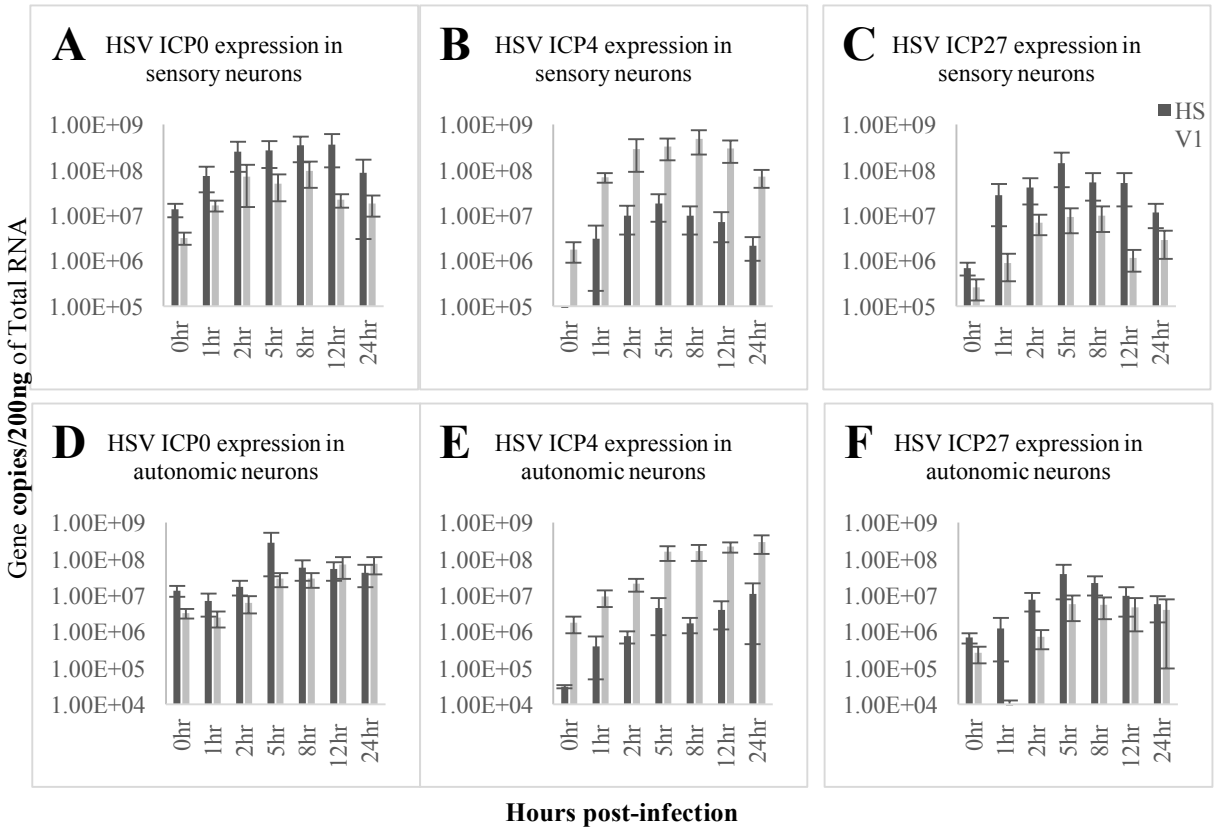


A) Both HSV-1 and HSV-2 expressed TK without remarkable difference in sensory neurons. B) HSV-2 expressed higher quantity of TK gene after 12 hours pi, correlating with higher HSV-2 viral load.

HSV IE genes expression in primary adult cultured DRG and MPG

Immediate early (IE) gene expression in sensory and autonomic neurons was also quantified during the first 24 hours pi (Fig. 15). No significant differences were detected in ICP0 or ICP27 expression although HSV-1 transcript levels were slightly higher than HSV-2. In contrast, HSV-2 ICP4 expression was significantly higher than HSV-1 at all time points in both DRG and MPG (Fig 15. B and E). In sensory neurons, the peak of ICP4 expression was detected at 8 hpi, followed by a decrease in expression. In autonomic neurons, however, HSV-2 ICP4 expression continued to increase throughout the 24 hour time period, correlating with the sustained TK expression and viral DNA load. These results suggest that ICP4 may be responsible for persistent HSV-2 infection of adult autonomic MPG neurons.

Fig 15. *In vitro* immediate early (IE) gene expression. ICP0, 4 and 27 expressions in mice primary cultured adult sensory (DRG) and autonomic (MPG) neurons

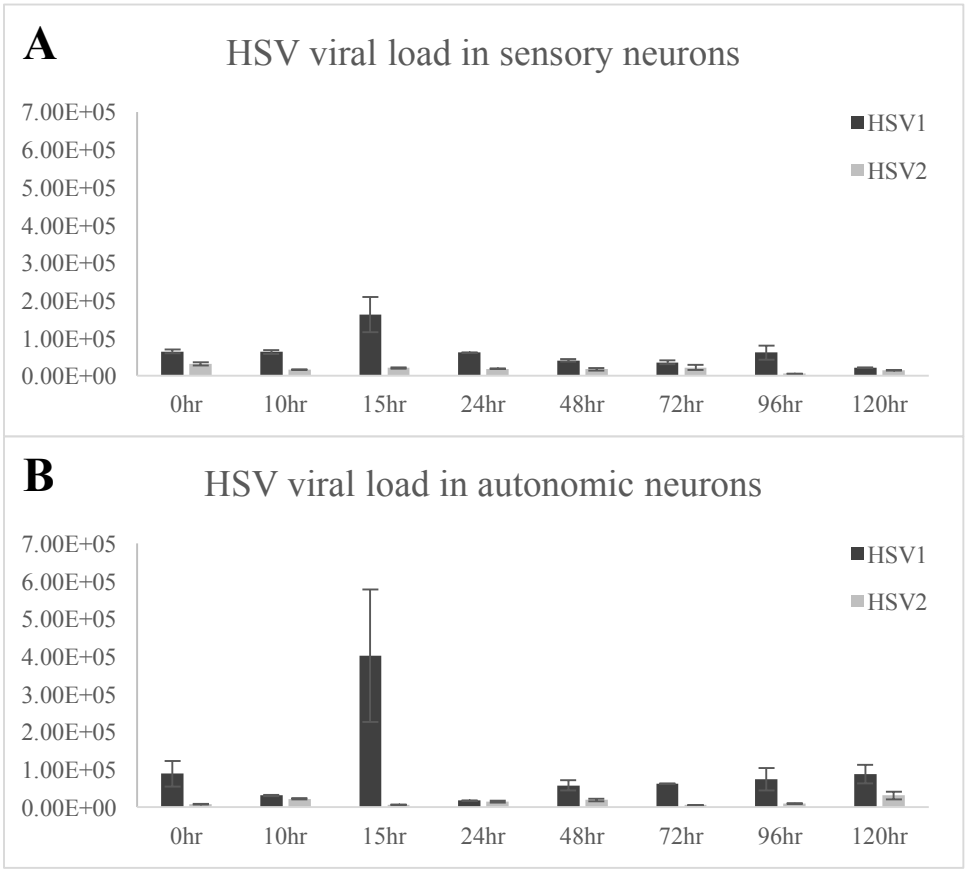


A) HSV-1 and HSV-2 ICP0 expression in sensory neurons showed same pattern with peak between 8 and 12 hours pi then reduced. B) HSV-2 ICP4 expression in sensory neurons exhibited higher quantity than HSV-1. C) HSV1 and HSV-2 ICP27 in sensory neurons showed same pattern of productive infection. D) HSV-2 ICP0 expression was gradually increased compared to the HSV-1 which had peak at 5 hour pi. E) HSV-2 ICP4 expression kept increased with higher quantity compared to HSV-1. F) HSV ICP27 expression in autonomic neurons showed no remarkable differences.

HSV reactivation in primary adult cultured DRG and MPG.

To further analyze gene expression during HSV reactivation in the sensory and autonomic neurons, HSV reactivation was tested in primary cultured sensory and autonomic neurons. Adding 300uM of ACV in the media successfully inhibited HSV replication in both sensory and autonomic ganglia. After removal of ACV from media, HSV-1 and HSV-2 spontaneously reactivated. HSV viral load was quantified (Fig 16.). HSV-1 viral load in the sensory neurons surged at 15 hrs after removal of ACV, suggesting HSV-1 was capable of reactivation in the sensory (Fig 16A) and autonomic neurons (Fig 16B). However, HSV-1 viral load remained stable after the first episode of reactivation in both sensory and autonomic neurons. HSV-2 viral load in the sensory neurons remained stable after reactivation. The result in the sensory neurons might be derived from the fact that since only neurons were collected, the viral load in the neurons did not reflect the actual HSV-2 viral load released into media. Unlike sensory neurons, HSV-2 viral load in the autonomic neurons showed three peaks during the experiment.

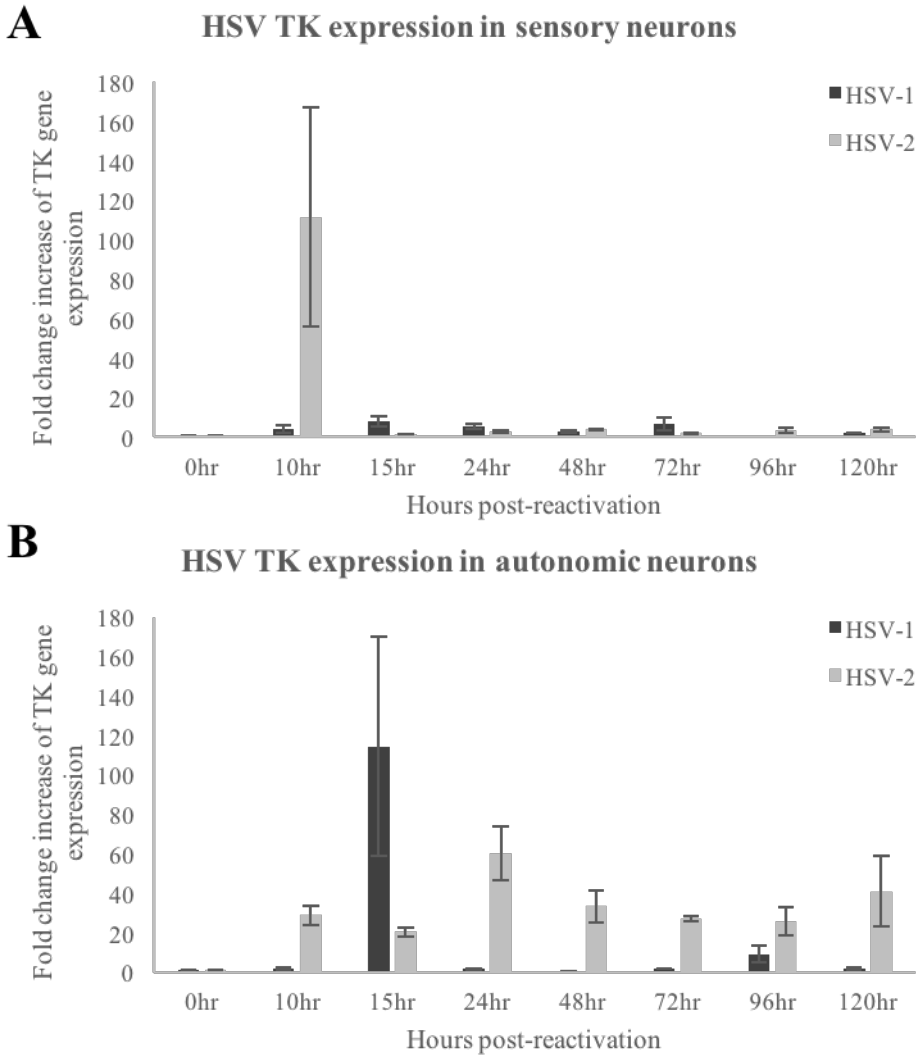
Fig 16. *In vitro* HSV viral load in the mice primary cultured adult sensory (DRG) and autonomic (MPG) neurons after spontaneous reactivation.



The viral load of HSV-1 and HSV-2 in the sensory and autonomic neurons were compared after induction of spontaneous reactivation by removal of Acyclovir. A) In the sensory neurons, HSV-1 successfully reactivated and peak of viral load was present at 15 hrs post-reactivation however, HSV-2 viral load in the sensory neurons was not changed a lot after reactivation in sensory neurons. B) In the autonomic neurons, the viral load of HSV-1 exhibited same pattern with in the sensory neurons. HSV-2 viral load in the autonomic neurons increased after 10hrs post-reactivation and persisted throughout the experiment.

HSV replication in sensory and autonomic neurons after spontaneous reactivation was shown by analyzing TK expression (Fig 17), since TK is expressed during active viral replication. In sensory neurons, both HSV-1 and HSV-2 replicated efficiently after reactivation. HSV-1 exhibited double peaks (at 15 and 72 hrs post-reactivation) of TK expression, correlating with the viral load, then remained stable. HSV-2 replication in sensory neurons showed a surge of replication (as shown by TK expression) at 10 hrs after reactivation then remained stable (Fig 17A). In the autonomic neurons, there was a peak of HSV-1 replication at 15 hrs post-reactivation, similar to the sensory neurons, however HSV-2 could replicate more efficiently than HSV-1 through the experimental time points (B), contributing to the higher HSV-2 viral load in the autonomic neurons after reactivation.

Fig 17. *In vitro* HSV replication in the mice primary cultured adult sensory (DRG) and autonomic (MPG) neurons after spontaneous reactivation.

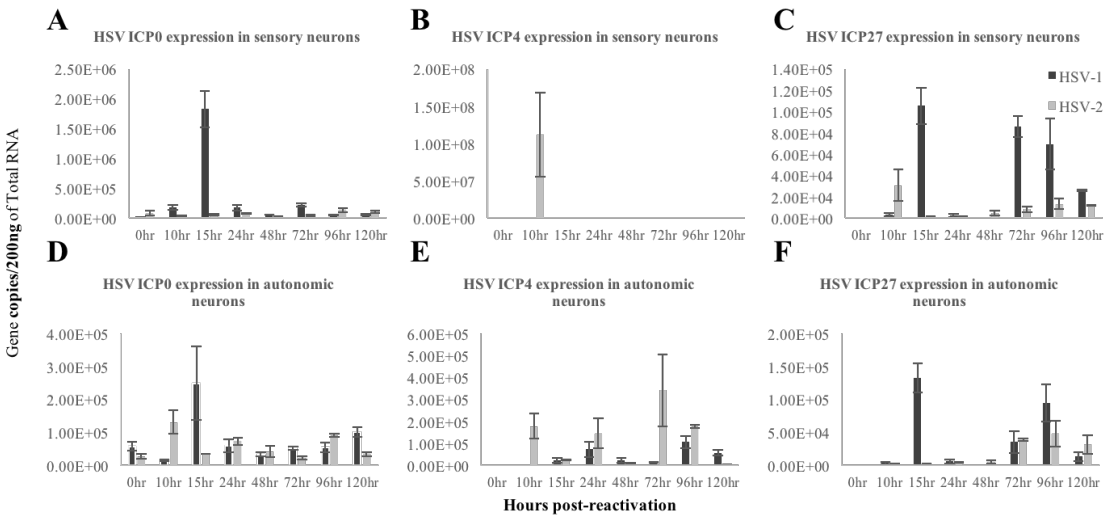


A) In the sensory neurons, HSV-1 replication was present with the peaks at the 15 and 72hrs post-reactivation. HSV-2 successfully replicated in the sensory neurons after reactivation and peak of replication was observed at 10 hrs post-reactivation. B) In the autonomic neurons, HSV-1 replication was peaked at 15hrs then remained low. In the autonomic neurons, HSV-2 started replication after reactivation then continued through the experiment.

IE genes expressions were also analyzed after reactivation in sensory and autonomic neurons (Fig. 18). HSV-1 ICP0 expression peaked at 15 hrs post-reactivation in both sensory and autonomic neurons, then remained stable. HSV-2 ICP0 expression was stable in the sensory neurons, while in autonomic neurons, HSV-2 ICP0 expression showed three peak points correlating with the increased HSV-2 viral DNA load after reactivation. HSV-1 ICP4 expression in the sensory neurons surged at 15 hrs post-reactivation, then stabilized. HSV-1 ICP4 expression in the autonomic neurons had double peaks but the expression was not correlated with HSV-1 DNA load in the autonomic neurons. HSV-2 ICP4 expression correlated with increased HSV-2 DNA load after reactivation, suggesting that ICP4 influenced efficient reactivation in neurons. It is not clear whether HSV-1 ICP4 expression in sensory or autonomic neurons had a neuron-specific pattern.

HSV-1 ICP27 expression in the sensory and autonomic neurons showed double peaks, at 15 and 96 hrs post-reactivation. HSV-2 ICP27 expression in the sensory neurons also had double peaks. However, in the autonomic neurons, ICP27 expression increased after 48 hrs after reactivation. Establishment of latent infection in the neurons, induced by the use of ACV, produced no detectable amount of ICP4 and ICP27. The only detectable IE gene was ICP0. The role of constant expression of ICP0 during latency in the neurons was not determined in this study. VP16 in the tegmental component of HSV. HSV-1 VP16 expression showed a similar pattern of fluctuation in the both sensory and autonomic neurons with a peak of expression at 15 hrs, after which it stabilized. HSV-2 VP16 expression in the sensory neurons was not altered during the experimental time points but in the autonomic neurons, there were dynamic increases of HSV-2 VP16 expression.

Fig 18. *In vitro* immediate early (IE) genes ICP0, 4 and 27 expressions in mice primary cultured adult sensory (DRG) and autonomic (MPG) neurons after spontaneous reactivation.

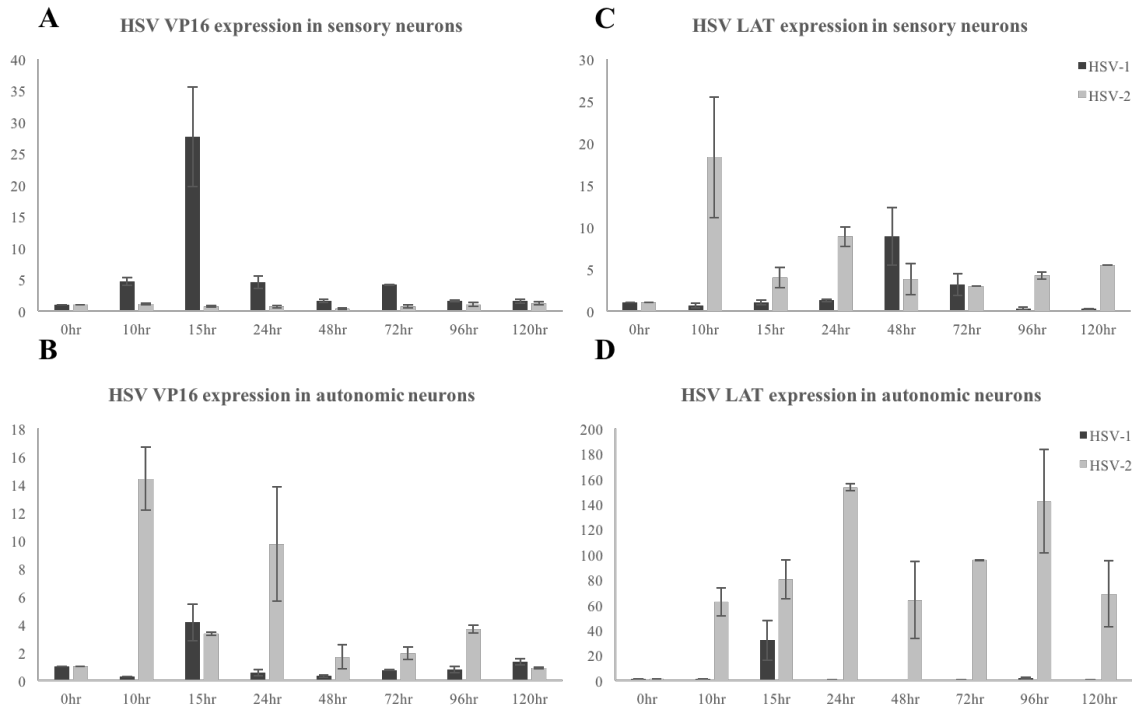


A) HSV-1 expressed ICP0 after reactivation in the sensory neurons. The peak of expression was present at 15hrs post-reactivation, same with the peak of viral load. HSV-2 ICP0 expression was present at the sensory neurons, however, the level of expression was lower compared to the HSV-1 B) ICP4 expression of HSV-2 was higher than HSV-1, while both HSV-1 and HSV-2 had peak of expression at 15hrs C) the expression of HSV-1 ICP27 had double peaks while HSV-2 expressed CIP27 at 10hrs then remained stable. D) ICP0 expression of HSV-1 and HSV-2 after reactivation had similar pattern in the autonomic neurons with the peak at the early time points. E) The fluctuation of ICP4 expression of HSV-1 and HSV-2 was present in the autonomic neurons, as with the ICP4 expression in the sensory neurons, the level of HSV-2 ICP4 expression was higher than HSV-1 at all time points. F) ICP27 expression of HSV-1 and HSV-2 showed similar pattern with the first peak at 15hrs then 96hrs.

The latency associated transcript (LAT) of HSV plays various roles in the establishment of latency and reactivation. HSV-1 LAT expression peaked at 48 hrs in the sensory neurons but in the autonomic neurons, the peak was present at 15 hrs post-

reactivation. The early onset of HSV-1 LAT expression in the autonomic neurons was correlated with increased HSV-1 load after reactivation. However, the delayed peak in the sensory neurons of HSV-1 LAT expression did not influence the HSV-1 load in the sensory neurons. HSV-2 LAT expression in the sensory neurons peaked early (10 hrs) then remained stable. Interestingly, latent HSV-2 in the autonomic neurons did not expressed LAT. However, after reactivation, latently infected HSV-2 started expressing LAT with double peaks at 24 and 96 hrs post-reactivation, implying that LAT might not be essential element of HSV in the establishment of latency in the autonomic neurons but required for efficient reactivation.

Fig 19. *In vitro* Late (L) gene expression. VP16 and latency associated transcript (LAT) expression in mice primary cultured adult sensory (DRG) and autonomic (MPG) neurons after spontaneous reactivation



A) HSV-1 VP16 expression had similar pattern with the viral load in the sensory neurons. HSV-2 VP16 expression was sustained but remained low. B) HSV-1 VP16 was correlated with the pattern of viral load in the autonomic neurons however, HSV-2 VP16 expression peaked within 24hrs then remained stable. C) HSV-2 expressed higher amount of LAT in the sensory neurons compared to the HSV-1. D) Minimal amount of HSV-1 LAT was present then peaked at 15hrs while HSV-2 did not express while latent infection, then expressed LAT after reactivation with the peaks at 24hrs and 96hrs post-reactivation.

Discussion

Both HSV-1 and HSV-2 infection in the female guinea pig genital model induced a similar pattern of disease during the acute phase (1 to 14 dpi). However, the pattern of reactivation differed between HSV-1 and HSV-2. Cumulative recurrences of HSV-2 in the guinea pig infection model were higher than HSV-1.

Both HSV-1 and HSV-2 reached the sensory neurons in the dorsal root ganglia (DRG), detectable from day 1 and producing a peak of HSV DNAat day 7 pi for HSV-1 and day 4 pi for HSV-2. Sensory neurons remained a stable HSV reservoir for the rest of study period. In autonomic ganglia, HSV-1 DNA was detected during the acute phase from 3 dpi to 14 dpi. At latent time points, no detectable HSV-1 DNA was present in autonomic ganglia. HSV-2 DNA in autonomic ganglia was present from 1 dpi and continued throughout the period of study. It should be noted that only HSV-2 DNA was detectable in autonomic ganglia at latent time points (30 and 60 dpi).

The pathway of HSV from the genital epithelium to sensory or autonomic ganglia was not determined in this study. Another researcher determined that when HSV-1 was inoculated into the mouse genital tract, HSV-1 spread into the autonomic enteric nerves system via nociceptive neurons, resulting in toxic mega colon and death of mice. HSV-1 infection in the sensory ganglia had limited expression of genes, thus not related with the death of the host. This result suggested that infection of autonomic nervous system with HSV had an impact on the pathogenesis of HSV (96). However, there was delay of HSV-1 transport into autonomic ganglia after genital infection compared to the sensory ganglia. It is not clear why autonomic axons transported HSV-1 into neuronal cell bodies slowly. HSV-1 in MPG might be derived from DRG but it was determined that spread of HSV into

other sites, such as the spinal cord, was independent of sensory innervation. When HSV-2 was inoculated into mice genitalia, HSV-2 DNA was present in a higher quantity in both sensory DRG and spinal cord. However, when HSV-2 was injected into the foot pad, which lacks autonomic innervation, HSV-2 viral DNA in the spinal cord was lower than the level of HSV-2 DNA after genital inoculation and spread of HSV into spinal cord was independent of sensory dorsal root ganglia (67). Thus, autonomic innervation selectively carried HSV into neuronal cell bodies and there was different mechanism that regulate HSV transportation in autonomic axon.

Autonomic neurons in the MPG exhibited different patterns of viral load and gene expression between HSV-1 and HSV-2. In animal infection study, both HSV-1 and HSV-2 infection created same pattern of acute disease, implying that primary replication of both HSV-1 and HSV-2, which is responsible for the acute severity of disease, happened equally at the site of inoculation. Both HSV-1 and HSV-2 viral DNA was detectable in sensory ganglia but in autonomic ganglia, only HSV-2 DNA was detectable at first day after inoculation. HSV-1 viral DNA was starting to be detected from 3 dpi. Thus viral transport mechanism might be varied in the different type of neurons. the mechanism of autonomic neurons, suppressing HSV-1 replication selectively was not determined in this study. If the mechanism underlying these phenomena was determined, this can be applied to the control of HSV infection. Thus further study to determine neuronal specific HSV suppressing factors.

Latency associated transcript (LAT) is the RNA molecule expressed during quiescent HSV infection in neurons. In this study, only latent HSV-2 DNA was present in the autonomic ganglia, MPG at 30 and 60 dpi. However latently infected HSV-2 did not

express LAT. In the previous study it was determined that spontaneous reactivation of LAT (-) HSV-2 was lower than rescued strain of HSV-2 (97). It was proposed that LAT was required for the efficient establishment of latency but latent viral load determined reactivation frequency (98). The HSV-2 viral load in the sensory ganglia was higher than HSV-1, so the greater frequency of HSV-2 recurrence might be derived from the latently infected HSV-2 in the sensory ganglia. However, it was determined that higher HSV-2 viral load in the sensory ganglia, TG was not correlated with the increased recurrences of HSV-1 after ocular infection (92). Thus the latent HSV-2 DNA in the autonomic ganglia might contribute the increased frequency of HSV-2 compared to HSV-1 even though no LAT expression was detectable. And the amount of LAT in the sensory ganglia, DRG couldn't explain the clinical observation of HSV-2 recurrences, since the amount of HSV-1 LAT expression was higher than HSV-2 throughout the study and especially only HSV-1 LAT expression was detectable at 60 dpi while no HSV-2 LAT expression was detectable but still HSV-2 reactivated to produce recurrent lesions. Primary cultured neurons provided more detailed information in the acute replication and reactivation of HSV-1 and HSV-2. In the sensory neurons, the pattern of viral load increase was similar between HSV-1 and HSV-2 however, there was a difference in the increase of viral load in the autonomic neurons. HSV-2 tends to persist in the autonomic neurons with the increased replication efficiency after 12hrs post-infection.

After induction of spontaneous reactivation in the sensory and autonomic neurons, HSV-2 in the sensory neurons remained stable, while HSV-2 in the autonomic neurons increased throughout the period of study with the increased TK expression. Interestingly, latently infected HSV-2 in the autonomic neurons did not express any genes with the

exception of ICP0. Expression of ICP0, 4 and VP16 in the sensory neurons was sufficient induce reactivation of HSV-1 (99). In the sensory neurons with latently infected with HSV-1, expression of these genes at the latent time point were present in this study, but HSV-2 expressed sustainable amount of ICP0 during establishment of latency. The role of ICP0 in the efficient replication and reactivation of HSV have been determined (100). The influence of inhibition of ICP0 expression in the autonomic neurons should be determined later for the understanding of HSV-2 reactivation in the autonomic neurons. VP16 is HSV encoded gene that controls activation of lytic genes with host cellular factor Oct-1 and HCF-1 (101). The expression of HSV-1 VP16 in the sensory ganglia was higher than HSV-2 however, HSV-2 VP16 expression in the autonomic neurons showed similar pattern of increase with HSV-2 viral load in the autonomic neurons, indicating that HSV-2 initiated lytic infection more efficiently in the autonomic neurons compared to the sensory neurons.

LAT expression have been identified as an essential element of HSV in the establishment of latency and efficient reactivation. The deletion of LAT from HSV significantly impact the HSV reactivation rate, not the viral load of HSV-1 and HSV-2 (43, 44). HSV-2 establish latent infection in both sensory and autonomic ganglia, shown by the latent viral DNA. However, LAT expression was not detectable in the autonomic ganglia. In the sensory neurons, both HSV-1 and HSV-2 expressed LAT and the pattern of LAT expression was differed according to the type of HSV. In the autonomic neurons, HSV-1 was still capable of expressing LAT while HSV-2 completely “shut down” LAT expression during latency. After induction of reactivation, both HSV expressed LAT. However, the level of HSV-1 LAT expression was minimal while HSV-2 expressed higher amount of LAT. This result suggested that LAT is required for the establishment of latency in the

neuron but the role of LAT in the maintenance of latency is different according to the type of neurons. The underlying mechanism how specific neuronal populations controlled LAT expression was not determined however, further study is required to elucidate the role of LAT in the autonomic neurons and the function in the reactivation and maintenance of latency.

Chapter 5. The influence of chemical sympathectomy in the reactivation of HSV after genital inoculation

Introduction

After genital inoculation of HSV, sensory and autonomic ganglia innervating genital area were identified as a site of HSV productive and latent infection as previously described elsewhere (93, 102, 103). However, there was fundamental HSV type specific differences in the viral load and gene expression in autonomic neurons compared to the sensory neurons, though the mechanism underlying was not elucidated. These differences were further confirmed with *in vitro* infection model. Thus, it was noted that gene expression and viral load changes in the autonomic neurons in the major pelvic ganglia (MPG) might be responsible for the increased reactivation frequencies compared to the HSV-1.

MPG was determined as a site of productive and latent infection of HSV after genital inoculation, shown by the presence of HSV DNA and gene expression analysis. Unlike the similarity of sensory neurons in HSV-1 and HSV-2 productive and latent infection, there was difference in the HSV latent DNA load and gene expression in the autonomic neurons. It was further determined that during the productive infection, the increase of HSV-2 DNA load was remarkable and gene expression exhibited differently accordingly to the type of HSV.

After inoculation of HSV into the ocular region, it was determined that parasympathetic neurons in the ciliary ganglia (CG) was responsible for the increased frequency of HSV-1 reactivation after ocular infection (92). In the genital infection study,

it was impossible to determine which autonomic neurons (sympathetic or parasympathetic) were responsible for the greater frequencies form of HSV-2 recurrences, due to the nature of MPG, which is composed of mixed populations of both sympathetic and parasympathetic neurons.

Thus, determining the target neurons in the autonomic ganglia, which is responsible for the HSV-2 recurrences, will provide more insight into the HSV research, especially how HSV establish latency and reactivate.

6 Hydroxydopamine (6-OHD) is synthetic catecholaminergic non-adrenergic toxin that can selectively destroy non-adrenergic neurons in the periphery or dopaminergic neurons in the brain by forming free radicals and inhibiting energy producing complex the mitochondria (104). 6-OHD can be selectively uptaken by the catecholamine containing neurons then accumulated in the neurons. Then the nerve terminal lost the ability of conducting action potential and synapse between neurons. The destructive effect of 6-OHD injection in newborn animals is irreversible and toxic, while it is reversible and nontoxic in adult animals. The adverse effect of 6-OHDA is tachycardia and increase in the blood pressure, when injected with high dose (105). Originally, 6-OHD has been used for the development of animal model for the Parkinson's disease, since this chemical can selectively destroy dopaminergic neurons in the brain (106).

The effect of 6-OHD in the immune system, in which lymphoid tissue, innervated by sympathetic fibers, is related with the anti-HSV immunity. The effect of 6-OHD was studied in conjunction with the immune cells generation and chemical sympathectomy influenced the generation of cytotoxic lymphocyte and HSV-specific memory cells (107). In the rabbit model, latently infected HSV-1 reactivation was investigated with 6-OHD.

Iontophoresis of 6-OHD to the eye, followed by the topical application of epinephrine showed enhanced induction of HSV-1 from latency (108). In the mice model, the use of 6-OHD resulted in the enhanced replication of HSV-1 in the SCG at the acute phase, but reduction of the subsequent prevalence of the establishment of latency (109), these result suggested that extra-neuronal stimuli could initiate reactivation of HSV. The use of 6-OHD after genital inoculation has not been determined so far in the limited knowledge of author's and excluding sympathetic neurons in the MPG on the HSV reactivation has not been performed yet. In the current study, reactivation of HSV-1 and HSV-2 after genital inoculation was determined after/before injection of 6-OHD, thus providing the evident showing that sympathetic neurons in the MPG might be responsible for the reactivation of HSV.

Results

Treatment of guinea pigs with 6-OHD

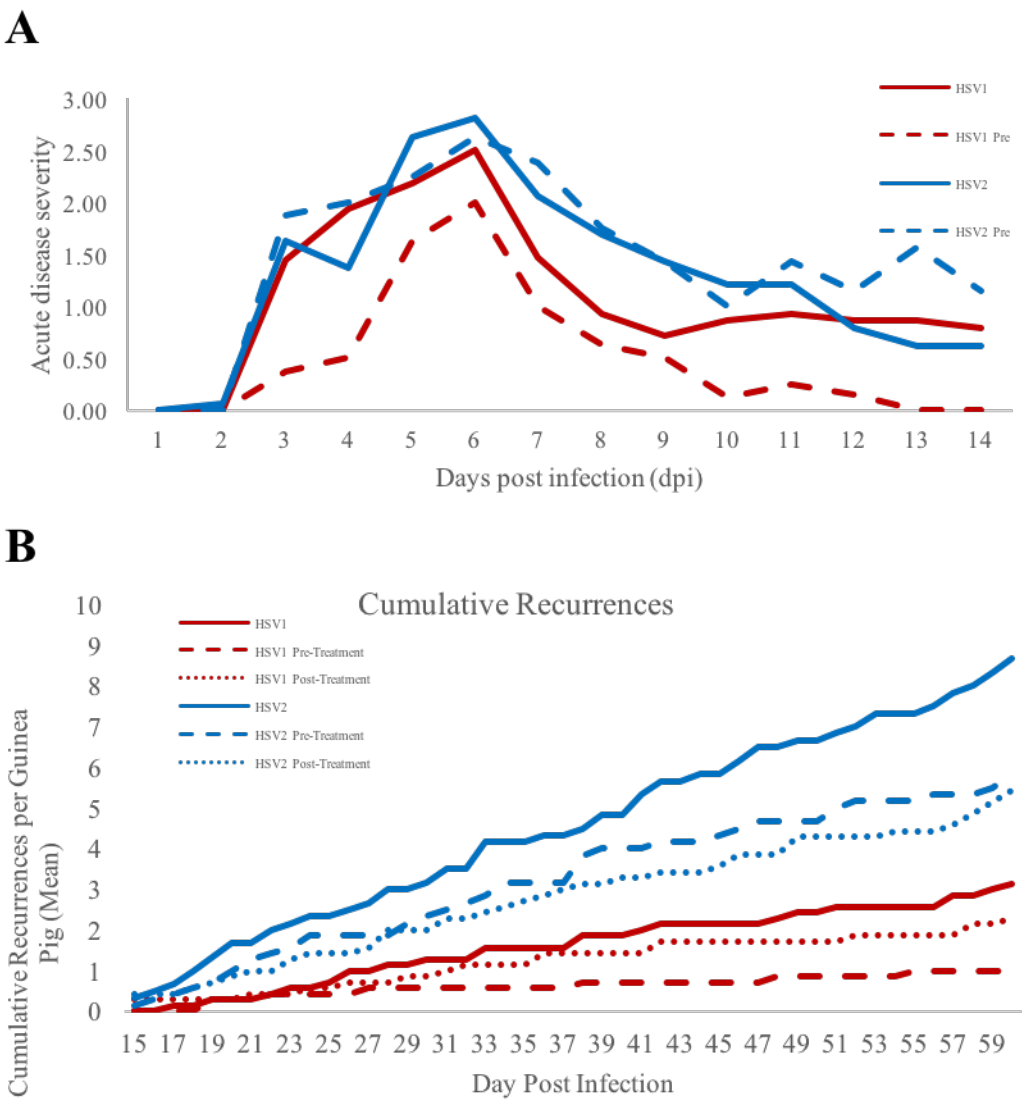
Previously it has been reported that injection of 6-OHD in mice with high dose produced some degree of sympathomimetic effect, such as tachycardia and hypertension (104). The dose of 50mg/kg intraperitoneal (IP) injection had no influence on the guinea pig behavior.

Influence of chemical sympathetectomy in the HSV reactivation frequencies

In acute infection phase, both pre- and post-infection HSV-2 group showed no remarkable difference in clinical signs (Fig 20. A). In HSV-1 infection group, pre-treatment group exhibited less severe form of acute disease. By preventing sympathetic neurons in MPG from HSV-1 infection, clinical outcome of HSV-1 infection differed, suggesting the importance of sympathetic neurons in HSV pathogenesis. In recurrent phase, both pre- and post-infection group exhibited less frequent reactivation compared to the control group. In HSV-2 infection group, both pre- and post-infection treatment of 6-OHD resulted in about 40% reduction of reactivation recurrences (Fig 20. B)

Thus it is reasonable to conclude that sympathetic neurons plays a role both in acute and latent phase of HSV infection however sympathetic neurons supported acute infection of HSV-1 not HSV-2, selectively.

Fig 20. Cumulative recurrences of HSV-1 and 2 after genital infection between 6-OHD treatment group and control group



Latent HSV viral load in sensory ganglia

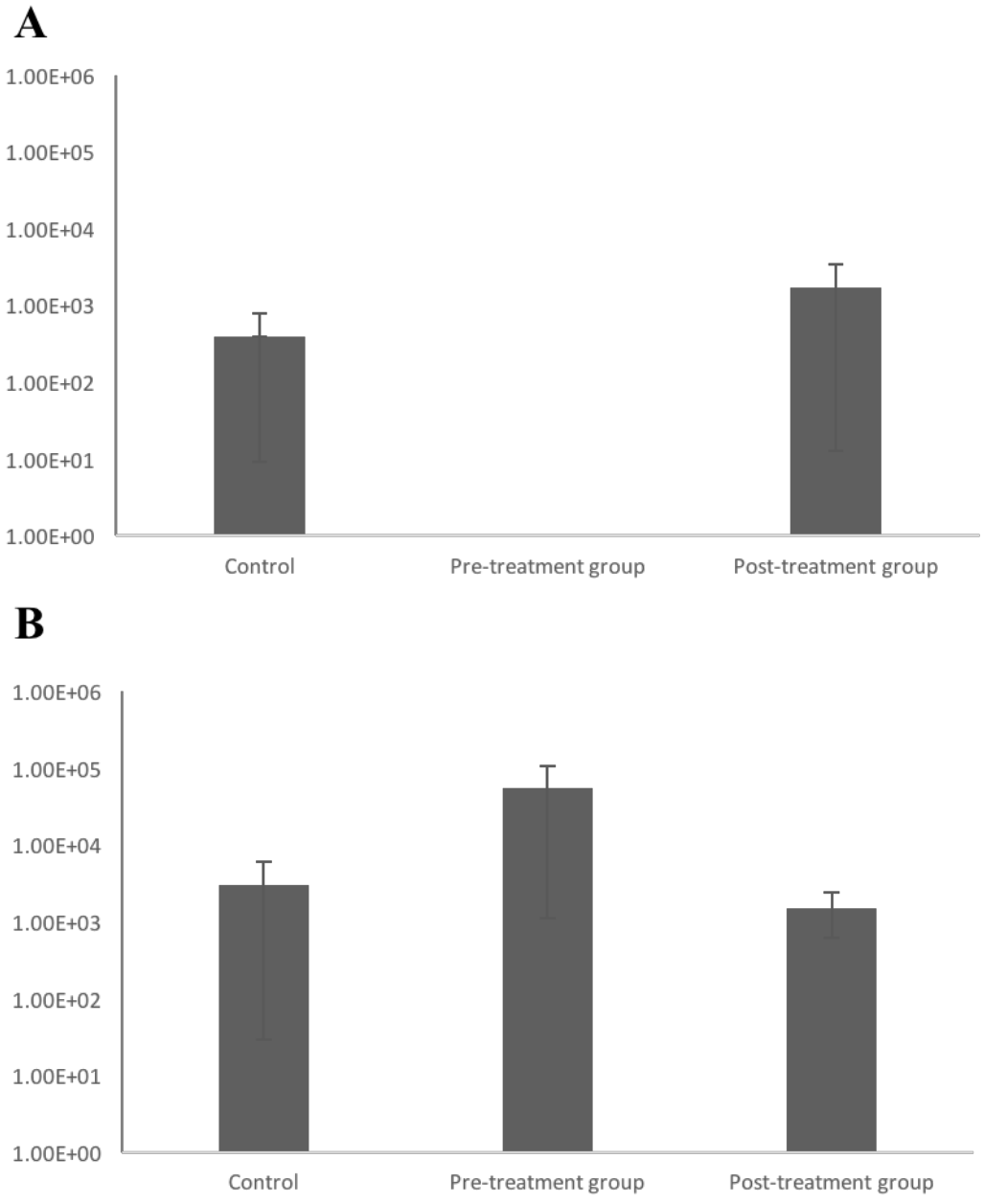
HSV latent viral load in the sensory ganglia was evaluated after genital inoculation in the group 1 (control group), Group 2 (sympathetectomy before HSV inoculation, pre-treatment group) and Group 3 (sympathetectomy after genital inoculation, post-treatment group). In the control group, HSV-1 latent DNA was detectable from two animals out of five animals. In the post-treatment group, HSV-1 was detected from three animals (out of five animals). However, in the pre-treatment group, there was no detectable HSV-1 DNA present. The mean HSV-1 load was the highest in the post-treatment group with the most animals have HSV-1 latent DNA (Fig 21).

When correlating the clinical reactivation profiles with the latent HSV-1 load in the sensory ganglia, there is a gap between frequencies and HSV-1 latent load in the sensory ganglia. The results of latent HSV-1 load in the sensory ganglia were not correlated with the decreased frequencies of HSV-1 reactivation. There were about 50% reduction of recurrences in HSV-1pre-treatment group and 30% reduction in post-treatment group.

HSV-2 latent load in the sensory ganglia was also determined. Unlike HSV-1, the highest HSV-2 latent load was detected at the pre-treatment group (three animals have latent HSV-2 DNA out of total five), although both treatment group exhibited higher load compared to the control group.

In the same with HSV-1 genital infection, the recurrences of HSV-2 were the highest in the control group. There was about 40% of reduction in the HSV-2 recurrences in the both treatment group, compared to the control group.

Fig 21. Latent HSV load in the sensory ganglia



A) HSV-1 latent viral load in the DRG. B) HSV-2 latent viral load in the DRG

Discussion

After genital inoculation of HSV-1 or HSV-2, neurons in the sensory and autonomic ganglia supported the productive and latent infection of HSV. However, there was a difference between sensory and autonomic neurons in the support of HSV infection. Autonomic neurons supported HSV-2 latent infection selectively while there was no difference between HSV-1 and HSV-2 in sensory neurons. Autonomic MPG is composed of both sympathetic and parasympathetic neurons. To further elucidate which type of autonomic neurons was responsible for the different reactivation frequencies, 6-hydroxydopamine (6-OHD) was inoculated into guinea pigs. The result of 6-OHD treatment showed that sympathetic neurons were responsible for about half of HSV-1 and HSV-2 recurrences. But in acute infection phase, there was no differences between treatment and control group in HSV-2 infection group. In HSV-1 infection group, there was reduction of acute disease severity of pretreatment group compared to the control group. This result indicated that sympathetic neurons contributed acute disease of HSV-1 not HSV-2.

Chapter 6. Discussion

Herpes simplex virus is wide spread pathogen that can infect human. The clinical signs of primary HSV infection is variable from asymptomatic to the formation of vesicular lesions around the site of invasion. In some instance, HSV infection can be fatal especially in the immunocompromised individual and transmission during delivery can result in fatal encephalitis that is usually ended up with poor prognosis.

The prevalence of HSV-1 is more than 70% with some degree of variations with the geographical location and HSV-2 is about 25% in the population. Most of human population have immunity against HSV, especially to HSV-1, but still the virus is actively circulating and causing problems in humans.

There are several factors involved in the high prevalence of HSV. Among the several factors, one of the viral factor is the nature of HSV, establishment of latency in the nervous system after invasion of host. HSV can latently infect host nervous system after peripheral invasion, thus, HSV is capable of evading the host immune surveillance and does not induce apoptosis of latently infected neurons then often it reactivates to produce recurrent lesions.

The establishment of latency and reactivation is the characteristic of HSV infection and several host and viral factors are involved. The mechanism of latency and reactivation has been extensively studied but still, there some questions remained unanswered.

One of the question is that though HSV-1 and HSV-2 are very similar viruses that can produce similar clinical sings in the mucous membrane such as formation of vesicular lesions and establish latency in the nervous system, the viruses behave differently in the

different anatomical location, especially in the process of reactivation. Generally HSV-1 reactivation is referred as “Above the waist” and HSV-2 is described as “Below the waist”. The factors determining this phenomenon, whether it is host or viral, is not elucidated yet.

Nervous system has been identified as a target of HSV latent infection and lots of researchers attempted to analyze and determine the mechanism of HSV pathogenesis with *in vivo* or *in vitro* system.

The most widely used model system for the HSV latency and reactivation research is sensory neurons. HSV infection in the sensory neurons revealed many aspect of mechanism required for the establishment of latency and reactivation and it was determined that there are certain types of neuronal population that can support productive or latent infection of HSV-1 or HSV-2 selectively. So the comparison between HSV-1 and HSV-2 should be required.

Many evidences have been found and indicated that autonomic neurons were also site of HSV infection however it was generally assumed that autonomic neurons equally contributed in the HSV pathogenesis as with sensory neurons and there are no differences in the pathogenesis of HSV-1 and HSV-2 in the neurons. Both sensory and autonomic neurons are differentiated from the common stem cell lineage but their physiological and anatomical function are distinctive. Therefore it is possible HSV infection can result in the completely different outcome compared to the sensory neuron infection. However, the systematic comparison between sensory and autonomic neurons after HSV infection has not been carried out yet. Therefore, the possibility of different outcome between the different neuronal populations, sensory vs autonomic, should be evaluated.

In this report, the differences in the pathogenesis of HSV after peripheral

inoculation between sensory and autonomic neurons were investigated and the result of this report will provide more insight into the HSV pathogenesis in the body.

Productive and latent HSV infection in the autonomic nervous system.

There are several clinical and experimental evidences indicating that autonomic nervous system can be infected with HSV and involved in the HSV pathogenesis.

Clinically, HSV infection in the autonomic nervous system resulted in the clinical signs such as urinary bladder distension, uncoordinated rectal sphincter muscle, hyperesthesia and sacral autonomic dysfunction after genital infection (89, 110, 111). In the ocular infection, autonomic nervous system infection after ocular infection resulted in the anterior uveitis or retinal inflammation (68, 112). And systemic autonomic dysfunction caused by HSV infection lead to the clinical signs such as gastrointestinal and amenorrhea (113). Most of autonomic dysfunction resulted from primary HSV infection and correlation between HSV reactivation in the autonomic nervous system with clinical disease are poorly defined, though recovery of infectious HSV from human ciliary ganglia indicated possibility of reactivation potency (65).

For HSV researches, especially establishment of latency and reactivation, rabbit, mice and guinea pig are the choice of laboratory animal species. Experimentally, HSV infection in the autonomic nervous system was confirmed in the mice, guinea pigs and rabbit (58, 87, 93, 114). In this report, autonomic neurons innervating eye and genitalia were also infected with HSV-1 and HSV-2, respectively.

The distinctive regulation mechanism of HSV-1 and HSV-2.

It has been a generally accepted idea that there are no differences between HSV subtypes so what is true for the HSV-1 is also true for HSV-2. However, growing evidences are indicating that there are different mechanisms regulating pathogenesis of HSV-1 and HSV-2. LAT has been known to function in the establish latency in the neurons but recently it was determined that LAT exon 1 determined neuronal tropism of HSV (47). Ocular infection of HSV-1 or HSV-2 exhibited different immune cells infiltration in the brains stem (115). Genital inoculation of HSV-1 or HSV-2 induced different immune responses on the mucosal membrane (116). After ocular infection, higher amount of HSV-1 shedding was present while HSV-2 shed more efficiently after genital inoculation in the mice (116). In this study, peripheral inoculation of HSV-1 or HSV-2 lead to the production of vesicular lesions around eye ball or genitalia as expected. HSV-2 inoculation in the eye ball presented more severe form of acute disease compared to the HSV-1. There were no significant differences in the acute severity of disease after HSV genital inoculation in the guinea pig used in this study, however genital infection of HSV-2 was more associated with nervous complications such as dissension of urinary bladder and hind limb paresis. The specific mechanism how HSV-2 produced more severe form of disease or nervous signs involvement compared to the HSV-1 infection was not clear. In several epithelial cells, similar pattern of replication was present in both HSV-1 and HSV-2 (117-119). Therefore, HSV primary replication in the epithelium is not the factor influencing the different clinical outcome of HSV infection. It is possible that the more severe form of HSV-2 acute disease might be resulted from the greater quantity of virus after replication in the sensory neurons. HSV viral DNA appeared on the sensory neurons from the first day after peripheral inoculation and HSV-2 replication in the sensory neurons and higher

quantity of HSV-2 load and more efficient form of replication were present in the sensory neurons after both ocular and genital inoculation. In the autonomic neurons HSV DNA started to be detected from the first day after inoculation and more efficient form of replication were also present. How HSV-2 replicated more efficiently in the neurons was not determined in this study. It has been generally accepted that both HSV-1 and HSV-2 function and behave similarly in the neurons. However, as shown in this study, there were differences in the gene expression and replication of HSV-1 and HSV-2 in neurons.

HSV infection in the sensory neurons and autonomic neurons.

HSV infection in the sensory neurons was of interest for the HSV researches especially for the establishment of latency and reactivation. In the sensory neurons, both HSV-1 and HSV-2 viral DNA changes showed similar pattern, though HSV-2 viral load was higher than HSV-1, implying both HSV-1 and HSV-2 readily infected sensory neurons after peripheral inoculation then persisted throughout the period of study. As stated above, certain type of sensory neurons supported selectively productive or latent infection of HSV (66). But overall infection of sensory ganglia exhibited similar pattern of viral load increase.

In the ocular infection model, higher titer of HSV-2 TK expression in the sensory neurons indicated that more severe form of acute clinical disease produced by HSV-2, resulted from the active replication and sequential higher amount of HSV-2 virus in the sensory neurons. However, in the latent infection period (30 to 60 days post infection (dpi)), TK expression in the sensory neurons continued but it was not correlated with the lower recurrence rate of HSV-2. HSV-1 LAT expression in the sensory neurons showed

higher titer compared to the HSV-2, especially in the latent time points. Therefore the increased frequencies of HSV-1 reactivation in the ocular infection model could be from the sensory neurons innervating the site of entry. However, there was inconsistency in the lytic gene expression. IE gene expression, which is repressed during latent infection and indicator of lytic infection, was analyzed and the result showed that the titer of HSV-2 IE gene expression was higher than HSV-1, especially at the latent time points.

After genital infection, HSV DNA was readily detectable in the sensory neurons in the DRG too. As with the ocular infection model, HSV-2 replicated more efficiently in the sensory neurons. However, still the titer of HSV-1 LAT expression exceeded HSV-2 LAT expression, showing inconsistency with the clinical observation of greater HSV-2 reactivation frequencies. Both HSV-1 and HSV-2 IE gene expression (ICP0) continued to 60 dpi but still HSV-2 ICP0 expression in the sensory neurons was higher. Therefore, HSV viral DNA load and gene expression in the sensory could partially explain why HSV-2 could cause more severe form of acute disease but have limitation in the explaining the differences in the reactivation frequencies between HSV-1 and HSV-2.

HSV infection in the autonomic nervous system – Sympathetic, parasympathetic and mixed autonomic neurons

In the previous study, higher titer of HSV-2 DNA in the sacral region of spinal cord, compared to the HSV-1, was present after foot pad inoculation of HSV, which is corresponding genital infection model. The result indicated that parasympathetic nervous system selectively transported HSV-2 into spinal cord (67). The selective support of

autonomic neurons in the HSV pathogenesis was further tested in our guinea pig model. Autonomic neurons in the sympathetic (SCG), parasympathetic (CG) and mixed autonomic ganglia (MPG) was infected after ocular or genital infection.

In the ocular infection model, it was determined that parasympathetic neurons in the ciliary ganglia (CG) selectively supported HSV-1 latent infection while sympathetic neurons showed less distinctive pattern of latent infection between HSV-1 and HSV-2.

Genital infection of female guinea pigs also confirmed autonomic neuronal infection of HSV after peripheral inoculation. Though HSV was readily detectable in the sensory neurons at all time points examined, Detection of HSV-1 infection in the autonomic neurons was limited only at acute infection phase (from 3 dpi to 14 dpi). There was no detectable HSV-1 DNA at latent time points, but HSV-2 DNA was persistently detectable in the autonomic neurons. The detectable amount of TK gene expression was present in autonomic neurons only fort HSV-2 and it was limited to to the first day pi. The whole mark of establishment of latency, LAT expression was analyzed and only HSV-1 LAT expression was detectable and it was limited at the acute phase, thus the following question arose. Is LAT essential for the establishment of latency in the autonomic neurons? And the non-LAT expressing form of HSV in autonomic neurons can reactivate from latency?

Influence of chemical sympathetectomy in the HSV pathogenesis

The mixed autonomic neuronal population in the major pelvic ganglia supported

HSV-2 productive and latent infection selectively. It was impossible to determine that which part of autonomic nervous system, whether sympathetic or parasympathetic neurons in the MPG, is responsible for the selective support of more frequent HSV-2 recurrences. The phenomenon of selective support of autonomic neurons in the MPG according to the type of HSV was determined with the use of chemical sympathetectomy. The use of chemical sympathetectomy provided the results of exclusion of sympathetic neurons from acute infection (shown by acute lesion severity) and latent infection (shown by reactivation frequencies).

There were differences in the pathogenesis of HSV in the acute phase infection. When limiting HSV-1 infection in the sympathetic neurons in the MPG, so parasympathetic neurons were solely infected, there was onset of less severe clinical disease compared to the control group, implying that sympathetic neurons supported acute infection of HSV-1. In HSV-2, there was no influence of chemical sympathetectomy, suggesting that the role of sympathetic neurons in the HSV-2 acute pathogenesis is questionable. Both genital infection of HSV-1 and HSV-2 produced onset of neurological signs. There was less severe form of neurological involvements in HSV-1 infection in the group with limited HSV entry into sympathetic neurons however, prevention of HSV-2 entry prolonged the duration of neurological signs. Thus it is clear that sympathetic neurons in the MPG supported HSV-1 productive infection but the role of sympathetic neurons in the HSV-2 productive infection is questionable.

Preventing efflux of reactivated HSV from sympathetic neurons in the MPG resulted in the decreased reactivation frequency in both HSV-1 and HSV-2. Therefore it is reasonable to conclude that sympathetic neurons contributed in the reactivation of HSV

after genital infection. However, there are still inconsistencies between HSV-1 and HSV-2. In the reactivation frequencies of HSV-1, both inhibition of entry and efflux reduced the frequencies of HSV-1. But limitation of HSV-1 entry had more impact in the reactivation frequencies than inhibition of efflux. It was reversal in case of HSV-2 reactivation. Unlike HSV-1, inhibition of efflux of HSV-2 from sympathetic neurons showed less frequencies of reactivation compared to the inhibition of entry group, though both groups had a reduction in the reactivation frequencies.

The different pathogenesis of HSV in the sympathetic, parasympathetic nervous system.

Sympathetic and parasympathetic neurons can be distinguished by the type of neurotransmitter by which neurons utilized for the transmission of neuronal signals and their physiological function. Adapting against the physical or emotional stress to maintain homeostasis is one of the most important function of autonomic nervous system. Activation of sympathetic nervous system generally lead to the release of epinephrine as a neurotransmitter and epinephrine is the one of the triggering factor for the HSV reactivation (120). Activation of parasympathetic nervous system produces acetylcholine at the nerve terminal. HSV-1 and acetylcholine receptor shared certain antigenic epitope, so the molecular mimicry enabled HSV-1 to utilize acetylcholine receptor (121). And there is indirect evidence that injection of acetylcholine induced reactivation of another alpha herpes virus, pseudorabiesvirus in the natural host, pig and mice (122, 123). But still the correlation between activation of parasympathetic neurons by acetylcholine and HSV reactivation was not determined.

It was determined that autonomic ganglia after ocular infection (87), and genital infection (93). The previous studies examined the possibility of autonomic neurons infection with HSV and probed the pathway of HSV spread. The limitations of previous studies were that differences between HSV-1 and HSV-2 were not examined due to the assumption that both HSV infection result in the similar outcome and the contribution of latently infected HSV in the recurrences was not evaluated.

The selective support of autonomic nervous system in the pathogenesis of HSV-1 or HSV-2 contributed different pattern of reactivation in the anatomical site in which sympathetic or parasympathetic nervous system innervated. Detection of HSV viral DNA from the first day after infection and persistence in the autonomic nervous system indicated both HSV-1 and HSV-2 can enter autonomic nervous system without any difference, so there is no difference in the sensory and autonomic nervous system receptor for the retrograde transportation of HSV to the neuronal cell body at the peripheral nerve terminal. The unknown neuronal type specific factor influenced HSV productive and latent infection.

Most interesting finding in this report was that autonomic neurons in the MPG supported latent infection of HSV-2, but LAT expression was dispensable for the establishment of latency.

LAT expression in the neurons has been a whole mark of HSV establishment of latency. It is not fully understood how LAT exert its function in the establishment of latency and reactivation process since there is no protein encoded in the LAT (124). microRNAs encoded in the LAT inhibited translation of protein level. There was limited evidence showing that expression of LAT ORF influenced the growth characteristic of HSV-1 in the protein level, not RNA (125), but still it is generally accepted that LAT functions in the

RNA level to facilitate establishment of latency and reactivation. No LAT expression was present in the both *in vivo* and *in vitro* model during latency. But expression of LAT resumed when HSV spontaneously reactivated in cultured autonomic neurons, implying that LAT might not be required for the establishment of latency in the autonomic neurons however LAT is not dispensable for the reactivation process. Deletion of LAT did not influenced establishment of latency in the neurons but still required for the efficient reactivation (126, 127) and reactivation frequencies were determined by the number of latently infected ganglia, rather than LAT expression (50). The percentage of latently infected neurons in the autonomic neurons in the autonomic ganglia was not determined in this report but since same titer of viruses were used, it is possible that number of neurons infected with HSV are the same between sensory and autonomic neurons. The specific elements in the autonomic neurons regulating establishment of HSV latency should be elucidated further. And determination of neuronal factor responsible for the phenomenon will provide insight for the better understanding of the mechanism of HSV establishment latency in the peripheral neurons and ultimately, for the control of HSV infection and reactivation.

Significance of findings

The burden of HSV infection in human is high since there is no available vaccine which completely prevent infection or reactivation. Host immunity against HSV could reduce the clinical onset caused by HSV reactivation but HSV could be shed from the latently infected population without visible lesion developed. The prevalence of HSV-1 and HSV-2 differed.

Direct contact of mucous membrane is the method of HSV transmission. The higher prevalence of HSV-1 (more than 70% even in civilized countries) and increase in the prevalence with the increase of age in the population are contributed from the nature of HSV-1. HSV-1 reactivation from the oropharyngeal regions permitted more readily transmissible form of HSV-1. HSV-2 is also prevalent in the human population but generally lower than HSV-1. Since the increase of HSV-2 prevalence is related with the period of puberty, it is generally accepted that HSV-2 transmitted via sexual contact.

Autonomic neurons supported acute and latent infection of HSV however autonomic neurons differ from sensory neurons in their ability of selectively supporting HSV reactivation.

It is still unknown that which determines these selective support of HSV reactivation. However, it is demonstrated that autonomic neurons could contribute the different reactivation of HSV in the specific anatomical location therefore selective support of autonomic neurons determines the efficiency of transmission, and ultimately contributed in the prevalence of HSV-1 and HSV-2 in the human population.

Chapter 6. Supplementary data

Effect of Black Tea Extract (BTE) and Curcumin on the Growth of Herpes Simplex Virus (HSV) Type 1 and 2 in Primary Neuronal Culture

Introduction

Herpes simplex virus (HSV) is ubiquitous pathogen that can infect human. HSV infection is followed by establishment of latency in the nervous tissue and latent HSV often reactivate to cause recurrent disease (128). Currently, there are antivirals are available but mutant virus that can tolerate current medication is arising (129) and the antivirals are only effective to reduce the clinical severity of disease but has no effect of eliminating latent HSV. Thus more effective therapeutic strategy is required for the control of HSV infection. Several natural compounds and metabolites from plants have been identified to possess inhibitory effects against pathogens including viruses. Among them, polyphenol group, such as *Theaflavin* extracted from black tea extract (BTE) has been known to possess anti-bacterial and anti-viral activity. An in vitro study using bovine rota virus showed that *Theaflavin* from black tea could inhibit infectivity of bovine rota virus, human immunodeficiency virus (HIV-1), influenza A virus infection and herpes simplex virus type 1 (130-132). Curcumin is a phenolic compound from curry spice turmeric and known to have antiviral activity against herpes simplex virus. It was initially proposed that curcumin could inhibit the histone acetyltransferase coactivator proteins which are essential molecules for HSV infection but curcumin could reduce HSV-1 infectivity independently of the histone acetyltransferase coactivator proteins (133). Curcumin also successfully protected mice from genital inoculation of HSV-2 (134). Curcumin has inhibitory effect against IMPDH *de novo* synthesis, which is suggested as the target of antiviral and anticancer compound (135).

BTE and curcumin have been tested against several pathogens and proved to be effective but the effect of the natural compounds was tested *in vitro* and mechanism by which natural compounds exert the function was not determined. In the previous study conducted by Cantatore et al., found that black tea extract have anti-HSV activity when tested in human epithelial cell lines (136).

For HSV, the site of latency for HSV is nervous tissue but anti-HSV effect of BTE and curcumin has not been tested on the neurons yet. In the current study, anti-HSV effect of BTE and curcumin was tested with primary sensory neuronal culture.

Material and methods

Preparation of Black tea extract (BTE) and curcumin.

BTE and curcumin were purchased from Sigma-Aldrich. BTE was dissolved in DMEM with 10% FBS and 1% pen-strep to produce 14mM stock. Stock of BTE was 10 fold serially diluted and kept in -20 degree until use. Curcumin was dissolved in 100% dimethylsulfoxide (DMSO) to produce 60uM stock solution.

Primary neuronal culture.

Six week old Swiss-Webster mice (Harlan) were humanely euthanized using CO_2 chamber and trigeminal ganglia (TG) were collected into Neurobasal A medium, supplemented with 2% of B27 Supplement and 1% of penicillin-streptomycin (all from Life Technologies). Ganglia were dissociated with papain, dispase and collagenase, followed by mechanical trituration by pipette. The neurons of the TG were enriched by Optiprep gradient (Sigma Aldrich). Neurons were plated onto 24-well plates coated with Matrigel (BD Biosciences).

Virus inactivation assay.

To determine the direct effect of BTE or curcumin against HSV-1 and HSV-2, one moi (multiplicity of infection) of HSV-1 or HSV-2 were mixed with various concentration of BTE or curcumin then incubated for one hour at 37 °C. The mixture was inoculated into primary neuronal culture for one hour after adsorption period media was replaced and neurons were incubated for two days. After 48 hours neurons and media was collected and analyzed with standard plaque assay and quantitative PCR assay specific for HSV.

Virus adsorption inhibition assay.

To determine if BTE or curcumin are capable of inhibiting HSV entry into target cells, primary neuronal culture was treated with various concentration of BTE or curcumin for 15 minutes. After treating neurons with BTE or curcumin, media containing one moi of HSV-1 or HSV-2 was inoculated into neurons then incubated for one hour. After one hour of incubation, media was replaced. 48 hours later, neurons and media was collected and analyzed with standard plaque assay and quantitative PCR specific for HSV to evaluate the effect of BTE or curcumin on the inhibition of HSV entry into target cells.

Quantitative PCR .

HSV load in the samples was determined by quantifying HSV thymidine kinase (TK) gene. DNA was extracted from neuronal culture using DNazol (Invitrogen) according to the instruction of manufacturer. Primer and probe sets specific for HSV-1 and HSV-2 TK gene were used to determine viral load in culture neurons described previously (92). qPCR was carried out using universal polymerase mixture (Biorad) on Viia7 thermocycler (Applied Biosystem) All values were normalized to 200 nanogram (ng) of DNA.

Plaque assay.

Collected neurons and media were 10 fold diluted serially and inoculated into 24 well plate of Verocell. After 1 hour of incubation, media was replaced with DMEM containing human IgG to limit horizontal spread of HSV. 48 hours later, 24 well plate was stained with crystal violet then plaques were counted.

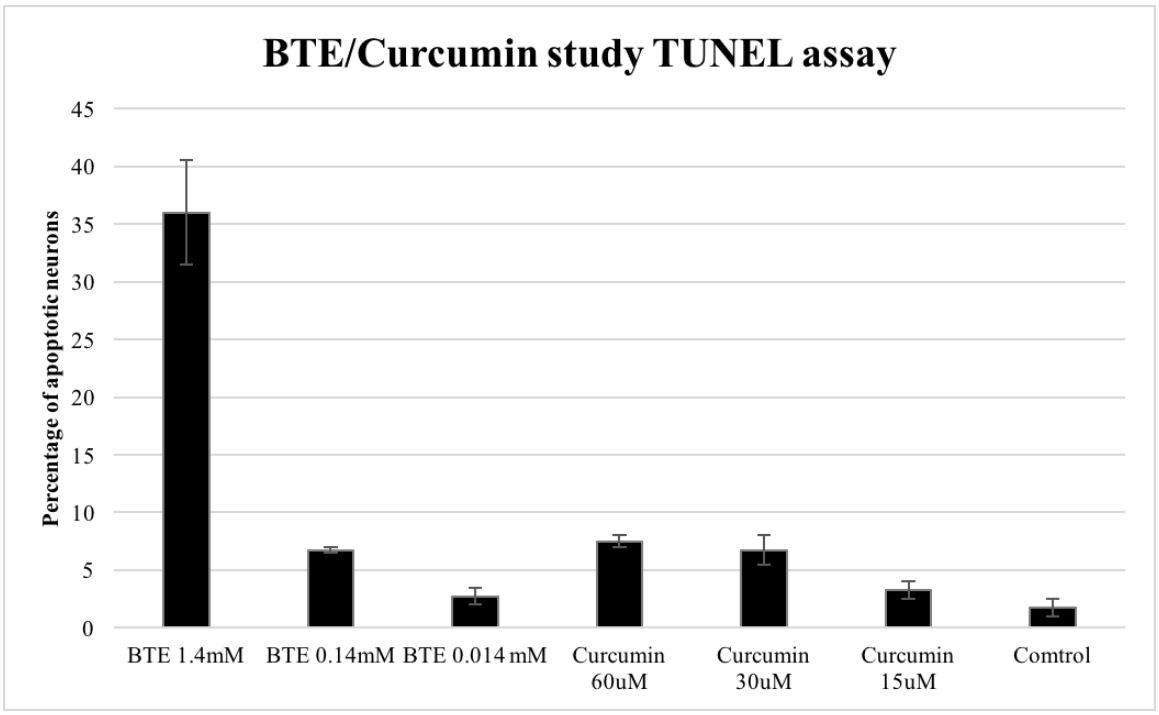
TUNEL assay.

To evaluate the influence of BTE or curcumin on the neuronal apoptosis, Neurons were treated with BTE or curcumin with various concentrations for 1 hour then incubated for two days. After incubation period, neurons were fixed with 2% paraformaldehyde and stained with TUNEL assay kit (Promega).

Results

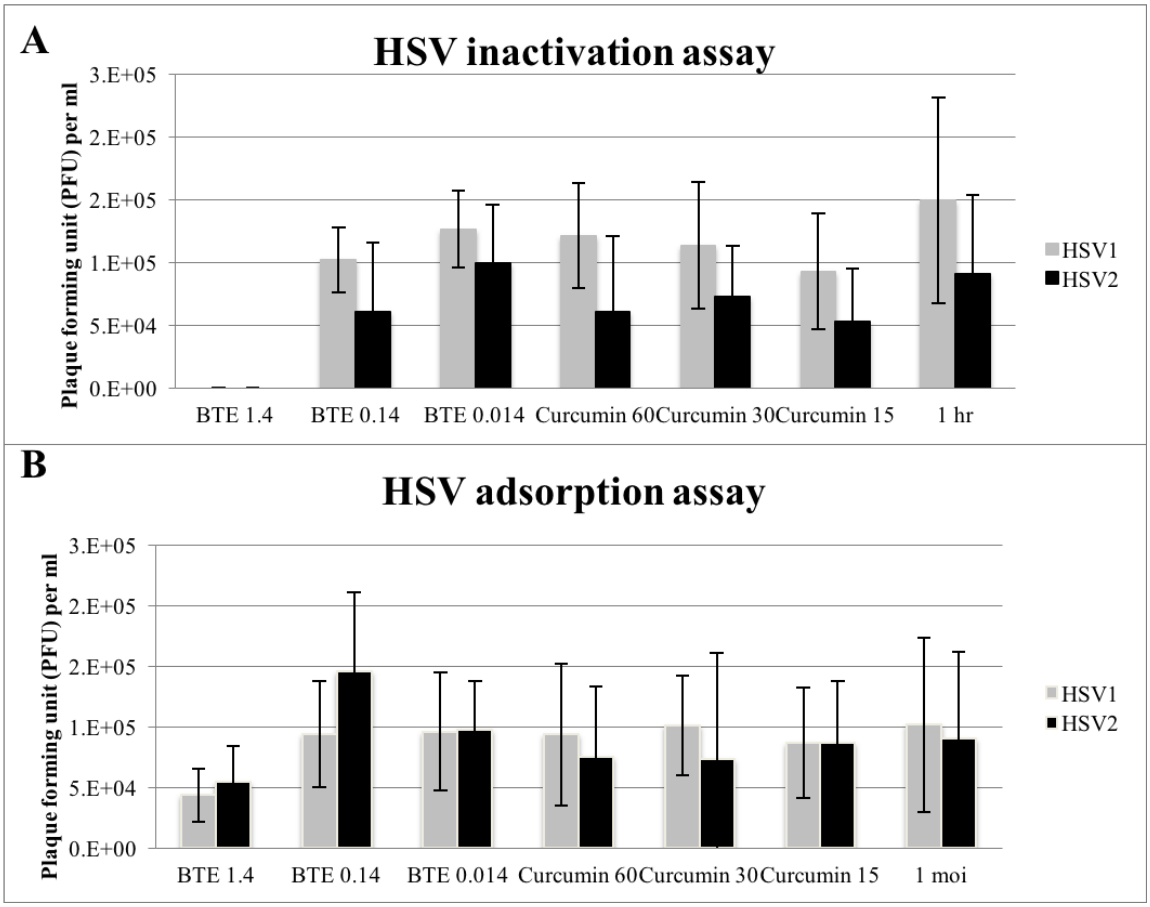
Neurons incubated with BTE or curcumin were monitored daily to evaluate cytotoxicic effect of BTE or curcumin. With use of the highest concentration (1.4 mM) of BTE, major cytopathic effect (CPE) was observed (degradation of axon, swelling of neuronal body etc). TUNEL assay was used to determine the degree of apoptosis induced by BTE or curcumin. The use of BTE at highest concentration induced considerable degree of apoptosis in neurons. But below that concentration no CPE was present. Use of curcumin on the primary neuronal culture showed moderate CPE at highest concentration of curcumin (60uM). But it was not prominent in TUNEL assay and as in case of BTE, no CPE was observed below the highest concentration of curcumin (Fig. 22).

Fig 22. The result of TUNEL assay


BTE/Curcumin study TUNEL assay
Percentage of apoptotic neurons
45
40
35
30
25
20
15
10
5
0
BTE 1.4mM
BTE 0.14mM
BTE 0.014 mM
Curcumin 60uM
Curcumin 30uM
Curcumin 15uM
Comtrol

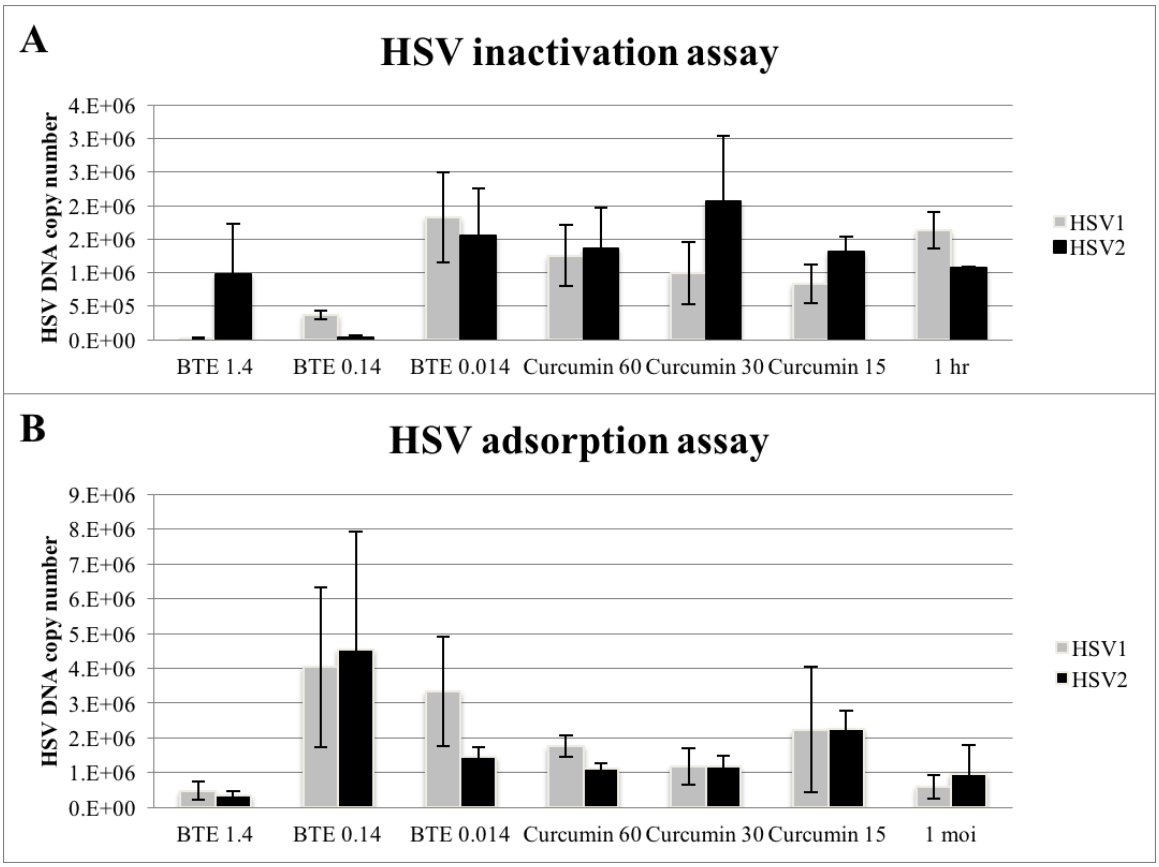
As shown with classical plaque assay, BTE exhibited potent anti-HSV activity for both HSV-1 and HSV-2 at the highest concentration in virus inactivation assay (Fig 23. A). But below 1.4 mM concentration of BTE, no reduction of HSV infectivity was present regardless of the type of HSV. Curcumin failed to inactivate infectivity of HSV at any concentration tested, suggesting that BTE at highest concentration was effective for reducing HSV load. But virus adsorption inhibition assay showed no remarkable differences, implied treating neurons prior to the HSV inoculation with those natural compounds failed to interfere virus attachment into neurons (Fig 23. B).

Fig 23. The result of HSV inactivation and adsorption inhibition assay


A
HSV inactivation assay
Plaque forming unit (PFU) per ml
3.E+05
2.E+05
2.E+05
1.E+05
5.E+04
0.E+00
BTE 1.4
BTE 0.14
BTE 0.014
Curcumin 60
Curcumin 30
Curcumin 15
1 hr
HSV1
HSV2
B
HSV adsorption assay
Plaque forming unit (PFU) per ml
3.E+05
2.E+05
2.E+05
1.E+05
5.E+04
0.E+00
BTE 1.4
BTE 0.14
BTE 0.014
Curcumin 60
Curcumin 30
Curcumin 15
1 moi
HSV1
HSV2

Viral load in the neurons was tested to determine if BTE and curcumin could inhibit HSV replication (Fig. 24). As shown by qPCR assays, BTE at highest concentration could reduce HSV-1 viral load in neurons but failed to reduce HSV-2 viral DNA. However since BTE at 1.4 mM concentration induced considerable neuronal apoptosis, the reduction of HSV-1 viral load might be derived from neuronal death.

Fig 24. HSV load in the inactivation and adsorption inhibition assay.



Discussion

In the previous study, BTE and curcumin exhibited potent anti-HSV activity on Vero cell or human epithelial cells (136). Although the exact mechanism of how these natural compounds exert anti-HSV activity was not clear, BTE and curcumin were not cytotoxic to epithelial origin cells. But in the current study, BTE and curcumin exhibited cytopathic effect on neurons, suggesting cytotoxicity of BTE and curcumin was cell type specific. And on epithelial cells, BTE could interfere virus adsorption effectively but not in this study. Nectin and other co-molecules have been known as receptors for HSV entry (27). The differences in the receptor distribution between epithelia cells and neurons were poorly defined so far. Since little known about how BTE and curcumin can interfere HSV attachment on epithelial cells, the difference between epithelial cells and neurons should be further determined.

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