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The role of glycoprotein K (gK) in the ocular and neuropathogenesis of herpes simplex virus-type 1 (HSV-1)

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THE ROLE OF GLYCOPROTEIN K (gK) IN THE OCULAR AND
NEUROPATHOGENESIS OF HERPES SIMPLEX VIRUS-TYPE 1 (HSV-1)

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

In

The Interdepartmental Program in
Veterinary Medical Sciences through the
Department of Pathobiological Sciences

By
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B.S. University of Missouri, 1998
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May 2012

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ABSTRACT

Herpes simplex virus type 1 (HSV-1) is a DNA virus that is a ubiquitous pathogen of humans. The hallmark of the HSV-lifecycle is infection of a mucosal surface with spread to sensory neurons where the virus establishes a latent infection with periodic recurrences for the life of the host. The most common course of symptomatic disease with HSV-1 is the typical mucocutaneous lesion that is self-limiting. HSV-1 can also cause acute encephalitis and ocular pathology on reactivation. Both of these manifestations of the disease have severe consequences. Although ocular infection is less frequent, the extensive prevalence of HSV makes it one of the most common infectious causes of blindness in developed countries. Herpetic encephalitis causes significant morbidity and mortality, particularly in the immunosuppressed or untreated individual. HSV-1 specifies at least 11 glycoproteins that are expressed in the infected cells. The viral glycoproteins are the first interface of the virus with a host cell and are therefore vitally important to virus pathogenesis. Glycoproteins B, C, D, H, and L have been extensively studied and found to be involved in initial attachment, fusion, and entry of the virus into a host cell. Recent work in this laboratory has shown that glycoprotein K (gK) is also involved in the fusion machinery of the virus, however, little was known about the role of gK in the pathogenesis of the virus. To assess the role of gK in pathogenesis, mutant viruses were used in an experimental infection in a mouse eye model. In this investigation, gK was found to have a critical role in the pathogenesis of the virus in the eye as well as in spread of the infection to neurons and subsequent development of latency. Following this work, a primary neuronal culture system was established to further define the role that gK played in neuronal infection and transport. Using this system, gK was found to be a critical

determinant for neuronal transport and pathology. Since gK deficient viruses appear unable to infect neurons, defining this phenotype may assist in the eventual development of a successful vaccine strain of the virus.

CHAPTER I

INTRODUCTION

STATEMENT OF PROBLEM AND HYPOTHESIS

Herpes Simplex Viruses (HSV) are ubiquitous and represent the etiologic agents responsible for a multitude of human ailments including mucocutaneous oral and genital diseases, ocular disease, and viral encephalitis. The hallmark of the HSV-lifecycle is infection of a mucosal surface with spread to sensory neurons where the virus establishes a latent infection with periodic recurrences for the life of the host (Whitley, Kimberlin et al. 1998a). The most common course of symptomatic disease with HSV-1 is the typical oral mucocutaneous lesion that is self-limiting. Disease with HSV-1 is often considered a quality of life issue rather than a potentially severe infection. However, HSV-1 can also cause acute encephalitis and ocular pathology on reactivation and the impact of HSV can be significant on healthy individuals as well as immunosuppressed people (Whitley 2001b). Although ocular infection is less frequent than mucocutaneous infection, the marked prevalence of HSV in the population makes it one of the most common infectious causes of blindness in developed countries (Liesegang, Melton et al. 1989a; Liesegang 2001). During the life cycle of HSV in its host, there are a number of distinct events including initial attachment of the virus to a host cell membrane, fusion and entry of the virus, viral protein and DNA production, assembly of capsids and DNA packaging of the progeny virions, and acquisition of the final envelope and egress of the virus from the

cell. Many of these individual facets of the virus life cycle are areas of intense investigation. In many cases the constituents of this process are known. Herpes simplex virus has a relatively large genome, for a virus, with approximately 152,000 bases (Kieff, Bachenheimer et al. 1971). This allows HSV-1 to encode approximately 90 known gene products and at least 84 transcriptional units participating in the production of proteins (Roizman, Knipe et al. 2007).

In particular, HSV-1 specifies at least 11 glycoproteins that are expressed in the infected cells (Roizman and Knipe 2001a). The viral glycoproteins are the first interface of the virus with a host cell and are therefore vitally important to virus pathogenesis. Glycoproteins B, C, D, H, and L have been extensively studied and found to be involved in initial attachment, fusion, and entry of the virus into a host cell (Furth, Whitbeck et al. 1997; Whitbeck, Peng et al. 1997; Laquerre, Argnani et al. 1998; Warner, Geraghty et al. 1998; Krummenacher, Rux et al. 1999; Whitbeck, Muggeridge et al. 1999). These proteins can control host cell range and even species range of infection.

The dogma often encountered with viruses with large genomes and many encoded proteins is one of, one protein equals one function or one area of function, such as virion attachment, entry, assembly, or egress. However, smaller viruses with smaller genomes often have multifunctional proteins. In HSV, glycoprotein K (gK) appears to be multifunctional. Glycoprotein K has been shown to have roles in virion egress, envelopment, and viral fusion and syncytia formation (Ruyechan, Morse et al. 1979; Bond and Person 1984; Pogue-Geile, Lee et al. 1984; Debroy, Pederson et al. 1985; Jayachandra, Baghian et al. 1997; Laquerre, Argnani et al. 1998; Foster, Melancon et al. 2004a; Melancon, Luna et al. 2005; Chouljenko, Iyer et al. 2009; Chouljenko, Iyer et al.

2010; Chouljenko, Kim et al. 2012). Recent work in this laboratory (cited above) has shown that glycoprotein K (gK) is also involved in the fusion machinery of the virus, however, little was known about the role of gK in the pathogenesis of the virus. The overall hypothesis of the investigations described in this thesis, has been that gK plays a significant role in viral pathogenesis. This hypothesis predicts that due to its many and varied effects in the virus, a virus lacking gK will have a significant negative impact on the pathogenesis of the virus in neurons and by extension ocular disease.

STATEMENT OF RESEARCH OBJECTIVES

The goal of this research was to investigate the role of Herpes Simplex Virus Type 1 glycoprotein K (gK) in relation to viral pathogenesis in the eye, nervous system and individual neurons. The specific aims of this research were:

1. To assess the virulence and replication characteristics of gK-null viruses in cell culture and the mouse eye model.
2. To develop a reproducible primary cell culture system amenable to examination via microscopy and molecular techniques such as PCR.
3. To use the system developed in aim 2 to examine specific role of gK in neuronal infection and anterograde and retrograde transport in neurons without the influence of a host organism to potentially confound results.

Overall, the results obtained from this research indicate that:

1. Glycoprotein K is critical to spread in the eye and for spread, neuroinvasiveness, and the establishment of latency in an animal.
2. Glycoprotein K is a critical determinant in the infection of neurons at the level of the axonal terminus.
3. Glycoprotein K is necessary for spread of the virus from a neuron to epithelial cells in an anterograde manner.

The work is presented in individual chapters in a manuscript format having a specific title for the central theme of each chapter.

Chapter 2: The Herpes Simplex Virus Type-1(HSV-1) Glycoprotein K(gK) is Essential for Viral Corneal Spread and Neuroinvasiveness.

Chapter 3: A Mutant Herpes Simplex Virus Type-1 (HSV-1) McKrae Lacking the Glycoprotein K (gK) Gene is Unable to be Transported in Axons in either the Anterograde or Retrograde Manner.

LITERATURE REVIEW

History of Herpesviruses

Herpes was possibly first described in descriptions of lesions as early as the third millennium BCE in a Sumerian tablet and the Ebers papyrus, 1500 BCE (Whitley 2001a). Hippocrates used the Greek for “herpes”, or “herpein” to describe a lesion that creeps or

crawls along the skin (Wildy 1973) and Pliny (the Elder) mentioned a syndrome that could likely be shingles and called this syndrome “zoster” (Beswick 1962). These lesions could have been the result of herpesvirus, other cutaneous infections or even cutaneous neoplasms; however, herpes has been part of the human experience since prior to recorded history. The three primary subfamilies of mammalian herpesviruses are projected to have arisen about 200 million years ago and the two types of simplex virus diverged as much as eight to ten million years ago, yet *Homo sapiens sapiens* are only approximately 200,000 years old (McGeoch, Dolan et al. 1985; Gentry, Rana et al. 1988).

Herodotus is credited with realizing the association of fever and cutaneous lesions in HSV (Whitley 2001a). While the Greek physician Galen recognized that recurrent HSV lesions develop at the same sites in the second century A.D. (Whitley 2001a). By the 18th century, study of the virus had progressed and the first description of genital disease associated with herpes virus was published in *De Morbis Veneris* by John Astruc, physician for King Louis XIV of France, after studying French prostitutes (*Puellae publicae*) (Astruc 1736). In 1814, Bateman accurately describes both herpes labialis and herpes preputialis as vesicular to ulcerative diseases often with heat, tingling, and pain associated with the vesicles and spontaneous resolution and reoccurrence of the disease (Bateman 1814). In the late 19th century, Vidal demonstrated that inocula from lesions could transmit vesicular lesions from patient-to-patient and from one anatomic area to another on the same patient, thereby proving the infectious nature of the virus (Vidal 1873; Longson 1978). Gruter, using rabbit studies, rather than human, showed that HSV could be transmitted from one rabbit to another in likely the first use of an animal model in the study of herpes (Gruter 1924). He is also given credit for first isolation of the virus

by most of the virology community. In 1930, Andrewes and Carmichael showed that recurrent disease could occur in people with significant levels of neutralizing antibodies, which was contrary to popular beliefs in virology at the time (Andrewes 1928; Andrewes 1929; Andrewes and A. 1930).

By 1939, Burnet and Williams had resolved much of the current view on herpetic latency; that the virus remains latent but various stimuli may awaken it from the latent state to cause recurrence and lesions (Burnet 1939). As tissue culture techniques emerged and evolved, isolation of other families of human herpesviruses became possible. During the 1950's, Varicella Zoster Virus (VZV, chickenpox) and Cytomegalovirus (CMV) were isolated (Weller and Stoddard 1952; Rowe, Hartley et al. 1956; Craig, Macauley et al. 1957). By the end of the 1990's T-cell lymphocyte culture allowed the isolation of Human Herpesviruses 6A, 6B and 7 and Representational Differential Analysis allowed the isolation of Human Herpesvirus 8 (Salahuddin, Ablashi et al. 1986; Lopez, Pellett et al. 1988; Frenkel, Schirmer et al. 1990; Chang, Cesarman et al. 1994). In 1962, Schneweiss demonstrated two distinct serotypes of Human Herpesvirus (Schneweiss 1962). In 1971, the first International Committee on Taxonomy of Viruses (ICTV) established the genus of *Herpesvirus* and by 1979 the committee had elevated the herpesviruses to a family (*Herpesviridae*) and established the two serotypes of herpes simplex virus (HSV) as Human Herpesvirus 1 and 2 (Davison 2010).

Taxonomy of Herpesviridae

Initially, identification of viruses, including Herpesviruses, was based on epidemiologic factors, such as host range, and biologic properties, such as clinical course

of disease and character of lesions. Developing technologies have shaped the classification of viruses. With electron microscopy, Herpesviruses could be classified on the basis of morphology. Biologic and chemical tests also allowed for characterization of a virus based on antigens and biochemical properties. Therefore, all herpesviruses have an icosahedral capsid of 162 capsomeres that surrounds a core filled with DNA and the capsid is surrounded by a protein-rich tegument and a lipid envelope (Davison 2010). Furthermore, biologic properties, like the establishment of latent infection in the natural host, were included in the initial classification of herpesviruses into the *Herpesviridae* family by the ICTV in 1979 (Matthews 1979; Davison 2010). Features of natural host species, cell types infected, reproductive cycle, and antigens were used to further subdivide the herpesviruses into the subfamilies of *Alpha-*, *Beta-* and *Gammaherpesvirinae* (Roizman 1973; Roizmann, Desrosiers et al. 1992; Van Regenmortel, Fauquet et al. 2000). During this time, a formal system for naming herpesviruses was founded with various changes occurring over the following years, but maintaining that herpesviruses are named after the family or subfamily to which the primary host belongs; with the notable exception that human herpesviruses would maintain human as a designation rather than *hominid* (Roizman 1973; Fenner 1976; Matthews 1979; Roizman, Carmichael et al. 1981; Francki, Fauquet et al. 1991; Roizmann, Desrosiers et al. 1992; Davison 2010). Since the ancestral *Herpesvirinae* subfamilies are projected to have appeared about 200 million years ago, it is not surprising that DNA analysis has found many distantly related species of viruses in animals (McGeoch, Dolan et al. 1985). With the discovery of many new herpesviruses, the ICTV established the order *Herpesvirales* in 2009 (Davison 2010). This order

contains three families: *Herpesviridae*, containing the viruses of mammals, birds and reptiles; *Alloherpesviridae*, containing viruses of bony fish and frogs; and *Malacoherpesviridae*, containing the bivalve herpes virus OsHV1 (Davison 2010). The family *Herpesviridae* now contains the subfamilies of *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammapherpesvirinae*. Type 1 simplex virus is an alphaherpesvirus.

Due to the relatively rapidly mutating nature of viruses a couple definitions are helpful to consider when discussing taxonomy of a virus. The ICTV defines virus species as “a polythetic class of viruses that constitutes a replicating lineage and occupies a particular ecological niche” (Van Regenmortel 1990). Herpes viruses are considered a distinct species if “(a) their genomes differ in a readily assayable and distinctive manner across the entire genome and not merely at a specific site, and (b) if the virus can be shown to have distinct epidemiologic and biologic characteristics” (Roizmann, Desrosiers et al. 1992). Members of the *Alphaherpesvirinae* subfamily have the following features in common: variable host range, a generally short reproductive cycle in primary host, rapid reproduction and spread in permissive tissue cultures, efficient lysis of infected non-neuronal cells, and the ability to establish latent infections in neuronal cells, primarily in sensory ganglia. The genera Simplexvirus (including HSV-1), Varicellovirus, Marek’s disease-like virus, and Infectious laryngotracheitis-like virus are members of the *Alphaherpesvirinae* subfamily (Roizmann, Desrosiers et al. 1992; Van Regenmortel, Fauquet et al. 2000). Members of the *Betaherpesvirinae* subfamily have the following features in common: a more restricted host range, long reproductive cycle in host cells, slow infection progression and replication in tissue culture, cytomegaly

(infected cells becoming enlarged) and latency is usually maintained in secretory glands, lymphoreticular cells or kidneys. *Cytomegalovirus*, *Muromegalovirus*, and *Roseolovirus* are members of the *Betaherpesvirinae* subfamily (Van Regenmortel, Fauquet et al. 2000). Members of the *Gammaherpesvirinae* subfamily share the following characteristics: the host range is limited to the family or order of the natural host, these viruses replicate in lymphoblastoid cells in cell culture, some members of the subfamily cause lytic infection in epithelial and fibroblastic cells, usually specific for either B or T lymphocytes, and viral latency is usually maintained in lymphoid tissue. *Lymphocryptovirus* (EBV-Epstein Barr virus), and *Rhadinovirus* are members of the *Gammaherpesvirinae* subfamily (Van Regenmortel, Fauquet et al. 2000). Table 1.1 shows the family classification and common names of the nine known human herpesviruses (denoted by an *) as well as other commonly studies herpesviruses.

Table 1.1: The taxonomic classification of the nine known human herpesviruses (denoted by *) as well as other commonly studied herpesviruses; adapted from (Roizmann, Desrosiers et al. 1992; Van Regenmortel, Fauquet et al. 2000).

Table 1.1 Notable members of the Herpesviridae family		
Subfamily	Designation	Vernacular Name
Alpha-herpesvirinae	Human herpesvirus 1 (HHV-1)*	Herpes simplex virus type 1 (HSV-1)
	Human herpesvirus 2 (HHV-2)*	Herpes simplex virus type 2 (HSV-2)
	Human herpesvirus 3 (HHV-3)*	Varicella-zoster virus (VZV), chicken pox, shingles
	Cercopithecine herpesvirus 1 (CeHV-1)	Herpesvirus B, Simian Herpesvirus
	Gallid herpesvirus 1 (GaHV-1)	Infectious laryngotracheitis virus
	Gallid herpesvirus 2 (GaHV-2)	Marek's disease virus
	Suid herpesvirus 1 (SuHV-1)	Pseudorabies virus, Aujeszky's disease virus
	Felid herpesvirus 1 (FeHV-1)	Feline herpesvirus 1, Feline rhinotracheitis virus

	Ictalurid herpesvirus 1 (IcHV-1)	Channel catfish herpesvirus
	Bovine herpesvirus 1 (BoHV-1)	Infectious bovine rhinotracheitis virus
	Bovine herpesvirus 2 (BoHV-2)	Bovine mammillitis
	Equid herpesvirus 1 (EHV-1)	Equine HV-1, Equine abortion virus
	Equid herpesvirus 3 (EHV-3)	Equine coital exanthema
	Equid herpesvirus 4 (EHV-4)	Equine HV-4, Equine rhinopneumonitis virus
	Human herpesvirus 5 (HHV-5) *	Cytomegalovirus (CMV)
	Human herpesvirus 6A (HHV-6A) *	
	Human herpesvirus 6B (HHV-6B) *	Roseolovirus
	Human herpesvirus 7 (HHV-7) *	
Beta-Herpesvirinae	Murid herpesvirus 1 (MuHV-1)	Mouse CMV
	Murid herpesvirus 2 (MuHV-2)	Rat CMV
	Suid herpesvirus 2 (SuHV-2)	Inclusion-body rhinitis virus
	Cercopithecine herpesvirus 5 (CeHV-5)	African green monkey CMV
	Cercopithecine herpesvirus 8 (CeHV-8)	Rhesus monkey CMV
	Elephantid herpesvirus 1 (ElHV-1)	Endotheliotropic elephant herpesvirus-1
	Human herpesvirus 4 (HHV-4) *	Epstein-Barr virus (EBV)
	Human herpesvirus 8 (HHV-8) *	Kaposi's sarcoma associated herpesvirus (KSHV)
	Alcelaphine herpesvirus 1 (AlHV-1)	Wildebeest herpesvirus, Malignant catarrhal fever of European cattle
	Canid herpesvirus 1 (CaHV-1)	Canine herpesvirus
Gamma-Herpesvirinae	Ovine herpesvirus 2	Sheep-associated malignant catarrhal fever

Organization of the Herpes Viral Genome

The DNA of herpesviruses is arranged as a linear double strand. Rapidly after entry into the nucleoplasm of an infected cell nucleus, the DNA circularizes to form a double stranded circle. Herpesvirus DNA ranges from 120 kilobase pairs (kbp) to 250 kbp depending on the species of virus (McGeoch, Dalrymple et al. 1988a). Within individual viruses, length of the genome can also vary due to variable copy numbers of terminal and internal repeated sequences that can change the length within an individual virus by 10 kbp. Herpes simplex virus DNA is composed of approximately 152,000 bases with a weight of approximately 100×10^6 Daltons and 67-69% cytosine/guanine content (Kieff, Bachenheimer et al. 1971). Cytosine and guanine content of herpesviruses ranges from 31% to 75% across the entire genome (Roizman and Pellett 2001). Among the herpesviruses, the architecture of the genome varies in the presence and location of reiterated sequences. In HSV, there are sequences at each terminus of the genome that are repeated in an inverted orientation. These are juxtaposed internally within the genome. The genome is divided into two unique regions, the unique long (U_L or UL) and unique short (U_S or US) segments, separated by the apposed internal repeats (Fig 1.1) (Roizman, Knipe et al. 2007). The U_L region is approximately 108 kbp and encodes at least 56 proteins, while the U_S region is approximately 13 kbp and encodes 12 proteins (McGeoch, Dalrymple et al. 1988b). The repeated segments are designated the terminal long and short repeats (TR_L and TR_S respectively) and the internal long and short repeats (IR_L and IR_S respectively) (Roizman and Pellett 2001). These four components are able to invert relative to one another, making possible four different

isomers that have been demonstrated in infected cells in equivalent populations arranged in varying orientations (Roizman and Pellett 2001).

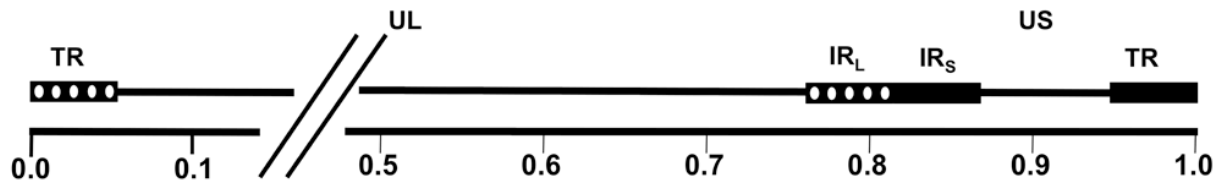


Figure 1.1: Schematic representation of the HSV genome. Schematic of the typical HSV genome showing the relative positions of the terminal repeats designated (TR), the unique long and short segments (designated UL and US respectively), and the internal repeats both long and short (designated IRL and IRS respectively). The character of the repeats (dotted or full black) denotes the positions of the related repeats.

The structure of most herpesvirus genes is a 50-200 bp promoter region followed by a TATA box and an initiation site 20-25 bases downstream from the TATA box. 30-300 bases later, this is followed by 5' untranslated leader sequence, a major open reading frame, a 3' untranslated segment 10-30 bases long and a polyadenylation signal with flanking sequences (Roizman and Pellett 2001). Occasionally genes lack the TATA box. Most genes in herpesviruses code for a single ORF, however, some have a second in-frame initiation site (Chou and Roizman 1986; Markovitz, Filatov et al. 1999). The majority of herpesvirus genes are not spliced. Herpesviruses can also produce noncoding RNAs, of which the HSV-1 latency associated transcript (LAT) is an example (Roizman and Pellett 2001). Herpesviruses encode a wide range (from 70-200) genes depending on the species of virus, with HSV-1 encoding approximately 90 known gene products and at least 84 transcriptional units participating in the production of proteins (Roizman, Knipe et al. 2007).

Herpes Virion Architecture

Herpes virus virions are composed of four structures: an electron-dense core (that contains the DNA), surrounded by an icosadeltahedral capsid composed of proteins, a protein-rich zone called the tegument, and an outer lipid envelope that contains glycosylated protein spikes (Fig 1.2) (Roizman 1974). Herpes virions range from 120nm to 300nm in diameter with the majority of this variability in size due to the makeup of the tegument and the state of the envelope (i.e.; an envelope that is not intact will allow more significant changes to the tegument during processing for electron microscopy) (Roizman 1974).

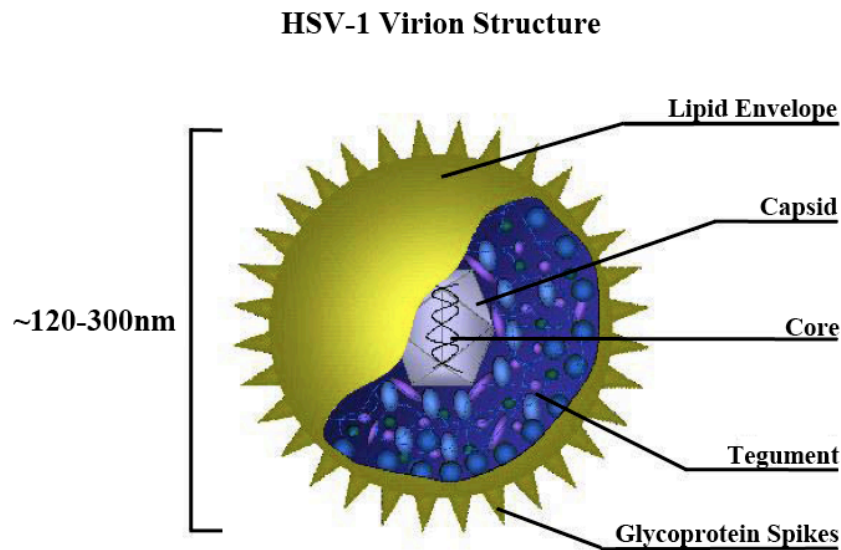


Figure 1.2: Herpesvirus virion structure. Virions of herpes viruses range from 120nm to 300nm in diameter (Roizman and Furlong, 1974). A virion consists of: an electron-dense core containing the viral genome, a protein capsid surrounding the core, an amorphous, proteinaceous tegument around the capsid, and an envelope derived from host cell membranes containing glycoproteins (Roizman and Furlong, 1974). Figure from (Melancon, 2003).

The Core

The core is the central-most structural component of a herpes virion, visible as an electron-dense area in electron micrographs. This structure is composed of the virus DNA packaged in the shape of a torus (doughnut) that has been shown in some herpes viruses to be suspended by a proteinaceous spindle that attaches the DNA to the inside surface of the capsid and contain a less-dense central cylinder (Falke, Siegert et al. 1959; Furlong, Swift et al. 1972; Nazerian 1974). The toroidal structure is 50 nm high, with an inside diameter of 18 nm and an outside diameter of 70 nm (Nazerian 1974). The arrangement of the viral DNA in the torus is unknown. In a host-cell nucleus, the virus DNA is arranged in a double-stranded circle (Roizman and Pellett 2001).

The Capsid

The capsid of all herpesviruses is composed of 162 capsomere proteins arranged in an icosahedron with triangulation symmetry of $T=16$, icosadeltahedron. This protein shell is approximately 15 nm thick and 126 nm in diameter and contains the DNA. The capsid is made of seven proteins pUL6, pUL17, pUL18, pUL19, pUL25, pUL35 and pUL38. The major capsid protein pUL19 (VP5) is the primary structural subunit of the capsid. This protein is arranged in monomers of 5 or 6 copies, pentons or hexons respectively. Of the 162 capsomeres, 150 are identical hexons with a copy of VP26 attached to the outer face (Baker, Jiang et al. 2006). Of the remaining 12 capsomeres, 11 are identical pentons situated at the 5 fold vertices (Zhou, He et al. 1995; Zhou, Chen et al. 1999; Newcomb, Thomsen et al. 2003). The last capsomere is a cylindrical, portal-like, structure made from 12 copies of the pUL6 protein (Newcomb, Juhas et al. 2001).

This portal functions as the channel for DNA entry into the capsid during encapsidation and for DNA release after infection. This portal is a cylindrical structure composed of twelve pUL6 molecules and functions as a channel through which DNA is introduced into and released from the capsid. Capsomeres make direct contact through the base of the capsomere. In addition to the direct contact, there is another component of the capsid, a heterotrimeric complex (triplex) that indirectly connects the capsomeres (Newcomb, Homa et al. 1999). The triplex consist of two copies of VP23 and one copy of VP19C and acts as a scaffold for the capsid proteins (Spencer, Newcomb et al. 1998). Another protein scaffold for the capsid proteins is pUL26/26.5, however this protein is lost when DNA is packaged into the core and is not present in mature virions (Homa and Brown 1997; Newcomb, Homa et al. 1999).

Three types of capsids (A, B and C) can be observed by electron microscopy. While the outer structures of the capsid are the same, the state of the DNA in the virus core and the presence of scaffolding proteins determines the types of capsids observed (Homa and Brown 1997). “A” capsids have a significant lack of DNA and protein in the capsid cavity due to failed DNA packaging. “B” capsids lack viral DNA but have the scaffolding proteins. “C” capsids contain all the viral DNA and scaffolding proteins, however, the presence of heterodimers of pUL17 and pUL25 on “C” capsids and not mature virions suggests that additional modifications occur during packaging and/or nuclear egress (Trus, Booy et al. 1996; Homa and Brown 1997; Cardone, Winkler et al. 2007).

The Tegument

The tegument is a proteinaceous region of variable thickness that lies between the capsid and envelope. In a mature HSV-1 virion, the tegument contains 24 proteins.

These proteins are pUL7, pUL11, pUL13, pUL14, pUL16, pUL21, pUL23, pUL36 (VP1/2), pUL37, pUL41, pUL46 (VP11/12), pUL47 (VP13/14), pUL48 (VP16), pUL49 (VP22), pUL50, pUL51, pUL55, pUS2, pUS3, pUS10, pUS11, ICP34.5, ICP0, and ICP4 (Mettenleiter 2004; Loret, Guay et al. 2008; Kelly, Fraefel et al. 2009). This layer appears fibrous on negative staining in electron micrographs and can be asymmetrically distributed (Morgan, Rose et al. 1959; Wildy and Watson 1962; Morgan, Rose et al. 1968). The asymmetrical distribution and thickness of the tegument appears to be dependent on location in the infected cell, with more symmetrical arrangement in perinuclear virions and more asymmetry in virions in cytoplasmic vesicles (Falke, Siegert et al. 1959). Extracellular virions also have asymmetric tegument, however, this may be a time-dependent phenomenon with the tegument more symmetrically organized at early times after egress (Grunewald, Desai et al. 2003; Newcomb and Brown 2009).

The tegument proteins are important in multiple stages of the virus life cycle. They function in the early stages of infection by recruiting the molecular motors of the cell to assist in entry and cellular transport and regulating viral and host gene expression, as well as having functions later in infection in virion egress (Kelly, Fraefel et al. 2009). The tegument has an ordered density surrounding the pentons of the capsid (Zhou, Chen et al. 1999). This is possibly due to the VP1-3 protein, which is a 336 kDa protein involved in nucleocapsid attachment to the nuclear pore and DNA release into the nucleoplasm (Knipe, Batterson et al. 1981; Batterson, Furlong et al. 1983; Ojala, Sodeik

et al. 2000). However, this protein is likely involved in multiple stages of the virus life cycle and is not essential for DNA packaging, since null mutants still accumulate DNA-filled capsids in the cytoplasm of infected cells (Desai 2000). Only 4 of the 24 tegument proteins are essential for growth in cell-culture (pUL36, pUL37, pUL48 and ICP4) (Roizman and Knipe 2001b; Mettenleiter 2002b; Loret, Guay et al. 2008; Kelly, Fraefel et al. 2009). Some tegument proteins are expressed in significantly, 1000 fold, greater quantities than others and recent investigations of null mutant viruses have shown that lack of one protein can often be compensated for by recruitment of other proteins (del Rio, DeCoste et al. 2005).

The Envelope

The envelope is the outer covering of the virus. The envelope is a lipid bilayer composed of altered cellular membranes that have the typical trilaminar appearance of lipid bilayers (Falke, Siegert et al. 1959; Armstrong, Pereira et al. 1961; Epstein 1962; Morgan, Rose et al. 1968). The envelope also contains numerous protruding glycoprotein spikes as well as non-glycosylated transmembrane proteins and membrane-associated proteins (Roizman, Knipe et al. 2007).

HSV-1 specifies at least 11 different glycoproteins, including; gpUL27 (gB), gpUL44 (gC), gpUS6 (gD), gpUS8 (gE), gpUS4 (gG), gpUL22 (gH), gpUS7 (gI), gpUS5 (gJ), gpUL53 (gK), gpUL1 (gL), gpUL10 (gM), and possibly gpUL49A (gN). These glycoproteins are expressed in varying amounts and copy number of a specific glycoprotein can exceed 1000 in a single virion. The glycoproteins form spikes and protrusions from the envelope that are variable in length and orientation, however, there appears to be a systematic distribution of the glycoproteins suggesting a functional

reason for their ordered arrangement (Grunewald, Desai et al. 2003). The envelope glycoproteins gB, gD, gH and gL have been shown to be essential for virion entry into a susceptible host cell. Non-glycosylated membrane proteins include pUS9, pUL20 and pUL45. Glycoproteins are important in membrane fusion with susceptible cells and mediate interactions with host cell membrane receptors and viral entry (Mettenleiter 2004). The envelope proteins interact in a complex manner to determine host-cell tropism and initiate entry into host cells. This is evidenced by the interactions of pUL20 and gK and subsequent interactions with gB, the primary fusogenic protein (Chouljenko, Iyer et al. 2009; Chouljenko, Iyer et al. 2010). Envelope proteins (notably gC) also play a role in modulating the host immune response to infection (Roizman, Knipe et al. 2007).

Table 1.2: HSV structural proteins by location in the virion architecture.

Capsid	Tegument	Envelope
pUL6	pUL4	pUL1 (gL)
pUL18 (VP23)	pUL11	pUL10 (gM)
pUL19 (VP5)	pUL13	pUL20
pUL25	pUL14	pUL22 (gH)
pUL35 (VP26)	pUL16	pUL27 (gB)
pUL38 (VP19C)	pUL17	pUL43
	pUL21	pUL44 (gC)
	pUL36 (VP1/2)	pUL45
	pUL37	pUL49.5 (gN)
	pUL41	pUL53 (gK)
	pUL46 (VP11/12)	pUS4 (gG)
	pUL47 (VP13/14)	pUS5 (gJ)
	pUL48 (VP16)	pUS6 (gD)
	pUL49 (VP22)	pUS7 (gI)
	pUL51	pUS8 (gE)
	pUL56	pUS9
	pUS2	
	pUS3	
	pUS10	
	pUS11	
	ICP0	
	ICP4	

Table 1.2: Adapted from (Roizman, Knipe et al. 2007; Diefenbach, Miranda-Saksena et al. 2008b).

The Herpes Simplex Virus Lifecycle

For the herpesvirus lifecycle, like all biological cycles, circular and stepwise progression through the cycle will bring one back to the selected start point. At its most basic, the herpesvirus life cycle starting with a mature viral particle, begins with initial attachment to a host cell membrane and is followed by fusion with the host cell membrane, entry into the host cell, virion transport to the nucleus, production of viral proteins and viral DNA, creation of progeny viruses and release as a mature virion from the infected cell (Roizman 1974). For HSV-1 this initial infection is generally contained within the oral mucosa. For the mucosal epithelial infection, this infection follows the basic life cycle until cleared by the immune system. However, HSV-1 or 2 infections, may enter the sensory neurons innervating the infected area and start a more complex life cycle, involving much more distant transport to the nucleus (retrograde transport) and a state of latency not observed in epithelial cell infections (Cunningham, Diefenbach et al. 2006; Saksena, Wakisaka et al. 2006). This latent state can result in life-long recurrent infections (Whitley, Kimberlin et al. 1998b). In the next few sections, primarily an infection lifecycle as observed in an epithelial cell will be discussed with the differences in neuronal infection covered later.

Virus Attachment and Entry

Entry is a three-stage process involving interactions between the viral membrane proteins and host-cell surface receptors. The gamut of membrane proteins on the surface of a herpesvirus can determine the species and cell types that a virus can infect (Roizman and Pellett 2001). The first stage of viral attachment and entry is initial virus attachment to a

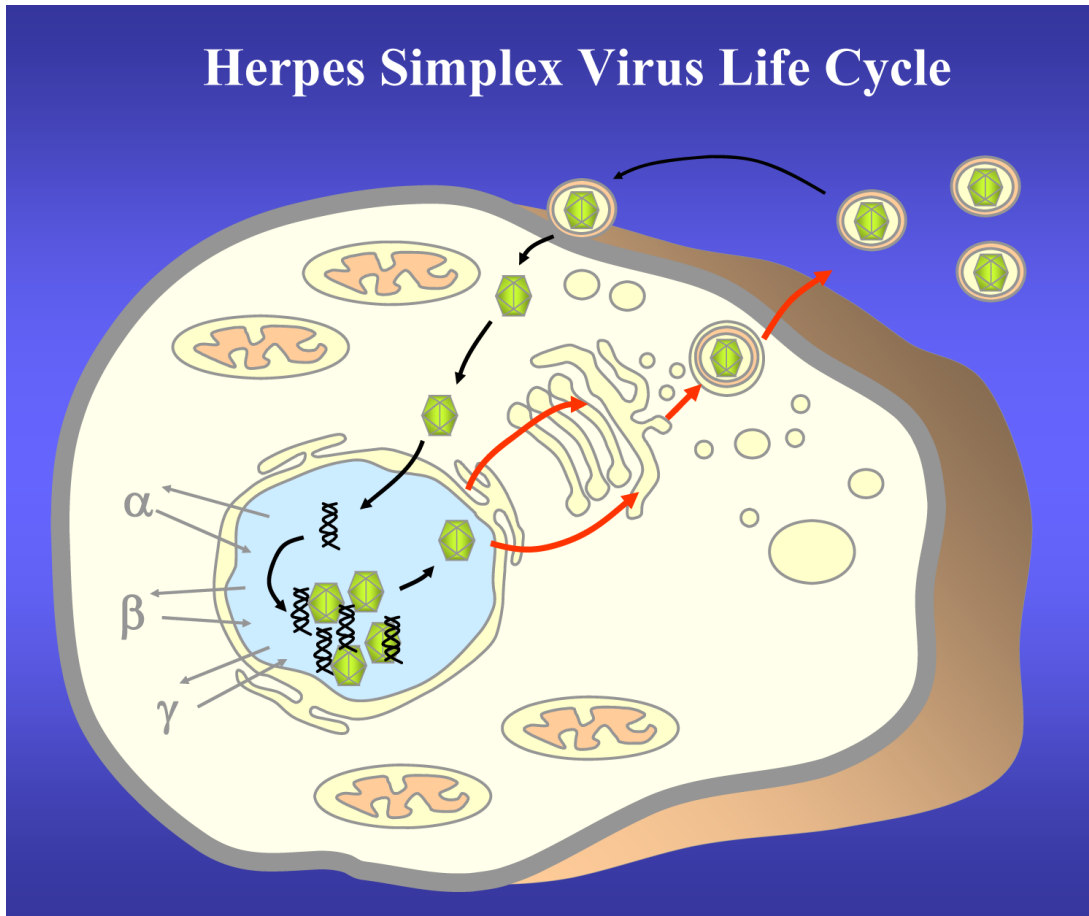


Figure 1.3: The Herpes Simplex Virus Life Cycle. Schematic cartoon of the overall herpes virus life cycle showing entry, transport to the nucleus, DNA release, coordinate gene expression, DNA replication and packaging, and virion egress. This schematic demonstrates the envelopment/deenvelopment model of egress. Figure from (Melancon, 2003).

potential host cell. The second stage is interaction of cellular membrane receptors with a viral protein that initiates the final stage of fusion of the two membranes and entry of the viral capsid and tegument into the host cell's cytoplasm.

Virus attachment begins with an interaction between viral proteins and the glycosaminoglycan (GAG) chains of proteoglycans that are richly distributed on the surface of mammalian cells (Spear 2004b). The viral proteins involved in this interaction are predominantly gB and gC, which have a predilection for heparan sulfate as the

proteoglycan of choice (Shieh, WuDunn et al. 1992; Spear 2004b). Interestingly, this initial attachment step is reversible and while greatly assisting infection of the host cell, it is not essential for virus entry or replication as cells lacking heparan sulfate can be infected and gC deficient viruses can infect and replicate albeit at 10 fold lower efficiencies than normal (Heine, Honess et al. 1974; Banfield, Leduc et al. 1995).

This initial attachment, while not essential, aids in the positioning of glycoprotein D (gD) to start an irreversible reaction that will lead to virus-cell fusion. Glycoprotein D interacts with its cellular receptors and as a result undergoes a conformational change that activates the virus membrane-cell membrane fusion machinery. This fusion complex involves multiple viral membrane proteins including: gB, gH/gI and recently discovered interactions with gK and UL20 (Cai, Gu et al. 1988; Forrester, Farrell et al. 1992; Roop, Hutchinson et al. 1993; Spear 2004b; Chouljenko, Iyer et al. 2009; Chouljenko, Iyer et al. 2010).

Binding and Entry Receptors

Cellular receptors of gD include: the herpesvirus entry mediator A (HVEM or HveA), nectins, and 3-O-sulfated heparan sulfate (Fig 1.4) (Roizman, Knipe et al. 2007). HveA is a member of the tumor necrosis factor (TNF) receptor family. This receptor is present on many mammalian tissues and cell types, including leukocytes, T and B lymphocytes, epithelial cells, and fibroblasts (Spear, Eisenberg et al. 2000; Spear 2004b). However, this receptor is not present on neurons (Spear 2004b). The natural ligands for this receptor include LIGHT and lymphotoxin-alpha (Mauri, Ebner et al. 1998). These ligands are involved in immune signaling and may be involved in HSV interaction with,

or evasion of, immune cells (Kwon, Kim et al. 2003). Studies have shown that HveA is significantly involved in HSV infection of activated T-cells, however, the T-cell is not thought to be a primary target of HSV and HveA has been shown to not be the primary mediator of entry in other cells, so the significance of this interaction is uncertain (Montgomery, Warner et al. 1996).

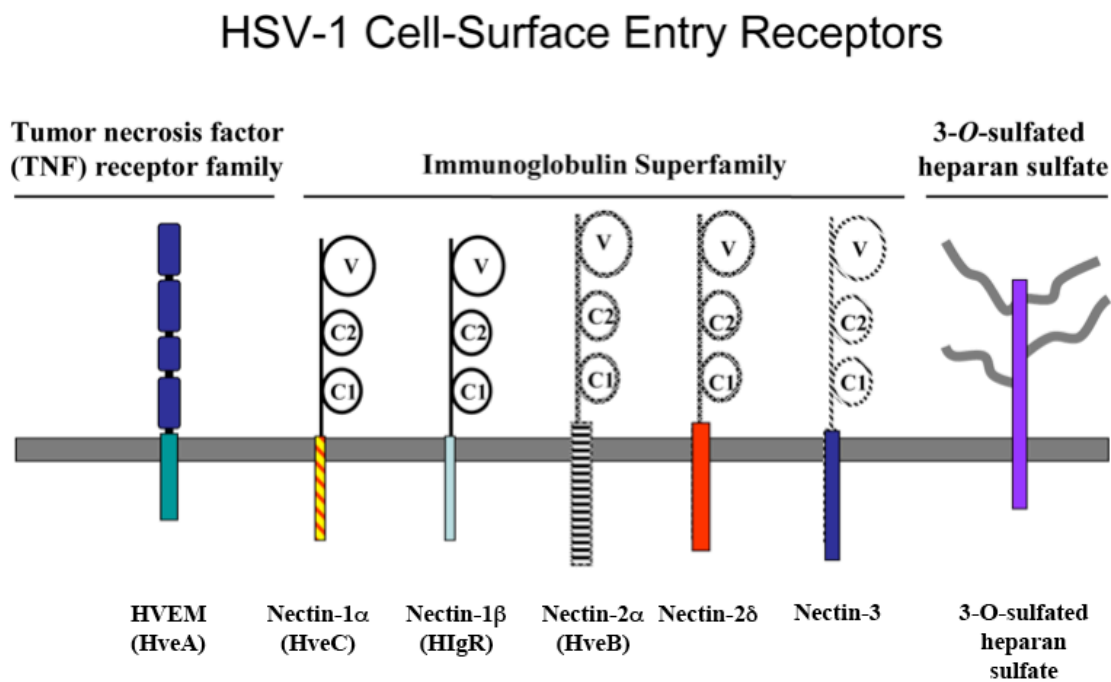


Figure 1.4: Herpes Simplex Virus entry receptors. The three classes of cell surface receptors for HSV entry: the tumor necrosis factor (TNF) receptor family consisting of HVEM, the immunoglobulin superfamily consisting of the nectins, and 3-O-sulfated heparin sulfate. Figure from (Melancon, 2003).

Members of the immunoglobulin superfamily may also serve as receptors. Nectin-1 α (α) (HveC), nectin-1 β (β) (Herpesvirus-like Immunoglobulin receptor HIgR, and nectin-2 α (HveB) are members that can mediate HSV entry. Nectin-1 α and nectin-1 β are expressed on epithelial cells, fibroblasts and neural cells, all cells that reside in the

human tissue targets of HSV infection (skin, brain, and spinal ganglia), as well as hematopoietic cells (Cocchi, Lopez et al. 1998; Cocchi, Menotti et al. 1998; Geraghty, Krummenacher et al. 1998). Nectin-1 α and β can mediate entry of all HSV-1 strains, HSV-2, Pseudorabies virus (PrV) and bovine herpes virus 1 (BHV-1) (Geraghty, Krummenacher et al. 1998). Nectin-2 α can mediate the entry of HSV-2, PrV, and certain mutants of HSV-1 but not wild-type HSV-1 (Warner, Geraghty et al. 1998; Lopez, Cocchi et al. 2000). Nectin-1 and nectin-2 are also related to the poliovirus receptor (Takai and Nakanishi 2003). Another member of the immunoglobulin family, the paired immunoglobulin-like type 2 receptor PILR α has been shown to bind to gB for fusion of the virus (Wang, Fan et al. 2009). PILR α is expressed on hematopoietic cells and some non-hematopoietic cells yet it remains to be determined if this receptor is a significant contributor to HSV infection of ocular, epithelial, or neuronal cells (Farooq, Valyi-Nagy et al. 2010).

In the host organism, the nectins are intercellular adhesion molecules localized to intercellular junctions (Takahashi, Nakanishi et al. 1999). The nectins are highly conserved throughout mammals and are expressed in the target tissues of herpesviruses, namely epithelial cells, fibroblasts and neurons (Takahashi, Nakanishi et al. 1999). The nectins are also similar in respect to structure, function, and the ability to mediate HSV entry across many species (Shukla, Dal Canto et al. 2000; Milne, Connolly et al. 2001). The distribution of these receptors and importance in intercellular junctions suggests that these proteins probably play a role in both virus entry and cell-to-cell spread (Roizman and Knipe 2001b).

3-*O*-sulfated heparan sulfates may mediate entry of HSV-1 and these proteoglycans are broadly distributed on human cells (Shukla, Liu et al. 1999). In addition to direct membrane fusion, HSV-1 may enter a cell by endocytosis depending on the cell type. In non-neuronal cells, endocytosis may occur in a pH-dependent manner (Garner 2003; Nicola, McEvoy et al. 2003) or pH-independent manner (Milne, Nicola et al. 2005). However, in neurons entry has been proven only in a pH-independent membrane fusion type process (Nicola, Hou et al. 2005).

Virus-to-Cell Fusion

The final stage of virus entry is fusion of the viral envelope with the host cell membrane and release of the capsid and tegument into the host cell cytoplasm (Morgan, Rose et al. 1968). In cell culture, gD, gB and gH/gL are essential for virus-to-cell fusion and entry into a host cell (Sarmiento, Haffey et al. 1979; Ligas and Johnson 1988; Forrester, Farrell et al. 1992). Glycoprotein D binding to cellular receptors likely induces conformational changes in gB that activate the virus fusion machinery (Heldwein, Lou et al. 2006; Roller, Dollery et al. 2008; Atanasiu, Whitbeck et al. 2010). Glycoproteins H, L and K as well as the non-glycosylated membrane protein UL20 interact to assist and modulate this fusion reaction (Spear 2004a; Chouljenko, Iyer et al. 2009; Chouljenko, Iyer et al. 2010). For entry via an endocytotic pathway, the first stage of endocytosis appears to occur independent of a gD receptor, however, the viral glycoproteins gB, gD, gH/gL are necessary and low endosomal pH may assist in conformational changes contributing to fusion (Nicola, McEvoy et al. 2003; Nicola and Straus 2004; Dollery, Wright et al. 2011). While these two pathways may occur in the same cell using similar

viral protein constituents, these pathways are spatially distinct. After the fusion of the virus and target cell membranes, the viral capsid, containing the viral DNA, and some of the tegument is released into the cytoplasm (Maurer, Sodeik et al. 2008). In studies of PrV, pUL11, pUL47, pUL48, and pUL49 were lost from the capsid while pUL36, pUL37 and PUS3 remained with the capsid (Granzow, Klupp et al. 2005; Luxton, Haverlock et al. 2005; Antinone, Shubeita et al. 2006; Copeland, Newcomb et al. 2009). The progression of the virus through the cytoplasm to the nucleus may also involve two of these remaining tegument proteins, UL36 and UL37 (Krautwald, Fuchs et al. 2009; Roberts, Abaitua et al. 2009).

Host Protein Shutoff

As the virus progresses toward the host cell nucleus, the virus also begins the inhibition of host cell protein synthesis. The virion host shutoff (VHS) comes from a structural element of the tegument and does not require protein synthesis in the newly infected cell (Nishioka and Silverstein 1977; Fenwick and Walker 1978; Nishioka and Silverstein 1978b; Nishioka and Silverstein 1978a). VHS inhibits the production of host cell proteins via two mechanisms; it inhibits synthesis and processing of host mRNA and it speeds the degradation of mRNAs. These effects are applicable to the virus, however, once viral protein synthesis is occurring, it is in an overwhelming amount that makes the effect of VHS on host cell protein production much more significant (Roizman, Knipe et al. 2007). VHS functions to induce the cleavage of mRNA and cause 5' degradation of mRNA (Elgadi, Hayes et al. 1999; Karr and Read 1999). VHS also forms a complex with the translation factor eIF-4H, to decap cellular mRNA from the 5' end (Roizman

and Knipe 2001b). Since VHS activity does not rely on production of *de novo* synthesis of viral proteins, VHS preferentially targets cellular mRNA early in infection and may facilitate the transition of viral gene expression through the A, B and C genes as it accumulates and shortens the life of viral mRNA (Kwong and Frenkel 1987; Oroskar and Read 1987). Although VHS accumulates late in infection, it does not degrade viral mRNA, likely due to an interaction between VP16 and VHS at late times during infection that blocks the degradation of RNA by VHS (Lam, Smibert et al. 1996). It is likely that this interaction halts the VHS function so that progeny virions may be assembled and released as mature infectious virions.

Virion Transport to the Host Cell Nucleus

After fusion of the viral envelope and deposition of the capsid and some of the tegument into the cytoplasm, the capsid and some of the tegument are transported to the cell nucleus. One of the tegument proteins that does not disassociate from the capsid is VP16 (α -tiff). This tegument protein is actually transported along with the capsid to the nucleus. The capsids are transported to the nuclear pores along microtubules by the molecular motors dynein and members of the kinesin protein superfamily (KIFs) (Sodeik, Ebersold et al. 1997; Dohner, Wolfstein et al. 2002; Garner 2003; Diefenbach, Miranda-Saksena et al. 2008b; Lyman, Curanovic et al. 2008). Some experiments have shown that microtubules adjacent to the cell plasma membrane are disrupted following entry, which suggests that normal microtubule formations are likely altered by the capsid and tegument proteins effects on microtubules (Ward, Avitabile et al. 1998). Additional specifics of microtubule motor transport of HSV will be covered later in the section

detailing transport in neurons. After transport, capsids accumulate at the nuclear pore complexes. Capsid binding to the nuclear pore complexes can be prevented with antibodies to parts of the nuclear transport machinery (Ojala, Sodeik et al. 2000). Once close to the nucleus, capsids seem to dock to the nuclear pores and the capsid is destabilized and DNA is released, or extruded, from the capsid into the nucleus (Sodeik, Ebersold et al. 1997; Ojala, Sodeik et al. 2000). The tegument protein VP1/2, encoded by the UL36, gene is involved in this process through interactions with the nuclear pore complex (Batterson, Furlong et al. 1983; Batterson and Roizman 1983; Ojala, Sodeik et al. 2000). Temperature sensitive HSV-1 mutants of UL36 cannot release DNA into the host cell nucleus (Knipe, Batterson et al. 1981; Batterson and Roizman 1983). The pUL25 capsid protein has been shown to interact with nucleoporins CAN/Nup214 and hCG1 and also binds to the pUL6 portal protein and the tegument protein pUL36 (Batterson, Furlong et al. 1983; Cardone, Winkler et al. 2007; Newcomb, Booy et al. 2007; Jovasevic, Liang et al. 2008; Roberts, Abaitua et al. 2009). It is likely that these proteins play a coordinated role in capsid-nuclear pore binding and viral DNA release into the nucleus.

Gene Expression in Infected Cells

Once the viral DNA has penetrated the host-cell nucleus, the HSV infection begins in earnest with the expression of viral proteins and eventual synthesis of viral DNA. During the infection, HSV-1 expresses greater than 80 gene products in an organized pattern and timing of these proteins. Proteins can be broken into three groups in terms of relative timing of expression; these are in order of expression: alpha (α), beta

(β), and gamma (γ) (Honess and Roizman 1974). Transcription of viral DNA occurs in the nucleus and the virus uses the host RNA polymerase II for transcription of viral genes (Alwine, Steinhart et al. 1974; Costanzo, Campadelli-Fiume et al. 1977). Upon entry into the host cell, the tegument protein VP16 is released from the tegument and binds to the host cell factor C1 and this complex enters the nucleus and binds to Oct-1 (Katan, Haigh et al. 1990; Kristie and Sharp 1990). This complex is a transactivator of α genes (La Boissiere, Hughes et al. 1999). Shortly after a cell is infected, HSV-1 DNA enters the nucleus and localizes to cellular ND10 structures, where VP16 causes the transactivation and transcription of the α genes giving VP16 the name (α -TIF) for the α gene transactivating factor (Post, Mackem et al. 1981; Ishov and Maul 1996; Maul, Ishov et al. 1996). Viral α gene expression peaks at about 2-4 hours post infection. There are six α genes (ICP0, ICP4, ICP22, ICP27, ICP47 and Us1.5 and five of these can stimulate β gene expression (Fig 1.5). ICP4 is required for all viral gene expression after the α genes and it also down regulates some α genes, including self down regulation and ICP0, as well as the gene products of ORF P and ORF O, which are pre- α genes (Clements, Watson et al. 1977; Dixon and Schaffer 1980; Kristie and Roizman 1984; Faber and Wilcox 1986; Godowski and Knipe 1986; Kristie and Roizman 1986; Gelman and Silverstein 1987; Muller 1987). ICP0 is a non-specific transactivator that can induce α , β , and γ genes (Everett, Orr et al. 1991). The expression of HSV-1 β genes, whose primary functions are viral DNA replication and nucleotide metabolism, peaks from 4-8 hours after infection (Roizman and Knipe 2001b). The β genes are further subdivided by the timing of expression into β_1 genes, which are expressed directly after α genes, and β_2 genes, which are expressed somewhat later (Roizman and Knipe 2001b). The final

group, the γ genes, peak after viral DNA replication has already begun. These genes require the presence of ICP4, ICP27 and ICP8 for efficient transcription. The γ genes predominantly include structural proteins, glycoproteins and tegument (Roizman and Knipe 2001b). The HSV-1 C genes are subdivided similarly to the β genes, with γ_1 genes being expressed earlier, even as early as during α gene expression (and are sometimes therefore called “leaky-late”); however, expression peaks after DNA replication. The γ_2 genes are not expressed until after DNA replication has begun (Wagner 1985).

Glycoprotein K is a typical example of the γ_2 genes.

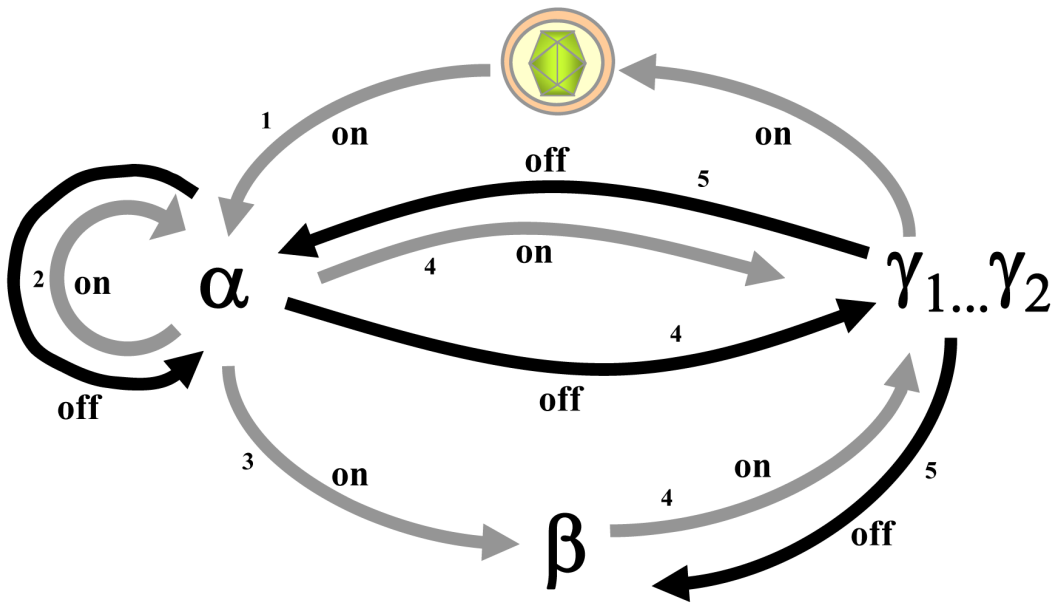


Figure 1.5: Map of the complex coordinate gene expression in Herpes Simplex Virus. 1) α -TIF, from the tegument activates initial transcription of α genes. 2) Autoregulation of gene expression. 3) Activation of β gene expression by α genes. 4) Activation of γ gene expression by α and β genes. 5) γ genes stop expression of α and β genes late in infection (Roizman and Knipe, 2001).

Viral DNA Replication

After entry of the viral DNA into the nucleus, the linear DNA forms a circle using the DNA ligase and XRCC4, a DNA repair protein, that then acts as the template for DNA synthesis (Strang and Stow 2005; Muylaert and Elias 2007). DNA replication begins after β genes are expressed and translated. Some of the β gene protein products assemble at the ND10 nuclear structures, in punctate prereplicative sites, where the viral DNA has already localized (Ishov and Maul 1996; Uprichard and Knipe 1996). The rolling circle of replication starts at the origins of replication using the origin binding protein (pUL9) (Elias, O'Donnell et al. 1986; Olivo, Nelson et al. 1988). UL9 binds to the viral DNA replication origin sites, unwinds DNA, and recruits ICP8, the ssDNA binding protein. Then these proteins recruit the remaining proteins in the helicase-primase complex and the DNA polymerase into a replisome (Muylaert, Tang et al. 2011). Then the replication of viral DNA begins, initially as a theta structure, then progresses to a rolling circle form by the replisome that generates leading and lagging strands of replicated DNA (Skaliter, Makhov et al. 1996) and eventually creates many head-to-tail concatamers (Jacob, Morse et al. 1979). The concatamers of viral DNA are contained in replication compartments consisting of viral DNA and replication complexes (Quinlan, Chen et al. 1984). Seven viral proteins are necessary for viral DNA replication: the DNA polymerase (UL30), the DNA polymerase accessory protein (UL42), the origin-binding protein (UL9), the single-stranded DNA binding protein (ICP8) and the helicase-primase complex (composed of UL5, UL8 and UL52) (Purifoy, Lewis et al. 1977; Conley, Knipe et al. 1981; Challberg 1986; Wu, Nelson et al. 1988). It is likely that host cell factors and enzymes such as DNA ligase and topoisomerase II are also necessary. Although only

one origin of replication is necessary for the replication of viral DNA, there are three possible origins of replication: *oriS* (present in two copies) and *oriL* (Frenkel, Locker et al. 1976; Locker, Frenkel et al. 1982; Mocarski and Roizman 1982; Stow 1982; Vlazny, Kwong et al. 1982; Weller, Spadaro et al. 1985; Knopf, Spies et al. 1986; Lockshon and Galloway 1986; Deb and Doelberg 1988).

Capsid Assembly and DNA Packaging

During DNA replication, the γ genes are transcribed and the proteins are made in the cytoplasm of the infected cell. The capsid components of the virus must be then be transported back to the nucleus for assembly. The triplex proteins (two copies of VP19C and one VP23) are brought into the nucleus already assembled in the cytoplasm, since VP23 does not transport into the nucleus alone (Nicholson, Addison et al. 1994; Rixon, Addison et al. 1996). The major capsid protein (VP5) is transported into the nucleus with the triplexes or a scaffolding protein pre-VP22a (Roizman, Knipe et al. 2007). The UL26/UL26.5 genes are responsible for multiple proteins involved in capsid formation and assembly. These proteins are the result of two overlapping genes and multiple cleavage forms of the UL26 gene. Pre-VP22a, the major scaffolding protein for capsid formation is the product of the UL26.5 gene and the UL26 gene is responsible for VP21, another scaffolding protein, and VP24, a protease that cleaves the major scaffold protein (Roizman, Knipe et al. 2007). Once the capsid and scaffold proteins are in replication compartments in the nucleus, the remainder of capsid assembly occurs. Assembly likely begins at the portal ring, that is made from 12 copies of the pUL6 protein (Newcomb, Homa et al. 2001; Newcomb, Juhas et al. 2001). The VP5 and pre-VP22 complexes self

assemble, likely guided by the self-assembling nature of the pre-VP22a and guided by alignment with the portal ring. As more pentons, hexons and triplexes are brought together the capsomeres form into a sphere-like procapsid (Newcomb, Homa et al. 1996; Baines 2011). Once this procapsid is formed, the viral DNA is loaded into the procapsids by a three-part enzyme, the product of the UL28 gene (Beard, Taus et al. 2002). This enzyme is also responsible for the cleavage of the replicated viral DNA concatamers into single unit monomers of the viral DNA ready for packaging into the capsids. During the DNA packaging process, the scaffold is also removed by the protease action of VP24 which allows the procapsid to assume a more stable and resilient icosahedral shape (Newcomb, Homa et al. 1996).

Three types of capsids have been identified for HSV; A, B, and C capsids. All three capsid types are formed of similar pentons, hexons and triplexes and are approximately 120 nm in diameter (Gibson and Roizman 1972; Newcomb, Trus et al. 1993). C-capsids are capsids that have gone through maturation fully as described and can proceed to become infectious viruses after egress. B-capsids contain VP21, VP22a, and VP24 but do not contain viral DNA (Gibson and Roizman 1972; Newcomb, Trus et al. 1993). A-capsids contain no scaffolding proteins and no DNA and are thought to be an aborted attempt at DNA packaging (Sherman and Bachenheimer 1987; Newcomb, Homa et al. 1996).

Virion Egress

To complete maturation of the virus and make an infectious particle, the encapsidated virion must acquire the tegument and envelope and exit the infected cell.

Two models are currently considered for this process. The first model has the virion acquire its final tegument and final envelope as it buds through the nuclear membrane and the mature virus particle exits the cell through the secretory pathway. The alternate model involves acquisition of a primary envelope as the encapsidated virion passes through the nuclear membrane followed by deenvelopment and final tegumentation and envelopment occurs in the trans-golgi network (Roizman, Knipe et al. 2007).

For either model, two viral proteins appear to be involved in the process of primary egress from the nucleus; pUL31, a nuclear phosphoprotein embedded in the nuclear membrane of infected cells (Reynolds, Ryckman et al. 2001; Fuchs, Klupp et al. 2002), and pUL34, a protein in inner and outer nuclear membrane of infected cells that uses a C-terminal membrane anchor (Klupp, Granzow et al. 2000). For both models, the first step for virion egress terminates with the virus enveloped and present in the perinuclear space between the two nuclear membranes (Vlazny, Kwong et al. 1982). Furthermore, the inner nuclear membrane is bound by a protein-rich nuclear lamina and it appears that this layer must be disrupted to allow intranuclear capsids access to the inner nuclear membrane (Scott and O'Hare 2001). The viral proteins of UL31 and UL34 can bind, reorganize and destabilize lamin A/C, which is the primary component of the nuclear lamina (Reynolds, Liang et al. 2004a; Reynolds, Liang et al. 2004b; Simpson-Holley, Baines et al. 2004). The UL11 protein has also been shown to increase the efficiency of this stage of envelopment, however it does not appear to be essential (Baines and Roizman 1992; MacLean, Dolan et al. 1992).

Once this primary envelopment occurs via budding through the inner nuclear membrane, the two models diverge. In the first model, the virion is basically complete at

this point and, while some maturation may still occur, the virion retains its envelope and tegument from this envelopment stage and exits the cell via the secretory pathways (Darlington and Moss 1968; Johnson and Spear 1982; Johnson, Webb et al. 2001b; Baines, Parsch et al. 2004). This exit strategy takes advantage of the continuity of the perinuclear space with the endoplasmic reticulum and secretory paths.

In the alternate model, the enveloped virion in the perinuclear space must de-envelope from its primary envelope as it fuses with the outer nuclear membrane (Fig 1.6). The virion is de-enveloped and loses some, or all, of its tegument as the virion enters the cytoplasm (Enquist, Husak et al. 1998; Mettenleiter 2000). Re-envelopment then occurs in the cytoplasm, specifically in the trans-Golgi network (TGN), compartments where additional tegument is also added (Roizman, Knipe et al. 2007). This has been termed a “de-envelopment, re-envelopment” model. The majority of evidence currently supports the latter model. By electron microscopy, primary enveloped particles have been shown fusing with the outer nuclear membrane and unenveloped, cytoplasmic virions have been shown budding into cytoplasmic vesicles (Gershon, Sherman et al. 1994; Granzow, Klupp et al. 2001; Mettenleiter 2002b). The lipid composition and ultrastructural appearance of the extracellular virions resembles the cells plasma membrane rather than the nuclear membranes (van Genderen, Brandimarti et al. 1994). Furthermore, UL31 and UL34 proteins are present in perinuclear virions, but are not able to be detected in extracellular virions. While, the tegument proteins of UL46 and UL49 genes are present in cytoplasmic or extracellular virions but are not found in perinuclear virion particles (Klupp, Granzow et al. 2000; Mettenleiter 2002b; Reynolds, Wills et al. 2002).

The major argument against the “de-envelopment, re-envelopment” model, currently, is the lack of a defined mechanism for de-envelopment. Glycoproteins involved in the fusion machinery of the virus do not affect this pathway, as shown by experiments with deletion mutants (Cai, Person et al. 1987; Jayachandra, Baghian et al. 1997; Steven and Spear 1997; Granzow, Klupp et al. 2001). Current investigation focuses on the possibility of redundant pathways including multiples of the glycoproteins or as yet unidentified players in this interaction.

In the “de-envelopment, re-envelopment” model, capsids acquire tegument proteins as they pass through the nucleus and cytoplasm, likely in the manner of protein-protein interactions with the capsid and other tegument members (Mettenleiter 2002b). The tegument is composed of at least 15 different proteins in HSV. While initially thought to be structureless, the tegument has been shown to possess some icosahedral structure in its inner-most layer around pUL36, a large tegument protein that interacts with the major capsid protein, VP5, (Machtiger, Pancake et al. 1980; McNabb and Courtney 1992; Newcomb, Trus et al. 1993; Zhou, Chen et al. 1999; Mettenleiter 2002b). The tegument likely plays a structural role, linking the capsid to the envelope, and has important functions in the early phase of infection, through the actions of the tegument proteins UL41 (vhs), UL48 (α -tiff), and Us3 (a protein kinase) (Mettenleiter 2002b; Roizman, Knipe et al. 2007). While a lack of UL36 or UL37 tegument proteins ceases virus maturation, this maturation can proceed without UL13, US3, UL41, UL46, UL47, or UL49 (Rafield and Knipe 1984; Purves, Longnecker et al. 1987; Desai 2000; Desai, Sexton et al. 2001; Klupp, Granzow et al. 2001; Roizman and Knipe 2001b; Mettenleiter 2002b). Therefore in cultured cells, while many tegument components are not necessary, the tegument in its full form likely contributes to an efficient infection in the host.

The final stage of production of an infectious particle involves acquisition of a mature virion envelope and exiting the host cell. After tegumentation of the virion in the “de-envelopment, re-envelopment” pathway, the HSV-1 capsid must bud into the system of cytoplasmic vesicles called the Trans-Golgi Network (TGN) (Fig1.6). This network is a collection of Golgi-derived endosomes. This final envelopment stage is not well characterized (Mettenleiter 2002b). It is likely that this step requires some interactions between the outer layer of the tegument and some of the viral membrane proteins that will be resident on the mature virion. Deletion of glycoproteins E (gE) and I (gI) or gM does not affect the formation of mature virions in pseudorabies virus (PRV-an alphaherpesvirus closely related to HSV) or HSV. However deletion of all three glycoproteins inhibits virus spread (and by extension production of mature virions) in PRV but not HSV (Enquist, Husak et al. 1998; Brack, Dijkstra et al. 1999; Browne, Bell et al. 2004). Simultaneous deletion of gD, gE and gI causes an envelopment defect in HSV (Farnsworth, Goldsmith et al. 2003). However, any multiple glycoprotein deletion may also destabilize other tegument or envelope interactions that may appear as solely an envelopment defect.

The most dramatic secondary envelopment effects are observed with gK or pUL20 deletions, in which there is marked accumulation of unenveloped and aberrantly enveloped capsids in the infected cell cytoplasm with corresponding decreases in extracellular infectious virus (Jayachandra, Baghian et al. 1997; Foster, Melancon et al. 2004b). In the normal course of infection, this final step of envelopment is proposed to be followed by final egress of the virus from the host cell via the normal cellular secretory transport machinery, resulting in a new, extracellular, infectious virus.

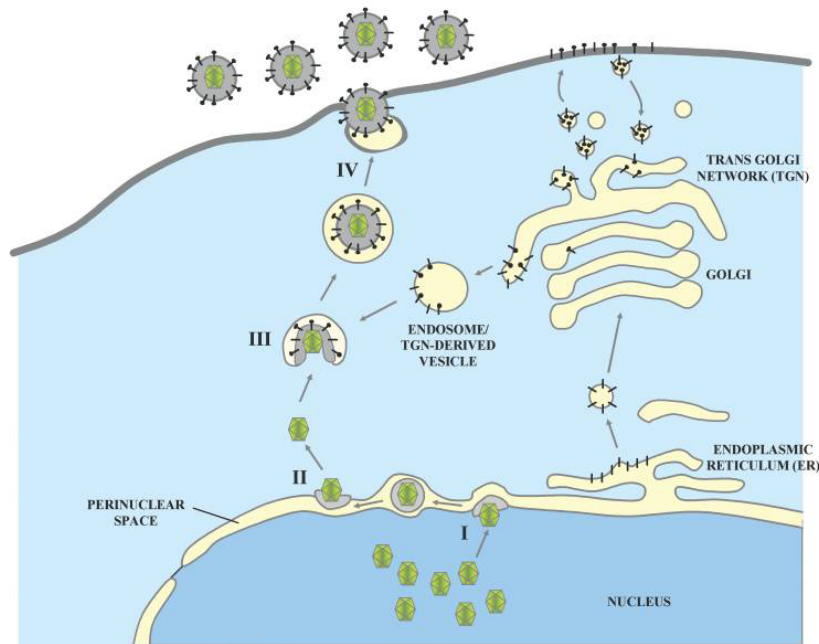


Figure 1.6: Schematic of HSV-1 virion morphogenesis and egress in the de-envelopment/re-envelopment model. (I) Mature capsids bud through the inner nuclear membrane into the perinuclear space. (II) De-envelopment of perinuclear virions at the outer nuclear membrane. (III) Re-envelopment of cytoplasmic capsids by budding into Trans-Golgi-Network derived cytoplasmic vesicles. (IV) Final egress to the extracellular space. Figure from (Melancon, Luna et al. 2005).

Latent Infection

Latent infection is more of an alternate pathway in the virus life cycle rather than a stage of the life cycle. The signals that push a herpes virus into a latent infection rather than a typical lytic infection are poorly characterized, however, latency and reactivation are well-documented phenomena clinically (Roizman, Knipe et al. 2007). In herpes viruses, latent infection generally follows a primary infection and spread in some tissue type with latency occurring in a secondary tissue. Herpesviruses tend to favor latency in one or a few cell types depending on the family of herpesvirus. For HSV-1 primary

infection is generally in a mucocutaneous epithelial tissue with the latent infection occurring in the sensory ganglionic neurons that innervate this tissue. This is followed by periodic reactivations and subsequent reoccurrence of clinical symptoms at the tissues of initial infection. Latency in HSV is characterized by the viral DNA being held in the host-cell nucleus, in a circular episome form, associated with the nucleosomes and near total silencing of gene expression. The latency-associated transcripts (LATs) are expressed giving multiple RNA species through splicing (Roizman, Knipe et al. 2007). Reactivation generally occurs following some type of neuronal or primary infected tissue stress; including UV radiation, intense heat, trauma or others, or a systemic stress and/or immunocompromise to the individual infected. Once this occurs, the infection cycle follows in a manner similar to that described above. Latency will be covered in greater detail later in this chapter in sections pertaining to neuronal infection.

HSV Glycoproteins and Their Putative Functions

Herpes simplex virus type-1 encodes and uses at least eleven virally encoded glycoproteins and possibly a twelfth in the mature, wild type virion: gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM, and potentially gN (UL49.5). HSV-1 also has multiple non-glycosylated, but still membrane associated proteins: pUL20, pUS9, pUL24, pUL43, and pUL34. Herpesvirus membrane and membrane-associated proteins function in multiple important roles; virus fusion to a potential host cell, virus entry via host cell membrane fusion, virion morphogenesis, egress from the nucleus and infected cell, cell-to-cell spread and virus-induced fusion of host organism cells (Roizman and Knipe 2001b; Mettenleiter 2002b; Mettenleiter 2002a; Spear and Longnecker 2003;

Spear 2004b). At least 11 membrane proteins are present on the surface of the virion envelope (Roizman, Knipe et al. 2007). These proteins are vitally important in the virus life-cycle as they are the first component of the virus that interacts with the host cell that will be infected and can shape the interactions available to the virus both in the already infected cell (egress, morphogenesis, and immune interactions) and the prospectively infected cell (i.e.: cell tropism, virus-cell fusion and entry).

Glycoprotein B (pUL27)

Glycoprotein B (gB) is the product of the HSV-1 UL27 gene. This gene encodes a 904 amino acid (aa) protein that exists in its mature form in the viral envelope as a homotrimer. This glycoprotein has a single cytoplasmic domain of 696 aa that has multiple N-glycosylation sites, a short, 69 aa transmembrane domain and a 109 aa carboxyterminal cellular domain (Claesson-Welsh and Spear 1986; Claesson-Welsh and Spear 1987; Highlander, Goins et al. 1991; Spear 1993; Laquerre, Person et al. 1996; Whitley 2001a). Resolution of the crystal structure has shown that it possesses features of class I, in the form of an α -helical coiled-coil core, and class II, in the form of extended β hairpins with hydrophobic tips, viral fusion proteins (Heldwein, Lou et al. 2006). The β hairpin structures are homologous to the Vesicular stomatitis virus (VSV) G fusion protein and studies have shown that the hydrophobic tips of the loops are vital for HSV-1 gB function in fusion (Hannah, Heldwein et al. 2007). Glycoprotein B is highly conserved among many herpesviruses and in HSV-1 this protein plays an important role in virus induced cell fusion, as the suspected primary virus-cell membrane interactor (Spear 2001; Spear 2004b). HSV-1 deletion mutants of gB cannot enter cells

(Cai, Person et al. 1987). The role of gB in membrane fusion can also be demonstrated by amino acid substitution or truncation mutations of the carboxyl terminus that result in extensive fusion (Bzik, Fox et al. 1984; Cai, Gu et al. 1988; Cai, Person et al. 1988; Baghian, Huang et al. 1993; Gage, Levine et al. 1993).

Glycoprotein B is part of the four glycoprotein group, gB, gD, gH, and gL, necessary for cell fusion in transient co-expression assays and carboxyl truncations of gB result in extensive fusion in this system (Foster, Melancon et al. 2001; Haan, Lee et al. 2001; Klupp, Fuchs et al. 2002; Pertel 2002). These proteins appear to operate in a sequence with gD in Phase I, gH/gL in Phase II, and gB in Phase III of fusion (Gianni, Fato et al. 2006; Subramanian and Geraghty 2007).

Glycoprotein C (pUL44)

Glycoprotein C (gC) is the product of the HSV-1 UL44 gene. This gene encodes a 511 aa protein that is a precursor of gC (Frink, Eisenberg et al. 1983). This protein has a single 453 aa extracellular domain, with a 23 aa transmembrane domain and a short cytoplasmic tail of 10 aa (Homa, Purifoy et al. 1986). In the mature form, found in the envelope, gC has heavy N-linked and O-linked glycosylation (Wenske and Courtney 1983). While not necessary for infection, gC increases efficiency of infection and can be found in over 1000 copies on the virus surface (Ruyechan, Morse et al. 1979).

Glycoprotein C can cause initial binding of the virus to host cell heparan sulfate at the N-terminal 120 aa binding site (Tal-Singer, Peng et al. 1995). HSV-1 gC can increase binding by nearly 10-fold to host cells. HSV-1 gC also contributes to the viral infection, in the host, by binding to the complement component C3b (Friedman, Cohen et al. 1984;

Eisenberg, Ponce de Leon et al. 1987; Huemer, Larcher et al. 1993; Huemer, Nowotny et al. 1995). This can function to protect the virus from the effects of complement mediated neutralization (Lubinski, Wang et al. 1998; Lubinski, Wang et al. 1999). This is an important virulence factor in the natural infection and gC deficient mutants can demonstrate 5000 fold titer loss due to the rapid inactivation from complement (Kotwal, Isaacs et al. 1990; Isaacs, Kotwal et al. 1992; Friedman, Wang et al. 1996; Sahu, Isaacs et al. 1998). Glycoprotein C also carries a second domain that blocks the complement component C5 from binding to C3 providing a second block on the potential complement neutralization of HSV (Roizman, Knipe et al. 2007).

Glycoprotein D (pUS6)

Glycoprotein D (gD) is the product of the HSV-1 US6 gene. This gene encodes a 394 aa multifunctional protein. Glycoprotein D has a 315 aa extracellular domain with three sites for glycosylation (Watson, Weis et al. 1982; McGeoch, Dolan et al. 1985). This protein also has short transmembrane anchoring, 22 aa, and cytoplasmic, 32 aa, domains (Minson, Hodgman et al. 1986). This glycoprotein is very important to attachment, and by extension, and fusion/entry of the virus, serving as a ligand for all known cellular entry receptors, including HVEM, nectin-1, nectin-2 and others (Spear, Eisenberg et al. 2000). This protein is necessary for virus entry and cell-to-cell fusion. At the extracellular N-terminus of the protein, there is an immunoglobulin like hairpin loop that contains the binding sites for all entry receptors other than nectin-1 and this small area also determines the ability to bind to nectin-2 (Carfi, Willis et al. 2001; Spear and Longnecker 2003; Yoon, Zago et al. 2003; Zago and Spear 2003). Potential host cells

that express gD are also resistant to infection by initiating endocytosis and degradation of the virus; however gD expression alone in some cells can cause a pH-dependent cellular fusion (Campadelli-Fiume, Arsenakis et al. 1988; Campadelli-Fiume, Avitabile et al. 1988; Johnson, Burke et al. 1990).

Glycoproteins E (pUS8) and I (pUS7)

Glycoprotein E (gE) and glycoprotein I (gI) are the products of the HSV-1 US8 and US7 genes, respectively. The US8 gene encodes a 550 aa protein, while the US7 gene encodes a 390 aa protein (Lee, Para et al. 1982; Longnecker, Chatterjee et al. 1987). In the mature virion, these proteins are present in a heterodimer, containing one copy of each protein. This heterodimer can mediate cell-to-cell fusion in epithelial and neuronal tissues; however, neither is necessary for virus entry and infection (Johnson and Feenstra 1987; Mettenleiter, Schreurs et al. 1987; Neidhardt, Schroder et al. 1987; Johnson, Frame et al. 1988; Kudelova, Kostal et al. 1991; Card, Whealy et al. 1992b; Jacobs, Mulder et al. 1993a; Whealy, Card et al. 1993; Balan, Davis-Poynter et al. 1994a; Kritas, Pensaert et al. 1994; Mulder, Jacobs et al. 1994; Cohen and Nguyen 1997; Tirabassi, Townley et al. 1997; Dingwell and Johnson 1998; Kimura, Wang et al. 1998; Tirabassi and Enquist 1998). In very permissive cells that do not form tight cell junctions, the heterodimeric complex is not necessary for cell-to-cell spread (Mettenleiter, Zsak et al. 1987; Zsak, Zuckermann et al. 1992; Dingwell, Brunetti et al. 1994; Dingwell, Doering et al. 1995; Tirabassi, Townley et al. 1997; Wisner, Brunetti et al. 2000). Furthermore, this complex is not necessary for infection of the apical surface of a cell by extracellular virus (Mettenleiter, Zsak et al. 1987; Dingwell, Brunetti et al. 1994). However, this complex is

necessary for spread in synaptically connected neurons (Whealy, Card et al. 1993; Mulder, Jacobs et al. 1994; Dingwell, Doering et al. 1995; Babic, Klupp et al. 1996b; Tirabassi, Townley et al. 1997; Tirabassi and Enquist 1998).

This protein complex appears to be specifically designed to assist in cell-to-cell spread at host cellular junctions. Viruses with the wild-type gE/gI complex preferentially sort daughter virions to the lateral cell surfaces and cell junctions of infected cells, while gE deletion mutants allow the accumulation of increased numbers of daughter virions in the supernatant, infected cell cytoplasm, and infected cell apical surface (Alconada, Bauer et al. 1996; Kimura, Wang et al. 1998).

The gE/gI complex also has a high affinity for the Fc portion of immunoglobulin G, which suggests a host immune evasion component to this complex. In the natural infection, this serves as a virulence factor, as this can disrupt antibody-dependent cellular cytotoxicity and prevent immune targeting of the virus or infected cell (Eberle, Dubreuil et al. 1995).

Glycoprotein G (pUS4)

Glycoprotein G (gG) is the product of the HSV-1 US4 gene. The US4 gene encodes a 283 aa protein. The function of gG is not precisely known, however, this glycoprotein has been shown to be non-essential for viral entry or infection. No phenotypic abnormalities are recognized in single gG deletion mutants in culture and the absence of gG only causes minimal attenuation of the virus in vivo (Atkinson, Barr et al. 1978). Using polarized cells in culture and in vivo infection of corneal epithelial cells, the gC binding to these cells was defective in gG deletion mutant viruses (Tran, Kissner

et al. 2000). It is possible that this protein is involved marginally or has redundant functions to another glycoprotein in assisting the binding and entry of the virus at the site of primary infection. Also, during the infection gG is incorporated in the nuclear and cytoplasmic membranes, suggesting a possible role for gG in nuclear egress, however deletion mutants are not defective for nuclear egress, suggesting a possible redundancy in this instance as well (Frame, Marsden et al. 1986; Sullivan and Smith 1987).

Glycoproteins H (pUL22) and L (pUL1)

Glycoprotein H (gH) and glycoprotein L (gL) are the products of the HSV-1 UL22 and UL1 genes, respectively. The UL22 gene encodes an 838 aa protein, while the UL1 gene encodes a 224 aa protein (Roizman, Knipe et al. 2007). Glycoprotein H has a singular 785 aa extracellular domain with 21 aa transmembrane domain and a short, 14 aa, C-terminal cytoplasmic tail. In the mature virion, these glycoproteins exist as a heterodimer. Since this complex is essential for viral entry and infection, both of these glycoproteins are essential (Roizman, Knipe et al. 2007).

Glycoprotein L has a unique form in the mature virion, in that it is not embedded in the membrane, but instead is dependent on the interaction with gH for incorporation into the virion (Dubin and Jiang 1995). In fact, gH appears similarly dependent on gL for its function since gH does not fold or process correctly in the absence of gL and gH remains in the endoplasmic reticulum and self-aggregates in cells only transfected with gH (Foa-Tomasi, Avitabile et al. 1991; Forrester, Sullivan et al. 1991; Roberts, Ponce de Leon et al. 1991). Furthermore, in cells infected with gL-null virus or gH-null virus, the alternate, or present protein, is in either case incorrectly processed and absent from the

viral envelope (Hutchinson, Browne et al. 1992; Roop, Hutchinson et al. 1993). The formation of the heterodimer requires no other viral proteins, suggesting a likely self-assembly or possibly the assistance of cellular components, however, gL is secreted into the extracellular fluid since it has no membrane targeting sequence (Roop, Hutchinson et al. 1993; Dubin and Jiang 1995).

Viruses lacking gH/gL are unable to enter cells, however, they can attach to the cell surface and are essential proteins. Therefore the gH/gL complex appears to be essential for later virus-cell membrane fusion and virus entry into cells.

Glycoprotein J (pUS5)

Glycoprotein J (gJ) is the product of the HSV-1 US5 gene. The US5 gene encodes a 92 aa protein with a predicted signal peptide and a transmembrane domain (Ghiasi, Nesburn et al. 1998). The precise function of gJ for the virus is not well known. Deletion of gJ causes no observable changes in virus growth or phenotype *in vivo* or *in vitro* and gJ deletion does not impair replication at the primary infection site or progression to the nervous system in mice (Balan, Davis-Poynter et al. 1994a). In viruses without gD or gJ, apoptosis of infected cells was not blocked as it would normally be in an infected cell (Roizman, Knipe et al. 2007). The UL27.5 gene encodes a protein that cross reacts to antibodies to gJ (Chang, Menotti et al. 1998). This protein accumulates in the cytoplasm of infected cells but its function is also uncertain (Roizman, Knipe et al. 2007). It is possible that these two proteins have a redundant function.

Glycoprotein K (pUL53)

Glycoprotein K (gK) is the product of the HSV-1 UL53 gene. The UL53 gene consists of 1017 bases and encodes the 338 aa protein precursor to gK (Debroy, Pederson et al. 1985; Pertel and Spear 1996). Glycoprotein K has two sites for N-linked mannose glycosylation and is extremely hydrophobic (Hutchinson, Goldsmith et al. 1992; Ramaswamy and Holland 1992). Glycoprotein K has been plagued with difficulty in determining its form and function.

Previous experiments have suggested two forms for gK. The predicted forms were a protein with four membrane-spanning domains with the N and C termini on the extracellular side of the membrane or a protein with three membrane-spanning domains and the C terminus located intracellularly (Debroy, Pederson et al. 1985; Ramaswamy and Holland 1992; Mo and Holland 1997). More recent experiments in this laboratory, Kousoulas laboratory LSU-SVM, have confirmed the four membrane spanning form of the protein as the overwhelmingly likely mature form of the protein (Fig 1.7) (Foster, Alvarez et al. 2003).

Originally, gK was determined to be necessary for viral replication in vivo, however, that has since been shown to be incorrect although gK null viruses have a greatly reduced ability to reproduce, due in large part to spread defects (MacLean, Efstathiou et al. 1991; David, Baghian et al. 2008). One attempt at producing a gK deletion mutant actually produced an insertion-deletion mutant with the N-terminal 112

aa of gK retained. The result was a syncytial phenotype with many aberrant particles and naked capsids trapped in the cytoplasm, which lead to the assumption that gK was a preventer of abundant syncytia formation (Hutchinson and Johnson 1995;

Hutchinson, Roop-Beauchamp et al. 1995). Later, a more precise deletion of gK provided an actual gK null virus. This virus displayed a phenotype of severely impaired egress, however, syncytia formation was not a noticeable feature of the gK-null phenotype (Jayachandra, Baghian et al. 1997; Foster, Melancon et al. 2004a; Melancon, Luna et al. 2005).

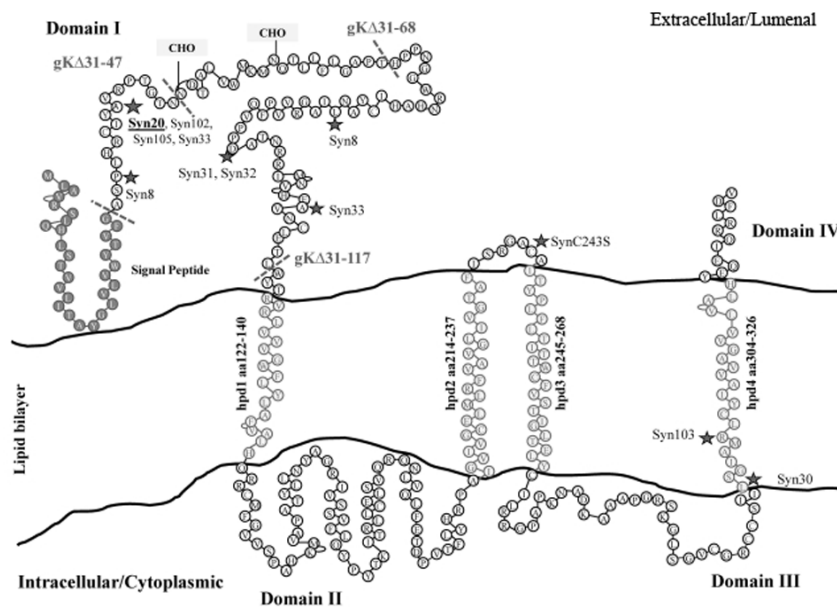


Figure 1.8: Schematic of the expected structure of gK situated in the membrane. Schematic shows locations of discrete domains and many characterized mutations. Figure and mutation characteristics from (Dolter, Ramaswamy et al. 1994; Foster, Kraus et al. 2003; Chouljenko, Iyer et al. 2009)

Some syncytial mutations are present in the non-glycosylated membrane protein UL20 (Baines, Ward et al. 1991; MacLean, Efstathiou et al. 1991), the membrane protein glycoprotein B (Bzik, Fox et al. 1984; Pellett, Kousoulas et al. 1985) and UL24 (Sanders, Wilkie et al. 1982; Jacobson, Martin et al. 1989) however, the vast majority of syncytial

mutations (especially those involving a minimum of deletions or substitutions) are contained within gK (Ruyechan, Morse et al. 1979; Bond and Person 1984; Pogue-Geile, Lee et al. 1984; Debroy, Pederson et al. 1985). The phenotype of syncytia formation would strongly indicate the presence of gK in the membrane as either a binding protein or a modulator of the primary fusion protein, gB.

A study of gK localization in the infected cell suggested that gK was located only in the perinuclear space and only contained unprocessed carbohydrates, compatible with a protein that does not pass through a Golgi-mediated maturation process or reach the surface of the cell membrane (Hutchinson, Roop-Beauchamp et al. 1995). Many of the earlier studies of the function of gK have occurred in transiently transfected cells and the phenotype observed for mutations of gK are aberrant, with gK retained in the perinuclear space or golgi, unless pUL20 is also transfected (Dietz, Klupp et al. 2000). It was later shown that pUL20 and gK are dependent on one another for transport through the Golgi network in a manner typical of wild-type virus (Fig 1.8) (Foster, Melancon et al. 2004b; Melancon, Fulmer et al. 2007). As shown for PRV and later HSV, gK appears to rely on the cotransfection of UL20 to restore processing of both proteins and to display their true phenotype (Foster, Alvarez et al. 2003). The interactions between gK and gB were later confirmed by the Kousoulas laboratory, showing that the amino terminus of gK, particularly amino acids 31-68, modulates gB mediated, virus-induced cell fusion and virion egress (Melancon, Luna et al. 2005; Chouljenko, Iyer et al. 2009).

Later glycoprotein K was shown to form a functional protein complex with the non-glycosylated membrane protein UL20 that interacted physically with gB (Chouljenko, Iyer et al. 2010). Furthermore, cleavage of the amino terminus of gK has

also been shown to prevent viral entry into otherwise permissible cells (Jambunathan, Chowdhury et al. 2011). Glycoprotein K along with its membrane partner pUL20 have been demonstrated to participate in a hierarchical relationship with a protein complex of the viral envelope that affects virion egress and envelopment (Chouljenko, Kim et al. 2012). It appears that gK and UL20 are intricately linked in the maturation and development of the virus and gK appears to be involved in many aspects of the virus life-cycle including; envelopment, morphogenesis, entry and cell fusion.

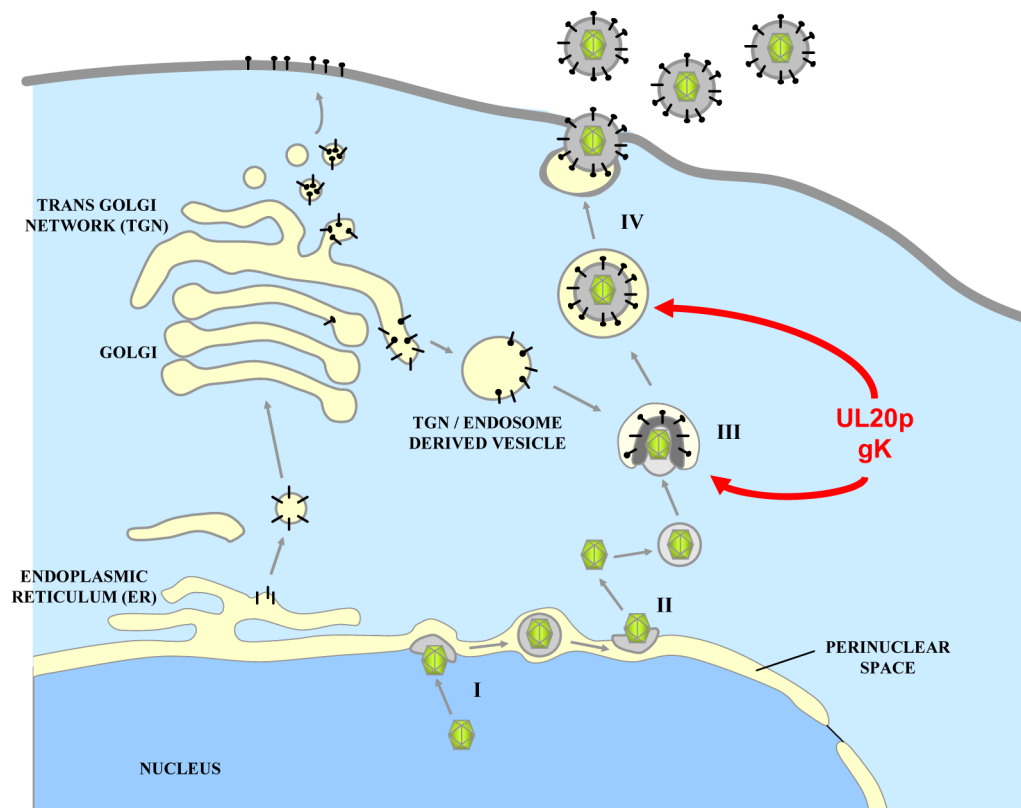


Figure 1.8: Schematic of HSV-1 virion morphogenesis and egress highlighting the areas of gK and UL20 function. Red arrows indicate the stages where gK and UL20 appear to function during egress. Figure from (Melancon 2003).

Glycoproteins M (pUL10) and N (pUL49.5)

Glycoprotein M (gM) and glycoprotein N (gN) are the products of the HSV-1 UL10 and UL49.5 (or UL49A) genes, respectively. The UL10 gene encodes a 473 aa protein, while the UL49.5 gene encodes a 91 aa protein (Baines and Roizman 1993). In the mature virion, gM is an integral membrane protein with eight predicted, hydrophobic transmembrane domains (Baines and Roizman 1993; MacLean, Robertson et al. 1993; Babic, Klupp et al. 1996b). In cell culture, gM deficient viruses replicated at 10 to 20 fold lower titers with smaller plaque morphology than wild-type (MacLean, Efstathiou et al. 1991; MacLean, Robertson et al. 1993). For gN, there are potential sites of O-linked glycosylation and for the homolog of gN in Pseudorabies (PRV), an α -herpesvirus related to HSV, the protein has been shown to be glycosylated in the mature virion (Jons, Granzow et al. 1996).

For HSV, gN has been shown to be present in the virion and linked to the tegument, however, it has not been shown to be glycosylated (Adams, Cunningham et al. 1998). Deletion of gN has shown that this protein is not essential for virus infection or replication, with only a two-fold reduction in titers and no phenotypic plaque differences in null mutants (Adams, Cunningham et al. 1998). Interestingly though neither protein is necessary for the life cycle of the virus, both proteins are highly conserved though the alpha-, beta-, and gammaherpesviruses and in many of these they form a dimeric complex (Jons, Dijkstra et al. 1998; Lake, Molesworth et al. 1998; Wu, Zhu et al. 1998; Mach, Kropff et al. 2000; Koyano, Mar et al. 2003). This may indicate redundancy or partial redundancy in the functions of these proteins, requiring knock-out of multiple proteins to determine their roles in the virus (Browne, Bell et al. 2004).

While the exact mechanism of action is unknown, it appears that gM and gN work together to inhibit virus induced cell fusion. It also appears that these proteins must work together to cause this phenotype. In cellular fusion assays, gM and gN cause a decrease in cell-cell fusion in experiments with the following: PRV gB, gD, and gH/gL, bovine respiratory syncytial virus F protein, or with HSV-1 gB, gD, and gH/gL or Moloney murine leukemia virus envelope protein. In these studies, the transfection of both gM and gN reduced fusion while the transfection of only gM did not (Klupp, Nixdorf et al. 2000; Koyano, Mar et al. 2003). This phenotype has even been shown to occur with human herpesvirus 8 (Koyano, Mar et al. 2003).

Glycoprotein M has also been shown to be located in the nuclear membranes and to be added to the viral particle as it passes through the nuclear membranes, possibly indicating a role for gM in nuclear egress and/or increasing pathogenesis of virions released during cell lysis, or those that do not undergo full transport through the Golgi network (Baines, Wills et al. 2007).

Membrane Protein UL20 (pUL20)

The UL20 protein is a membrane protein of HSV-1 that is not glycosylated. The ORF for this protein sits between the UL19 and UL20.5 genes and the expressed form is situated on the complementary strand of the viral genome giving a 222 aa protein. The UL20 protein was originally suspected to have two or three membrane spanning domains, however, it was more recently predicted to have four membrane spanning domains with the amino and carboxyl termini located cytoplasmically (McGeoch, Dalrymple et al. 1988a; McGeoch, Dalrymple et al. 1988b; Hofmann, James et al. 1993; Hirokawa, Boon-

Chieng et al. 1998). Later this form of the protein in the mature virion was partially confirmed at the same time that the dependence on UL20 for gK for proper trafficking through the cell and maturation was established (Foster, Melancon et al. 2004a; Melancon, Foster et al. 2004; Melancon, Fulmer et al. 2007). The interactions of gK and pUL20 were covered in more detail in the above section on gK; the involvement of pUL20 will be covered more briefly in this section.

During infection, gK and pUL20 are interdependent for proper processing and localization of the proteins. The lack of either of these proteins causes the entrapment of the other in the endoplasmic reticulum, while expression of both proteins together results in these proteins being present in the TGN, cell membrane and on the surface of released mature virions (Foster, Melancon et al. 2004b; Melancon, Fulmer et al. 2007). Furthermore, these proteins generally colocalize in these areas, suggesting a functional and physical linkage for these proteins (Foster, Melancon et al. 2004b; Melancon, Fulmer et al. 2007). In the virus life-cycle, pUL20 appears to regulate virus induced fusion since syncytia can be prevented in viruses with syncytial mutations in gB or gK if pUL20 is not also expressed (Foster, Melancon et al. 2004b; Melancon, Fulmer et al. 2007).

Epidemiology of Herpes Simplex Virus

Typical infection of a healthy, not immunosuppressed, individual extremely rarely results in a primarily fatal infection or contributes to a secondary infection that results in death. This course of infection more typically results in a short duration of mucocutaneous lesions, generally around the mouth, with latent disease established in the trigeminal ganglia and lifetime recurrences with periods of shedding. These factors along

with the age of the virus, likely a human (or human predecessor) pathogen for at least 8-10 million, contribute to the distribution of HSV-1 as endemic in developed and underdeveloped countries worldwide (Black, Hierholzer et al. 1974; Black 1975; McGeoch, Dolan et al. 1985; Gentry, Rana et al. 1988). Infection with HSV requires intimate contact of mucosal surfaces or damaged skin in a susceptible individual with someone who is shedding virus. Being an enveloped virus, HSV usually must be transmitted directly or with little exposure to the environment. Enveloped viruses are generally more susceptible to degradation of their infectiousness by interactions with the environment (e.g.: UV radiation, dehydration, lipids, solvents, etc). Although some new and old world monkeys can be infected with HSV-1 and some animals can be experimentally infected, there are no known animal reservoirs or carriers of the virus. There have, however, been reports of new world primates being naturally infected; however, these are rapidly severe courses of disease and have not been established as a true reservoir or reliable source of infection back into the human population (Costa, Luppi et al. 2011).

New world primates are far more susceptible to HSV-1 infection than old world primates. Reports of infection in primates, such as the gorilla (*Gorilla gorilla*), chimpanzee (*Pan troglodytes*), bonobo (*Pan paniscus*), and white-handed gibbon (*Hylobates lar*), indicate that the infection in old world primates usually follows the typical course of infection similar to that found in immunocompetent humans with lesions remaining on mucocutaneous tissues and not progressing to systemic infection (Matz-Rensing, Jentsch et al. 2003). In new world primates and prosimians, the infection is more similar to an aggressive infection found in immunocompromised humans

(Schrenzel, Osborn et al. 2003). Although old world primates are generally less susceptible, fatal systemic infections have been reported in the following new and old world primates: marmosets (*Callithrix penicillata*), white-faced saki (*Pithecia pithecia*), the common marmoset (*Callithrix jacchus*), the white-handed gibbon (*Hylobates lar*) and the orangutan (*Pongo pygmaeus*) (Huemer, Larcher et al. 2002; Schmutzhard, Merete Riedel et al. 2004; Kik, Bos et al. 2005; Landolfi, Wellehan et al. 2005). Most infections in primates have occurred in pet, zoo, or research settings where primates have a greater risk of exposure to a shedding human, however, naturally occurring infections can occur in wild populations and are suspected to be the result of exposure to contaminated food (Bruno, Liebhold et al. 1997; Juan-Salles, Ramos-Vara et al. 1997; Huemer, Larcher et al. 2002; Matz-Rensing, Jentsch et al. 2003; Lefaux, Duprez et al. 2004; Costa, Luppi et al. 2011). For HSV-1, humans are considered the reservoir for primate infections and due to the nature of transmission, primate transmission to a human is extremely unlikely and therefore, humans are considered the sole source of infection for other humans (Whitley and Roizman 2001).

Mucocutaneous Infection

The frequency and duration of contact contributes significantly to the chance of infection for a person (Whitley and Miller 2001). HSV-1 infection most often occurs by 5 years of age and is often fairly asymptomatic. The most common site of primary infection is the mucosa of the lips and mucocutaneous junction around the mouth (Whitley and Miller 2001; Whitley and Roizman 2001). After infection, virus is shed from the mouth and feces for 7-10 days after infection (Amir, Nussinovitch et al. 1997).

Fecal shedding is likely due to swallowed virus, as replication of HSV-1 in the epithelium of the digestive tract has not been reported as a prominent feature of the virus. By 4 to 7 days after neutralizing antibodies can be detected in the serum and these antibodies peak at about 3 weeks after infection (Buddingh, Schrum et al. 1953). Dissemination of HSV is often correlated with socioeconomic status with in some underdeveloped countries having as much as 95% seroprevalence by age 15 (Black, Hierholzer et al. 1974; Bader, Crumpacker et al. 1978). By 10 years of age the estimated seroprevalence in the United States is about 50% and about 70% in Europe and at 60 years of age both populations are at 70% to 85% depending on the study (Nahmias, Lee et al. 1990; Looker and Garnett 2005; Fatahzadeh and Schwartz 2007).

Due to the nature of HSV-1 being primarily acquired orally and HSV-2 primarily acquired genitally, seroprevalence to HSV-1 is found in greater numbers and at an earlier age than HSV-2, in which the seroprevalence increases after the age of the onset of sexual activity (Nahmias, Lee et al. 1990; Fleming, McQuillan et al. 1997). While HSV-1 is usually an oral infection and HSV-2 is a genital infection, there are increasing reports of both viruses at the alternate sites, however, genital infections with HSV-1 tend to be less severe and have a lower frequency of recurrence (Kalinyak, Fleagle et al. 1977; Reeves, Corey et al. 1981; Corey, Adams et al. 1983).

The hallmark of HSV-lifecycle is infection of sensory neurons where the virus establishes a latent infection for the life of the host (Whitley, Kimberlin et al. 1998a). However, alternate forms of a herpes simplex infection include: neonatal infection, disseminated infection, herpes gladiatorum, encephalitic infection, and ocular infection.

Neonatal Infection

Neonatal infections with HSV occur at a rate of 1 in 3000 deliveries and 1500 cases per year in the United States (Nahmias 1983; Nahmias 1989; Brown, Wald et al. 2003). Neonatal infections may occur via in utero, intrapartum or postnatally with intrapartum the most common and postnatal infection occurring in 10% of cases (Whitley 1994; Fatahzadeh and Schwartz 2007). Congenital infection with herpes simplex is rare and often results in myriad developmental abnormalities in the brain (Jones 1996; Whitley and Roizman 2001). Perinatal infection is much more common and may result in isolated disease and clearance or systemic disease and encephalitis (NoAuthor 1998; Fatahzadeh and Schwartz 2007). Neonatal herpes was shown to involve the skin, eye, and mouth in approximately 42% of cases and generally a treated infant will resolve the disease and recover normally (Whitley, Corey et al. 1988; Kimberlin, Lin et al. 2001).

Neonatal infections that result in encephalitis and/or disseminated disease and are often fatal with disseminated disease reaching 60% mortality even in treated infants (Fatahzadeh and Schwartz 2007). In the case of neonatal herpes encephalitis, mortality is lower than for disseminated disease, but the brain develops normally in less than half of these infants following recovery (Thompson and Whitley 2011).

Disseminated Infection

Disseminated herpes simplex infection is the result of either neonatal infection or immunosuppression in an adult. The immunosuppressed state has a greater risk for virus reactivation with aberrant spread of the virus to the internal organs including; the brain, liver or lungs (Ramsey, Fife et al. 1982). The clinical significance of this disseminated

spread is severe and the disease can be fatal even with therapy (Meyers, Flournoy et al. 1980; Siegal, Lopez et al. 1981; Ramsey, Fife et al. 1982).

Herpes Gladiatorum

Herpes gladiatorum is infection through damaged or abraded skin in a location that is not the typical mucocutaneous exposure (Fatahzadeh and Schwartz 2007). The incidence of this type of herpes infection is directly related to the potential for exposure in a shedding individual, with wrestlers, or athletes of similar unprotected contact sports, being especially susceptible (Fatahzadeh and Schwartz 2007).

Herpetic Encephalitis

The encephalitic form of disease with herpes simplex viruses is more attributable to the state of the patient rather than the strain of the virus. When compared to mucocutaneous infection, herpes simplex encephalitis is a rare event in the immunocompetent person, however, in neonates the primary infection may result in encephalitis and in immunocompromised adolescents and adults, the primary or recurrent infection can result in encephalitis (Fatahzadeh and Schwartz 2007). While rare in the healthy adult, herpes simplex encephalitis is still a significant cause of encephalitis in humans, as the most common cause of infectious encephalitis world wide and responsible for over 10% of the 7.3 million hospitalizations for encephalitis from 1979-1988 in the US (Stahl, Mailles et al. 2011).

HSV encephalitis usually causes fever, headache, neurologic deficits, and personality and cognitive disorders and has a 70% fatality rate when untreated (Wald and

Corey 2007). When untreated, surviving patients rarely return to a fully normal neurologic state and even some treated patients have persistent neurologic deficits (Wald and Corey 2007). HSV or inflammation resulting from HSV has also been implicated in the development of Alzheimer's disease (Honjo, van Reekum et al. 2009).

Ocular Infection

Whether through primary ocular infection, such as may occur with herpes gladiatorum or intimate contact, or recurrence resulting in spread through the trigeminal ganglia, HSV may cause ocular disease as well. Ocular HSV is one of the most common infectious causes of blindness in developed countries, with estimates of the incidence of herpes ocular disease ranging from 4.1 to 20.7 cases per 100,000 in developed countries (Liesegang, Melton et al. 1989b; Liesegang 2001). An estimated 400,000 people in the United States have had herpes simplex ocular disease, with about 50,000 new or recurrent cases occurring yearly (NoAuthor 1998).

Pathogenesis of Herpes Simplex Virus

Herpes simplex virus is an enveloped, double-stranded DNA virus that must infect a target cell and replicate in that cell's nucleus to complete its life cycle. Other than research settings, HSV is nearly entirely a human pathogen, with person-to-person contact responsible for the spread of the virus (Whitley 2001a). Herpes simplex virus is unable to penetrate heavily keratinized layers of the skin, therefore, initial infection occurs at mucous membranes, such as the lips or oral cavity, or at areas of abraded skin for HSV-1 and generally the genital tissues for HSV-2 (Roizman, Knipe et al. 2007). For

HSV-1, the initial infection and first round of virus replication typically occurs in the orolabial mucosa (Roizman, Knipe et al. 2007). After this initial round of infection, the virus spreads in subsequent rounds of infection, through this tissue and into sensory neurons innervating the infected tissue. For HSV-2 this is the dorsal root ganglia in the sacral spinal area and for HSV-1 this is typically in the trigeminal ganglia (Bastian, Rabson et al. 1972; Baringer and Swoveland 1973; Whitley 2001a).

The most common clinical form of the disease consists of a short to moderate duration of lesions at the initial site of infection, possibly with fever and generalized symptoms. Following this, the virus having infected sensory neurons, establishes a latent infection for the life of the host that results in periodic recurrences at or near the site of initial infection with generally reduced severity from the initial infection (Bastian, Rabson et al. 1972; Baringer and Swoveland 1973; Roizman, Knipe et al. 2007).

Alternate, and less common, sequelae to the initial infection include the development of ocular disease, disseminated disease and neurologic disease. With the exception of those arising from neonatal infections; disseminated, neurologic, and ocular disease are more common for HSV-1 than HSV-2 (Roizman, Knipe et al. 2007). Individuals with reduced immune capability, such as neonates, people concurrently infected with HIV, or people with other causes of immune suppression are at greater risk for these alternate manifestations of the infection (Fatahzadeh and Schwartz 2007).

At the site of initial mucosal infection, infected cells exhibit enlargement of the cell with condensation of the chromatin in the nucleus. Sometimes cellular chromatin is visibly moved to the periphery of the nucleus with formation of intranuclear inclusion bodies (Whitley 2001a). Once the cell goes fully into a lytic phase, there is

degradation and fragmentation of the nucleus and disruption of the plasma membrane. Occasionally cells will fuse the plasma membranes forming multinucleated giant cells (Pugh, Dudgeon et al. 1955; Wheeler and Abele 1966; Terezhalmay, Tyler et al. 1979). In the infected dermis there is an intense inflammatory response that may be complicated by secondary bacterial or fungal opportunistic infection. Immune cellular response due to the erosion of the mucosal surface and surface bacteria and fungi is often mixed with polymorphonuclear cells, macrophages, lymphocytes and plasma cells (Pugh, Dudgeon et al. 1955; Wheeler and Abele 1966; Terezhalmay, Tyler et al. 1979). If infected neurons enter the lytic phase of infection, they appear similar to infected epithelial cells although multinucleate giant cells are very rare (Dudgeon 1969). Neurons that enter the latently infected state may appear normal. In neuronal tissues, the immune reaction is generally restricted to lymphocytes and plasma cells with some macrophages. In mucosal tissues, usually the intensity and duration of the cellular destruction and inflammation decreases with recurrent infections (Whitley 2001a).

Mucocutaneous Infection

As stated above, primary infection with HSV-1 usually occurs in the orolabial, glossal, gingival or buccal mucosa. This infection is often accompanied by one or more of the following symptoms: vesicular or ulcerative lesions, localized pain and edema, fever, and sore throat (Amir, Nussinovitch et al. 1997). The primary infection can frequently be asymptomatic or of insufficient clinical significance to be reported as well (Whitley and Roizman 2001). When symptomatic in children, the clinical course is usually 2 to 3 weeks long with temperatures ranging from 101 F to 104 F. In young

adults, the primary infection can produce pharyngitis at the initial stage of the oral infection and tonsillectomy is often the treatment of choice when this occurs (Langenberg, Corey et al. 1999). Occasionally the primary infection is complicated by dehydration, likely due to an unwillingness to drink due to pain, and secondary bacterial infection (Langenberg, Corey et al. 1999).

With HSV-1, recurrent disease is frequently more noticeable with marked pain, burning, tingling or itching as a prodromal event (Spruance, Overall et al. 1977; Spruance and Crumpacker 1982; Spruance 1984). These symptoms are usually short, six hours or less, and are followed by formation of vesicles and the typical cold sore within 24-48 hours (Spruance, Overall et al. 1977; Spruance and Crumpacker 1982; Spruance 1984). Pain is generally most intense at the beginning of the cold sore formation with the pain resolving over 4-5 days and lesions resolving in approximately 7 days (Segal, Katcher et al. 1974; Ship, Miller et al. 1977). Reactivation can occur for a number of reasons including: trauma to the local area of initial infection, fever, exposure to UV radiation and stress (Segal, Katcher et al. 1974; Ship, Miller et al. 1977; Spruance, Freeman et al. 1991). The frequency of reactivation varies greatly among individuals, with a study reporting that approximately 30% of seropositive individuals experience recurrent infections with 40% of these having at least one recurrence per year (Wald and Corey 2007). HSV-1 can also be detected on the orolabial mucosa of seropositive individuals even without clinical disease, suggesting that recurrence, shedding, and possibly transmission, can occur as an asymptomatic, subclinical, event (Koelle and Wald 2000; Fatahzadeh and Schwartz 2007).

In HSV-2 the primary infection usually occurs in the genital epithelial or mucosal tissues. Generally with HSV-2, the most severe symptoms are associated with the primary infection and are characterized by macules and papules that turn into pustules and ulcers with lesions and viral secretion lasting about three week (Corey 1982; Corey, Adams et al. 1983). It appears that previous exposure and immunity to HSV-1 may mitigate HSV-2 infection, by decreasing the severity of the primary infection (Kaufman, Gardner et al. 1973; Corey 1981; Allen and Rapp 1982).

Clinical recurrence in HSV-2, unlike HSV-1, is typically less severe than the initial infection with asymptomatic shedding or milder pain, the development of vesicles, and shedding of the virus lasting less than 10 days total (Adams, Benson et al. 1976). However, with HSV-2 recurrence seems to be more frequent with several recurrences occurring per year and the possibility of transmission during asymptomatic or symptomatic disease (Corey 1982; Corey, Adams et al. 1983).

Neonatal Infection

Neonatal HSV infections occur at a rate of approximately 1 in 3000 births with an estimated 1500 cases of neonatal HSV per year in the United States (Nahmias 1983; Nahmias 1989; Brown, Wald et al. 2003). Neonatal infections may occur in utero, intrapartum or postnatally with intrapartum the most common and postnatal infection occurring in 10% of cases (Whitley 1994; Fatahzadeh and Schwartz 2007). Congenital, in utero, infection is rare with HSV but is often severe when it occurs (Jones 1996; Whitley and Roizman 2001). In utero infection causes cutaneous lesions, scars and chorioretinitis and can cause multiple developmental abnormalities including:

microcephaly, lissencephaly, hydranencphaly and microphthalmia (Hutto, Arvin et al. 1987). Intrapartum infection accounts for 75-80% of cases (Whitley 2001a). HSV-2 is often the culprit in intrapartum infection, although HSV-1 infection may also occur, when the fetus is exposed to maternal genital secretions during delivery or in the post-natal period from the mother or other infected individuals (Whitley 2001a; Wald and Corey 2007).

In cases of herpes encephalitis in the neonate, HSV-1 usually has a better prognosis than HSV-2 (Toth, Harder et al. 2003). The risk of fetal infection is directly related to the type of maternal genital infection at the time of delivery, with a transmission risk of approximately 30% if the mother has a primary infection and 3% or less risk of transmission to the fetus if the mother has a recurrent infection at the time of delivery (Brown, Benedetti et al. 1991). Risk of fetal infection is increased in the intrapartum period with closer proximity of a primary maternal infection to the time of delivery and with increased disruption or trauma to the mucocutaneous tissues (Nahmias, Josey et al. 1971; Parvey and Ch'ien 1980; Brown, Wald et al. 2003). Risk of transmission is reduced with maternal seroconversion prior to delivery and with caesarean delivery (Brown, Wald et al. 2003; Johnston, Magaret et al. 2008).

HSV infection in newborns can result in disseminated disease, central nervous system disease, or cutaneous disease. The type of disease is a result of the site and extent of viral replication. Infection of the newborn may result in isolated cutaneous disease, systemic (disseminated) disease, or encephalitis (NoAuthor 1998; Fatahzadeh and Schwartz 2007). Neonatal herpes was shown to involve the skin, eye, and mouth in approximately 42% of cases and generally a treated infant will resolve the disease and

recover normally (Whitley, Corey et al. 1988; Kimberlin, Lin et al. 2001). Without antiviral therapy, 25% of infants with only cutaneous disease still had neurologic developmental delays (Whitley, Nahmias et al. 1980; Kimberlin, Lin et al. 2001). Neonatal infections resulting in disseminated disease or encephalitis are much more severe. Even with antiviral therapy, in one study there was 57% mortality for cases of disseminated disease and 15% mortality for cases of encephalitis in infants (Whitley, Arvin et al. 1991). In this same study none of the infants with cutaneous disease died and 94% developed normally neurologically (Whitley, Arvin et al. 1991). While mortality is less in cases of infant with encephalitis versus disseminated disease, there are developmental abnormalities in more than half of these infants following recovery (Thompson and Whitley 2011). In the previously mentioned study, one year following recovery 36% of infants that had encephalitis and 59% of the infants surviving disseminated disease were developing normally (Whitley, Arvin et al. 1991).

Infants with cutaneous or disseminated disease usually present within the first 10-12 days of life (Kimberlin, Lin et al. 2001). Cases of encephalitis in neonates usually present slightly later, at 2-3 weeks after birth (Kimberlin, Lin et al. 2001). Cutaneous vesicles may be seen with all presentations of neonatal HSV infection but are frequently resolved by the time of presentation with more severe disseminated or encephalitic disease (Kimberlin, Lin et al. 2001; Thompson and Whitley 2011). With central nervous system infection and disseminated disease the initial symptoms are often non-specific, including fever or fluctuating temperature, respiratory distress, lethargy, inappetance, hypotension, shock, disseminated intravascular coagulation, apnea, and shock (Thompson and Whitley 2011). Disseminated disease often involves many of the visceral organs

including lungs, liver, adrenal glands, skin, eyes, and often the brain (Thompson and Whitley 2011). Severe pneumonitis, hepatitis, and coagulopathy are common complications of disseminated disease in neonates (Thompson and Whitley 2011). Lesions in the central nervous system of infected neonates are similar to those described later for adults with the exception that brainstem involvement appears to be less common in the neonate (Chu, Kang et al. 2002; Pelligra, Lynch et al. 2007). In the cerebrospinal fluid, there is mild to moderate pleocytosis, consisting predominantly of mononuclear inflammatory cells and normal to decreased protein levels (Koskiniemi, Vaheri et al. 1980; Cinque, Cleator et al. 1996).

Ocular Infection

A less frequent, however, very serious manifestation of HSV infection is herpetic ocular disease. Ocular disease with herpes simplex virus is usually due to HSV-1. Even at a prevalence of 149 cases per 100,000 people per year, corresponding to less than 1% of people exposed to the virus, HSV is the leading infectious cause of blindness in the United States with an estimated 400,000 people in the United States with herpes simplex ocular disease and approximately 50,000 new or recurrent cases occurring yearly (Liesegang, Melton et al. 1989b; NoAuthor 1998; Liesegang 2001; Tullo 2003).

The ocular manifestation of HSV is usually a result of spread of primary disease through the branches of the trigeminal ganglion rather than primary infection of the ocular area (Tullo, Easty et al. 1982; Theil, Derfuss et al. 2003; Tullo 2003; Pepose, Keadle et al. 2006). While a primary ocular infection could result from mechanical transmission to the eye, ocular herpetic disease is usually the result of recurrent, or more

accurately multiple recurrences, following a primary infection in the normal route. Superinfection, or infection with a second strain after previous exposure to the virus and incomplete immune protection, may account for some of the cases of ocular disease and recent studies suggest that this phenomenon may be more common than previously thought (Remeijer, Maertzdorf et al. 2002). In either case, after superinfection or multiple recurrences or reactivation events, the tissues of the eye, including the cornea and adjacent epithelial tissues, become infected and subsequent reactivations can cause a progressive degeneration of the cornea eventually leading to blindness (NoAuthor 1998; Liesegang 2001).

More rare causes of herpetic ocular disease can occur with primary or recurrent infection that involves the innervation of alternate structures in the eye; e.g.: those structures innervated by a nerve that is not part of the trigeminal. Reactivation of latent HSV, possibly from earlier localized or CNS infection, from the ciliary or superior cervical ganglion or Edinger-Westphal-nucleus can result in herpes infection in the iris (Pepose, Leib et al. 1996; Labetoulle, Kucera et al. 2000). Furthermore, reactivation of HSV from the suprachiasmatic or paraventricular nuclei or any other infection/reactivation event causing encephalitis can result in retinitis or retinal necrosis and spread of infection to the surrounding tissues (Gentry, Lowe et al. 1988; Atherton 2001). Following this round of replication, the trigeminal ganglion, which innervates the sensory structures around the eye can be infected and contribute to later bouts of more traditional recurrent herpetic eye disease.

With recurrent bouts of herpes keratitis, the cornea can become opaque leading to blindness and sometimes the cornea is replaced with a corneal transplant. Herpes may

spread from the patient's latently infected trigeminal ganglia to the new cornea and can even cause primary graft failure (Cockerham, Krafft et al. 1997; De Kesel, Koppen et al. 2001; Tullo 2003). Less commonly, a person without herpes keratitis may receive a transplant cornea that is harboring latent or persistent HSV and following a flourishing infection in the transplant, the patients previously naïve trigeminal ganglia (Easty, Shimeld et al. 1987; Gordon, Romanowski et al. 1991; Kaye, Lynas et al. 1991). This has recently lead to investigations into the possibility of non-neuronal sources of latency for HSV (Farooq and Shukla 2011).

Primary infection or recurrent shedding causes herpetic ocular disease in one or both eyes. The lesions of ocular infection with HSV include: blepharitis, conjunctivitis, dendritic or epithelial keratitis (superficial infection of the cornea), stromal keratitis (deep infection of the cornea), anterior uveitis, iridocyclitis, and retinitis (NoAuthor 1998; Liesegang 2001). These conditions can cause blindness through progressive inflammation and opacity of the cornea or more directly through destruction of the retina (NoAuthor 1998; Liesegang 2001). On the surface of the cornea, the infection usually begins as focal point that spreads to a web-like (dendritic) pattern (Maudgal and Missotten 1978). Pathologically, on the surface of the cornea, there are erosions and ulcerations of the epithelium with necrosis and inflammation by polymorphonuclear inflammatory cells, lymphocytes and plasma cell, sometimes with syncytial cells and intranuclear inclusions (Maudgal and Missotten 1978; Metcalf and Reichert 1979). These surface lesions may also be complicated with secondary bacterial or fungal infections.

As the lesions progress deeper to stromal keratitis, there is inflammation of the corneal stroma by polymorphonuclear inflammatory cells, lymphocytes, plasma cells and macrophages (Metcalf and Reichert 1979). Necrosis, neovascularization, fibroplasia and full-thickness penetration may also be observed (Metcalf and Reichert 1979). The infection can progress to deeper eye tissues and cause infection and inflammation of the anterior chamber, iris and retina (Holland, Togni et al. 1987; Liesegang 2001; David, Baghian et al. 2008). Lesions can even progress to full-thickness necrosis of the retina with mixed inflammatory infiltrates and herpes particles detectable in the cell layers of the retina, including the retinal pigment epithelium (Holland, Togni et al. 1987; Liesegang 2001). Acute necrosis of the retina in people is rare but more frequently observed in young patients when it occurs (Liesegang 2001).

Another possible consequence of herpetic ocular disease is hypoesthesia, or loss of sensation, in the cornea due to destruction or degeneration of sensory trigeminal nerves (Keijser, van Best et al. 2002). This can cause a decrease in tear production and resulting dry eye with increased chances for corneal abrasion, inflammation, secondary infection and even stimulation of additional herpetic recurrence (Keijser, van Best et al. 2002; Tullo 2003).

Central Nervous System Infection

Central nervous system (CNS) infection with herpes simplex virus encephalitis is a rare event in the immunocompetent adult, however, in neonates the primary infection may result in encephalitis and in immunocompromised adolescents and adults, the primary or recurrent infection more frequently results in encephalitis (Fatahzadeh and

Schwartz 2007). Although rare compared to oral mucosal infection, HSV-1 is one of the most common and potentially severe causes of sporadic encephalitis in adults (Lakeman and Whitley 1995). Herpes simplex encephalitis can happen to any age person, has no seasonal variability and is one of the most common causes of infectious encephalitis with an estimated incidence of 1 in 250,000 to 1 in 1 million people per year (Skoldenberg, Forsgren et al. 1984; Whitley and Schlitt 1991). From 1979-1988, herpes simplex encephalitis was the most common cause of infectious encephalitis in the US and was responsible for over 10% of the 7.3 million hospitalizations for encephalitis (Khetsuriani, Holman et al. 2007; Stahl, Mailles et al. 2011). HSV-2 is a more common cause of encephalitis in neonates, but HSV-1 is a far more common cause of herpes encephalitis in older age groups, accounting for 90% of cases (Ito, Watanabe et al. 2000).

Infection of the central nervous system most likely occurs via spread of the virus from the primary site of infection, usually mucous membranes, through the nerves that innervate these tissues, usually the trigeminal or olfactory nerves (Arduino and Porter 2008). This spread can occur following the primary infection or more frequently during a recurrence, after latency has been established. HSV encephalitis usually causes fever, headache, neurologic deficits, and personality and cognitive disorders and has a 70% fatality rate when untreated (Wald and Corey 2007). Herpes encephalitis has also been associated with senile-plaque-like beta amyloid deposits in the brain that are associated with the development of dementia and Alzheimer's disease (Honjo, van Reekum et al. 2009; Miklossy 2011).

Clinically, herpes encephalitis can present with altered consciousness, non-specific or randomly localizing focal neurologic deficits, focal seizures, and personality

or behavior changes (Cinque, Cleator et al. 1996). These lesions generally refer to the areas of the brain involved; however, there is no specific pattern for herpes encephalitis (Cinque, Cleator et al. 1996). Prior to full onset of symptoms, there is often a prodromal phase consisting of aphasia, fever, headache, nausea, and visual deficits that may continue until presentation (Cinque, Cleator et al. 1996). In the most severe, or untreated, cases, the disease can be rapidly progressive with severe cerebral edema, necrosis of vital brain structures, and death within 7 to 14 days (Cinque, Cleator et al. 1996).

CNS lesions of herpetic encephalitis include aseptic meningitis and acute encephalomyelitis either with or without necrosis (Dudgeon 1969). Herpes cases involving solely aseptic meningitis appear to be rare (Dudgeon 1969). Cases with aseptic meningitis often have few lesions with only infiltration of the meninges infiltrated by mononuclear inflammatory cells and a slight increase of mononuclear inflammatory cells in the cerebrospinal fluid (CSF) (Dudgeon 1969; Koskiniemi, Vaehri et al. 1980; Skoldenberg, Forsgren et al. 1984). Cases with encephalitis or encephalomyelitis range from moderate to severe with possible extensive cortical necrosis (Stahl, Mailles et al. 2011). Generally the temporal lobes are the most frequently affected in a bilateral, random, asymmetrical pattern (Dudgeon 1969; Stahl, Mailles et al. 2011). The frontal and parietal cortices are less commonly involved, however, all the brain can become involved (Stahl, Mailles et al. 2011). In cases of encephalitis/encephalomyelitis, the early CSF shows a mild to moderate increase in mononuclear cells with normal or slight increases in protein (Dudgeon 1969; Koskiniemi, Vaehri et al. 1980; Skoldenberg, Forsgren et al. 1984). Later, especially with necrosis, the CSF can show markedly increased protein and red cells as well (Dudgeon 1969).

Microscopically, herpes encephalitis lesions are characterized by focal to massive necrosis with hemorrhage and edema of the gray matter sometimes with intranuclear inclusions (Dudgeon 1969; Booss and Kim 1984). Neutrophils may be present in areas of necrosis and microglial infiltration, microglial nodules and perivascular cuffing is observed (Dudgeon 1969). Although lesions are often confined to the cortex, lesions can progress to the basal ganglia, lobar white matter, and the brainstem (Chu, Kang et al. 2002; Ueda, Miyasaki et al. 2003; Ono, Manabe et al. 2009). In the end stages there is gliosis and astrogliosis and there may be persistent post infectious encephalomyelitis, movement disorders, atypical pain syndromes, neurologic deficits and epilepsy (Schmutzhard 2001; Simmons 2002). HSV encephalitis may be diagnosed via PCR of brain biopsy tissue or CSF, ELISA of CSF, or viral isolation from brain biopsy tissue (Cinque, Cleator et al. 1996).

Infection in an Immunocompromised Host

Rather than being a distinct clinical appearance, primary or recurrent infection of an immunocompromised person follows pattern similar to those observed in healthy individuals with the exception that the infections are more severe, more common, have greater spread, and are more likely to develop into serious illness (Fatahzadeh and Schwartz 2007). Immune suppression may be due to immunotherapy, metabolic disturbances, malnutrition, or concurrent disease, such as acquired immunodeficiency syndrome (AIDS) or immune system neoplasia. In these patients, HSV infections may develop progression from the oral mucosa to severe disease involving the esophagus, gastrointestinal (GI) tract or respiratory tract (Montgomerie, Becroft et al. 1969;

Korsager, Spencer et al. 1975). In the case of recurrence of oral mucosal disease, the disease may spread extensively in the mouth, on the tongue and along the GI tract resulting in a disease that is more painful and takes significantly longer to heal, up to 6 weeks or more (Whitley, Levin et al. 1984; Cohen and Greenberg 1985; Epstein, Sherlock et al. 1990; Woo, Sonis et al. 1990). The incidence of reactivation can range from 60-95% in seropositive patients that receive immunosuppressive therapy for bone marrow transplant (Arduino and Porter 2008). The most common complication in immunosuppressed patients is severe, progressive, chronic mucocutaneous infection with recurrent infection leading to disseminated disease or CNS infection occurring rarely (Whitley, Levin et al. 1984; Whitley and Roizman 2001). When disseminated disease or CNS infection occurs it is similar to the conditions previously described. In the immunocompromised patient, HSV-1 is the prominent culprit, however, HSV-2 has been reported to cause meningoencephalitis in immunocompromised patients (Linnemann, First et al. 1976; Gateley, Gander et al. 1990).

Treatment and Prevention of HSV Infection

Generally the two broad strategies for control of an infectious agent are some type of preventative measure and then treatment of infected individuals. In the case of HSV, whether type 1 or 2, prevention has been difficult to accomplish. The ideal mechanism for prevention would be a vaccine that prevents the development of infection past a mild mucocutaneous course of infection, eliminating spread to the nervous system and possible recurrence. No vaccine has produced this level of protection to date (Roizman, Knipe et al. 2007). Vaccination may also be used in a therapeutic sense rather than a

prophylactic sense to prime the immune system to limit the severity and duration of clinical symptoms in primary or recurrent infection (Stanberry 2004).

Vaccines

Attenuated live strains, killed virus, viral subunits, and genetically modified, attenuated viruses have all been attempted. Attenuated live strains tended to produce infection at the site of infection as well as recurrent disease later (Roizman, Knipe et al. 2007). Killed virus vaccines have been unreliable at producing a decrease in the symptoms of recurrent infection versus placebo (Whitley 2001a). These vaccines have also been unable to provide substantial increases in neutralizing antibody titers or complement fixation of virus to defend against primary infection (Roizman, Knipe et al. 2007). Subunit vaccines utilize viral proteins without the DNA component to prevent the vaccine from causing an infection. Typically subunit vaccines have provided little mitigation of symptoms in the already infected and no definitive protection for the unexposed (Roizman, Knipe et al. 2007). A recent subunit vaccine has had some success with the mitigation of symptoms and prevention of primary infection.

Two somewhat similar HSV-2 based vaccines have recently been used in large, well controlled studies (Roizman, Knipe et al. 2007). They both contain a modified recombinant form of glycoprotein D, while one also contains a modified glycoprotein B (Wilson, Fakioglu et al. 2009). The vaccine containing only the gD modification has been shown to be protective versus primary infection as well as able to mitigate symptoms of recurrence in latently infected women, however, no discernable effect was observed in men (Roizman, Knipe et al. 2007; Wilson, Fakioglu et al. 2009). Given the

similarity in the two vaccines, the difference may lie in the adjuvant, which in the more efficacious vaccine induced T helper-1 rather than T helper-2 immune responses (Roizman, Knipe et al. 2007; Wilson, Fakioglu et al. 2009). The gender difference in this vaccine needs to be examined to provide a more efficacious vaccine; however, given the poor status of HSV vaccines to date this progress is promising.

Construction of a recombinant (genetically modified), attenuated, live vaccine strain of the virus is the most likely method to produce a vaccine that both limits the course of recurrent disease in the latently infected and prevents primary infection. Currently some strains with limited replication characteristics have been attempted as possible vaccine strains, however, limited replication can have negative consequences on the development of a robust immune response (Dudek and Knipe 2006; Roizman, Knipe et al. 2007). Development of vaccine strains that allow sufficient replication to induce an immune response while preventing the establishment of latency are likely the best candidates for an effective vaccine (Whitley, Kern et al. 1993; Roizman, Knipe et al. 2007).

Isolation

Another prevention strategy is to eliminate contact with seronegative individuals when seropositive individuals are shedding virus. Prevention using this strategy is difficult due to asymptomatic shedding. Asymptomatic shedding has been reported for HSV-2 and one study found that 33% of people were asymptomatic at the time of infection of their partner (Mertz, Schmidt et al. 1985; Barton, Wright et al. 1986). For asymptomatic shedding of HSV-1 in the oral cavity, one study found that as much as

70% of the population may shed HSV-1 at least once per month with many of these shedding six times per month without symptoms (Miller and Danaher 2008). Amounts of viral DNA that can be detected decrease during antiviral therapy, however, detectable DNA is still shed without symptoms during this therapy (Koelle and Wald 2000). Clearly the virus can be transmitted to a seronegative individual without the knowledge of the carrier.

Therapeutics

Therapeutic antivirals that can reduce the symptoms and duration of primary and recurrent infection are available for HSV. The nucleoside analogs such as acyclovir, ganciclovir, famciclovir and valacyclovir have specific activity against HSV. The first nucleoside analog used effectively to treat HSV was acyclovir (Elion 1977). In general these are analogs of the nucleoside guanosine, or are converted by cellular or viral enzymes to analogs of guanosine, that can substitute for guanosine in DNA replication (Wilson, Fakioglu et al. 2009). In these cases the deoxyribose sugar ring is altered or replaced by another molecular structure. For acyclovir this is specifically a non-ring, linear structure. Acyclovir enters the cell in this form and is phosphorylated to acycloguanosine-monophosphate by viral thymidine kinase (Coen and Richman 2007). It is then converted to the tri-phosphate form by cellular kinases and acycloguanosine-3P competes with GTP in DNA synthesis (Coen and Richman 2007). It inhibits the viral DNA polymerase and since the sugar end of acyclovir cannot be used to link the next nucleotide, it terminates the DNA strand replication (Furman, St Clair et al. 1984). Viral DNA polymerase also appears to be unable, or poorly able at best, to do any proof

reading/removal of the acyclovir. This is because the HSV DNA polymerase is irreversibly inactivated when it attempts to use the acyclovir-triphosphate (Furman, St Clair et al. 1984). Acyclovir and the other guanosine analogs appear to have a higher affinity for viral kinases and viral DNA polymerase thereby making them have a more potent anti-viral effect than an anti-cellular enzyme effect (Fyfe, Keller et al. 1978). The oral bioavailability of acyclovir is low, therefore other forms of guanosine analogs and pro-drugs, such as famciclovir and ganciclovir were created with greater flexibility in dosing (Coen and Richman 2007). Acyclovir and related compounds are fairly efficacious in limiting the severity of herpetic disease, however, resistant strains have developed (Bacon, Levin et al. 2003; Coen and Richman 2007). In cell culture, acyclovir can be used to establish a quiescent/latent infection state, therefore, it is likely that drugs like acyclovir may provide some measure of help in maintaining the latent state in sensory neurons (Danaher, Jacob et al. 1999).

Another inhibitor of DNA replication, and HSV antiviral compound, is foscarnet. This is a pyrophosphate mimic that can directly inhibit the action of the DNA polymerase by substituting for pyrophosphate (Coen and Richman 2007). This compound will also inhibit RNA polymerase. Foscarnet does not require the viral thymidine kinase for activation and is therefore active versus acyclovir resistant viruses (Wilson, Fakioglu et al. 2009). Since this compound is not as specific for HSV, it also possesses action against cellular polymerases and is more toxic (Wilson, Fakioglu et al. 2009). Cidofovir is a nucleoside analog, similar to acyclovir, however it uses cellular enzymes to be phosphorylated to an active form and, therefore, can be used versus acyclovir resistant strains (Wilson, Fakioglu et al. 2009). Since both foscarnet and cidofovir are more toxic

and effective against acyclovir resistant viruses, these are generally administered only in cases of resistance (Wilson, Fakioglu et al. 2009).

The Host Neuron and its Role in HSV Infection

Basic Neuron Structure

The anatomic structure of a typical neuron is composed of the cell body (soma) that contains the nucleus, an axon (which is the long tubular process that traverses the distance between the soma and the axon terminals, dendrites (which are multiple web-like projections of processes and synaptic bulbs (King 1994). Most neurons are multipolar with axons and dendrites projecting in multiple directions and dendrites coming directly off the soma. Sensory neurons, the primary neuronal target of HSV, are pseudounipolar with a single process that bifurcates to make the axon running to the receptors at one end and axonal termini at the other with the soma situated in the middle (King 1994). In a sensory pseudounipolar neuron, the dendrite extends to the innervated site and acts electrophysiologically like a dendrite bringing sensory stimuli toward the cell body (Zaichick, Bohannon et al. 2011). However, in other respects the structural composition, functional elements, and directionality of microtubules of this structure are more similar to an axon (Enquist, Husak et al. 1998; Zaichick, Bohannon et al. 2011). The synapse is formed from a synaptic bulb (a bulge in the axon terminus), the synaptic cleft and the post-synaptic structure being acted upon (dendrites, other axons or neuron cell bodies). The synaptic cleft is lined by the pre-synaptic membrane (part of the bulb which releases neurotransmitters) and the post-synaptic membrane (where neurotransmitters are bound and elicit their function) (King 1994). The synapse is an

attractive target for entry of a virus as there is abundant turn-over of membranes due to the vast recycling associated with release and reuptake of neurotransmitters (Salinas, Schiavo et al. 2010). For HSV specifically, nectin-1 is located at the synaptic junction for sensory neurons (Salinas, Schiavo et al. 2010).

Retrograde and Anterograde Transport

To discuss neuronal axonal biology and transport functions a few terms must first be defined. First directionality of transport within a neuron must be considered.

Retrograde transport is defined as transport from the axon terminus/ends of dendritic processes toward the nucleus/cell body. Anterograde transport is the opposite, transport from the nucleus/cell body to the axon terminus/ends of dendritic processes. When referring to a single neuron, this system is fairly straightforward. However, occasionally these terms are used to refer to the overall direction of travel from the point of a whole system that contains arcs of multiple neurons. In this case, retrograde transport refers to the overall movement toward the central nervous system (CNS), the brain or spinal cord. In this context, anterograde transport refers to movement from the CNS toward the peripheral tissues of the animal. Therefore, due to the architecture of neuronal pathways and individual neurons, retrograde transport at the level of the overall animal may require brief anterograde transport on an individual neuron level.

The axon is a long, tubular structure in which the majority of transport within neurons occurs. The axon is made predominantly of microtubules surrounded by the plasma membrane and contains abundant transport molecules, proteins, enzymes, and organelles that are being transported. Axons are myelinated by Schwann cells (a type of

glial cell) in the peripheral nervous system (PNS) and by oligodendroglial cells in the CNS with unmyelinated axons being supported and surrounded in groups in the PNS (King 1994). The axon structure is vital to the function of transport of molecules within the neuron.

Interestingly, intracellular transport is highly conserved among neurons and non-neuronal cells. Transport along axons occurs primarily along a scaffold of microtubule rails. These are made of heterodimers of alpha and beta tubulins and are approximately 25 nm in diameter (Hirokawa 1998; Hirokawa and Takemura 2005). Although, transport mechanisms are conserved, microtubules have polarity and in axons they run in the same direction while in dendrites and other cell types they may run in multiple directions. In axons, the plus end is directed to the periphery; which means that in axons anterograde transport is always directed toward the plus end and retrograde transport toward the minus end (Baas, Deitch et al. 1988; Burton 1988; Sharp, Yu et al. 1995). In epithelial cells the minus ends are oriented toward the apical surface of the cell and in fibroblasts they radiate around the nucleus in various directions (Hirokawa, Noda et al. 2009). Transport within axons is accomplished by two types of molecular motors. One is the kinesin superfamily of proteins (KIFs) and the other is dynein. Both of these proteins transport a large variety of molecules and, although they use different mechanisms to specify what molecules are transported, they both use ATP hydrolysis to drive transport (Hirokawa, Noda et al. 2009). Dynein forms a dynactin complex with multiple subunits to bind various molecules while KIFs use a wide variety of proteins in the family to accomplish binding and transport of varied proteins, enzymes and organelles (Vallee, Williams et al. 2004; Hirokawa, Noda et al. 2009).

Comprehensive analysis of mammalian genomes have found 45 KIF genes with 38 expressed in the brains of mice (Miki, Setou et al. 2001). There are 3 broad categories of KIFs; N-kinesins with the motor domain near the amino terminus, M-kinesins with the motor near the middle and C-kinesins with the motor near the carboxy terminus (Hirokawa and Noda 2008; Hirokawa, Noda et al. 2009). N-kinesins have plus directed motor activity and C-kinesins have minus motor activity. The majority of kinesins are N-kinesins with KIFs responsible for all anterograde transport currently identified (other than diffusion) and dynein responsible for the majority of retrograde transport (Miki, Setou et al. 2001; Gennerich and Vale 2009; Hirokawa, Noda et al. 2009). A few of the best characterized kinesin superfamily proteins and their known cargos will be listed. KIF1A has been shown to transport synaptic vesicle precursors containing synaptophysin, synaptotagmin and Rab-3A and binds its cargo through a pleckstrin-homology domain (Okada, Yamazaki et al. 1995; Klopfenstein, Tomishige et al. 2002). KIF1B β seems to share many functions with KIF1A and this was not a unique instance of some redundancy in these proteins (Hirokawa and Noda 2008). KIF1B α is a monomer used for transport of mitochondria (Nangaku, Sato-Yoshitake et al. 1994). KIF5 is a dimeric motor that consists of 3 subtypes (A, B, and C) that can form tetramers with KIF light chains. KIF5 has been shown to transport mitochondria, neurofilaments, syntaxin, syntabulin, GAP-43, synapsin and likely c-jun NH2-terminal kinase interacting proteins (which serve as scaffolds for MAP kinases (Hirokawa and Noda 2008). KIF5 can also play a role in retrograde transport (Hirokawa and Noda 2008).

The previous examples are of fast axonal transport, however, there is a second set of transport functions described as slow axonal transport. Generally slow axonal

transport carries structural proteins, like tubulins and neurofilaments, or glycolytic enzymes (Hirokawa and Noda 2008). KIF5 is the primary currently identified mediator of slow anterograde transport (Hirokawa and Noda 2008). There is increasing evidence that slow and fast transport actually occur at similar rates of forward progress, just that slow anterograde movement has pauses of significantly greater duration than in fast transport (Brown 2003). The determinants of what causes a pause, allows a return to movement or determines the length of pauses are not well known (Brown 2003; Hirokawa and Noda 2008). However, it appears that in slow axonal transport, there is a distinct on- or off-track state and in the off-track state, neurofilaments pause for prolonged periods of time without forward movement (Brown, Wang et al. 2005). Furthermore, myosin Va may play a role in the length of pauses in the delivery of neurofilaments by reducing time off-track (Alami, Jung et al. 2009).

Most of the retrograde transport that occurs in neurons, occurs via dynein. Less specific details are known about dynein in axonal transport compared to the kinesin superfamily. Dynein is a very large protein complex, 1.2 MegaDaltons (MDa), and is composed of two heavy chains and multiple light chains (Gennerich and Vale 2009). The motor action of dynein is performed by the heavy chains and the intermediate and light chains perform cargo attachment functions (Hall, Song et al. 2010). The heavy chains of dynein are encoded by a single gene and as few as two genes may encode the possible dynein light chains (Pfister, Shah et al. 2006; Ha, Lo et al. 2008). These may be arranged as isoforms to provide diversity of cargo binding domains. The intermediate chain of dynein has at least 6 splice variants that appear to be preferentially expressed in various neural tissues and appears to preferentially bind different tyrosine kinases (ie: TrK A or

TrK B) (Pfister, Shah et al. 2006; Lo, Kogoy et al. 2007; Myers, Lo et al. 2007; Ha, Lo et al. 2008). When considered simply, this disparity in complexity is likely due in part to the vast amounts of molecular material that must be transported to the axon terminus versus the much lower amount of material that needs to be transported back from the terminus to the cell body. Cellular components and neurotransmitters that must be recycled at the level of the cell body, must be transported in the retrograde direction. However, there is substantial evidence that protein synthesis occurs at the nerve termini and that there may be glial-neuronal cell signaling at protein and RNA levels at the axon terminus (Giuditta, Chun et al. 2008). This is interesting in that it would provide a faster response mechanism for the neuron to react at a local level, especially in long axons. Neurons would need to react at the cell body level to signals for axonal growth and development/regeneration. It is known that signaling endosomes do transport in a retrograde fashion (Ha, Lo et al. 2008). Signaling endosomes are created and transported when growth factors (such as neural growth factor NGF) or neurotrophic factors (such as brain-derived neurotrophic factor BDNF) activate tyrosine kinases at the ends of neuronal processes (Bhattacharyya, Watson et al. 2002; Delcroix, Valletta et al. 2003; Huang and Reichardt 2003; Heerssen, Pazyra et al. 2004). Other cellular constituents such as organelles are transported by the dynein motor complex as well (Vallee, Williams et al. 2004). Retrograde transport is also necessary for neuron survival and the plasticity of axons (Ha, Lo et al. 2008).

One of the prime functions of anterograde transport is the delivery of neurotransmitters/neurotransmitter precursors and synaptic vesicle constituents to the synapse. The synapse is formed from the pre-synaptic membrane (where the

neurotransmitters are released), the synaptic cleft, and the post-synaptic membrane (where neurotransmitter receptors reside). Neurotransmitters are released in response to the arrival of an action potential. The arrival of this action potential causes voltage gated calcium channels to open. Ca^{2+} floods into the synaptic bulb (some release from stored intracellular calcium storage may contribute) and this causes pre-synaptic vesicles to release their neurotransmitter into the synaptic cleft (King 1994). Then the transmitter binds to its receptor and causes its particular action.

Classically defined neurotransmitters include GABA (gamma aminobutyric acid) (considered an inhibitory transmitter), glutamate (considered excitatory), acetylcholine (functioning at the neuromuscular junctions), and dopamine and serotonin (which have more complex functions in the central and peripheral nervous systems) (Kawaguchi 1997; Lee, Schmidt et al. 2001; Chase and Koelle 2007; Wang, Van Bockstaele et al. 2008; Dave and Bordey 2009). Neurotransmitters can also be classified as monoamines (serotonin, dopamine, norepinephrine, epinephrine etc), amino acids (glutamate, GABA, etc) and neuropeptides (endorphins, enkephalins, etc) (Kawaguchi 1997; Lee, Schmidt et al. 2001; Chase and Koelle 2007; Wang, Van Bockstaele et al. 2008; Dave and Bordey 2009). Once the excitatory and/or inhibitory neurotransmitters are released, an action potential is generated if a threshold of excitation is reached. This propagates as an electrical signal along the axon. Once an action potential threshold is reached, these are generally considered an all or none phenomenon with the action potential propagating the entire length of the axon (King 1994). In many instances these neurotransmitters have channels for reuptake and recycling at the synapse, which reduces the demand for novel material from the cell body.

Effects of Action Potentials on Transport

Action potentials are primarily a membrane effect, while axonal transport occurs in the axonal cytoplasm. The majority of research appears to cover how axonal damage alters impulses, delivery of molecules via axonal transport or neurotransmitter release or recycling. However, there was some evidence that the mere generation of action potentials may have an effect on the transport of molecules through the axon. It appears that nerve impulse firing significantly reduced the retrograde transport of dynein in both velocity and total distance traveled (Lardong, Maas et al. 2009). Also, firing of action potentials altered the anterograde transport in axons. In one study of bullfrog dorsal root ganglia, nerve stimulation decreased the overall fast axonal transport temporally, but did not result in ultrastructural changes (Hammerschlag and Bobinski 1992). This was interpreted as neurons increase transport when conduction is not occurring to replenish the axon terminus (Hammerschlag and Bobinski 1992). Interestingly in neither study did tetrodotoxin (which blocks action potentials by blocking ion channels) have an effect on axonal transport (Hammerschlag and Bobinski 1992; Lardong, Maas et al. 2009).

Neuronal Transport as it Relates to HSV Infection

For HSV to complete its infectious cycle for a neuron, it must first come into contact with the axonal processes of a neuron. Then the virus enters the neuronal cell via pH-independent fusion of the viral envelope to the axonal process membrane (Qie, Marcellino et al. 1999; Nicola, Hou et al. 2005). Viral fusion and entry are thought to operate similarly to described earlier for non-neuronal cells using the coordinated interactions of glycoproteins gD, gB, gH/gL, gC and possibly gK (Cai, Gu et al. 1988;

Desai, Schaffer et al. 1988; Ligas and Johnson 1988; Hutchinson, Browne et al. 1992).

Some neurotropic viruses enter the neuron via endocytotic or clathrin mediated pathways, including Canine Adenovirus type-2 (CA_{AdV}-2) and Rabies virus, and through neuron specific sorting of endosomes can directly begin retrograde transport (Salinas, Schiavo et al. 2010). Rabies virus transport has been demonstrated in an enveloped state and CA_{AdV}-2 has been shown to be transported in intact endosomes (Salinas, Schiavo et al. 2010).

This may suggest a possible alternate, as yet undescribed, pathway for HSV that would have implications for glycoproteins and the tegument proteins potential involvement in early infection. After entry, the virus must be transported along the axon to the cell body and eventually the nucleus. HSV has been shown to rely on the dynein/dynactin molecular motor system using the microtubule network for retrograde transport in fluorescence studies of viral transport in neurons and in studies using inhibitors or destabilizers of dynein and the microtubule network (Topp, Meade et al. 1994; Bearer, Breakefield et al. 2000; Dohner, Wolfstein et al. 2002). The exact interaction remains to be determined for retrograde transport. Some proteins have been shown to bind dynein in vitro including pUL34 and pUL9 (Diefenbach, Miranda-Saksena et al. 2008b). These proteins are not present in the mature virion however, and may be used by the virus for transport of materials during assembly (Diefenbach, Miranda-Saksena et al. 2008b).

Viruses lacking the pUL35 protein, or the capsid protein VP26, have been shown to have greatly decreased retrograde transport in vivo, but in vitro retrograde transport to the nucleus is retained, suggesting that functional redundancies or more complex interactions may exist in this system (Desai, DeLuca et al. 1998; Antinone, Shubeita et al. 2006; Dohner, Radtke et al. 2006). Since the envelope is shed during viral fusion to the neuron,

it is assumed that interacting proteins will come from the capsid or tegument proteins closely associated with the capsid. However, if the virus were to enter the neuron via endocytosis it is unknown what role envelope proteins may play or if multiple pathways exist to instigate retrograde transport.

Once the virus reaches the nucleus, the virus is likely to go latent, as discussed earlier. During the reactivation of a latent infection or immediately if the virus stays in the lytic phase, the viral components must be created and assembled to produce progeny virus. This progeny virus is then transported in the anterograde direction to the axon termini to be released and infect epithelial cells at the initial site of infection. The vast majority of this process is considered to occur in the same manner as previously described for non-neuronal cells. The mechanism of transport and the character of the virion during transport in the anterograde direction is a highly controversial issue. Two competing theories predominate, these are: 1) transport of the capsid containing the DNA separate from the glycoproteins with final assembly occurring at the plasma membrane of the neuron at the axon terminus, sometimes called the “unmarried model”; 2) transport of a fully formed, enveloped virus that is created by budding through the TGN described earlier, sometimes called the “married model” (Enquist, Husak et al. 1998). These two models are in contention in large part due to disparate results from studies in two alphaherpesviruses, Pseudorabies virus (PRV) and HSV-1 (Diefenbach, Miranda-Saksena et al. 2008a; Liu, Goodhouse et al. 2008; Mettenleiter 2008; Mettenleiter, Klupp et al. 2009; Antinone and Smith 2010; Antinone, Zaichick et al. 2010; Maresch, Granzow et al. 2010; Osakada and Cui 2011).

Since the N-kinesin members of the KIF superfamily (such as the prominent members KIF1, KIF3, and KIF5) are the primary anterograde transporters in neurons, it is likely that one of these molecules is the major molecular motor to transport herpes viruses (Diefenbach, Miranda-Saksena et al. 2008b). Notably, since the exact character of transport is unknown, little is also known about what components of the described neuronal anterograde transport system are involved in the transport of herpes virions. Although many viral glycoproteins have been implicated in efficient egress of virions from epithelial cells, the role of these proteins in neuronal egress has not been studied extensively (Mettenleiter, Klupp et al. 2009). In PRV glycoproteins gE and gI are required for anterograde transport, but not retrograde transport (Card, Whealy et al. 1992a; Jacobs, Mulder et al. 1993b; Babic, Klupp et al. 1996a; Johnson, Webb et al. 2001a; Brittle, Reynolds et al. 2004; Ch'ng and Enquist 2005). HSV-1 gE has been reported to function in both retrograde and virus spread from epithelial cells to neurites but it is not necessary for direct infection of neurites or retrograde transport in axons (Balan, Davis-Poynter et al. 1994b; Saldanha, Lubinski et al. 2000; Wang, Tang et al. 2005; Brittle, Wang et al. 2008; McGraw and Friedman 2009).

Recently, the role of actin in herpes virus infection in neurons has been under increased scrutiny. In the growing axon, actin directs the growth of the axon by maintaining polarity in axonal growth cones (Roberts and Baines 2011). In the mature neuron, actin still plays a role in maintaining polarity at the axon hillock, where it acts as a buffer between the endoplasmic reticulum and cargoes that are destined for transport along the axon (Roberts and Baines 2011). This would suggest that HSV necessarily interacts with actin as it enters the nucleus during retrograde transport and as it leaves the

nucleus for anterograde transport during a reactivation. Very recent work has suggested that myosin Va, a molecular motor that interacts with actin, may bridge the gaps between axonal transport and the nuclear membrane, but these models are early in development and more work is necessary to confirm the hypothesis (Roberts and Baines 2011).

Latent Infection with HSV

While much is known about herpes viral latency, even more remains a mystery regarding the exact mechanisms of latency and reactivation. Latent infection must first be distinguished from chronic infection. In a chronic infection, a virus will express proteins and produce a low level of infectious progeny that is shed continuously albeit in amounts that may significantly wax and wane. In a true latent state, as defined for herpes viruses, the viral DNA is held in a circular episome within the host cell nucleus and a very slight level of DNA transcription occurs, however, there is no protein production (Roizman, Knipe et al. 2007). During latency, HSV creates a latency-associated transcript (LAT) from the viral DNA. The promoter for the encoded LAT is maintained in acetylated histones, or active areas, of the host DNA (Kubat, Amelio et al. 2004). In the latent stage of the infection, the lytic, or active infection, genes of HSV are maintained in areas of heterochromatin, which are regions of host cell DNA that are inactivated (Wang, Zhou et al. 2005). HSV DNA also avoids methylation by the host nucleus that could inactivate the virus (Roizman, Knipe et al. 2007). During the latent infection, the LAT is the only transcript expressed in abundance, however, very sensitive assays can detect small amounts of transcription of lytic genes (Kramer and Coen 1995; Kramer, Chen et al. 1998). This small expression of lytic genes may be a state of

leakiness or abortive attempts at reactivation (Bloom, Giordani et al. 2010). The exact reason for this expression is unknown. The LAT is an 8.3 kb sequence with several splice variants, of which a 2.0 kb sequence is the most common (Farrell, Dobson et al. 1991). Through evaluation of mutant viruses, the LAT is associated with efficient establishment of latency, reactivation, and blocking apoptosis (Farrell, Dobson et al. 1991). LAT deficient mutants have more significant pathology in the trigeminal ganglion with more necrosis of neurons and greater mortality in mouse studies (Roizman, Knipe et al. 2007). The LAT actually appears to have a protective effect for neurons that become infected.

In the normal course of HSV infection, the virus replicates at the initial site of epithelial infection and then infects the innervating sensory neurons. The virus travels in the anterograde direction through the axon of the neuron to the nucleus and establishes a latent infection. During the first 24-72 hours after infection of the ganglionic neurons, expression of lytic genes and the LAT are observed, however, the lytic genes must be rapidly suppressed to prevent a lytic infection, since neurons do not have an intrinsic invulnerability to lytic infection (Kosz-Vnenchak, Coen et al. 1990; Kramer, Chen et al. 1998). Although an exact mechanism is unknown, it appears that CD8⁺ T cells play a role in regulating infection in nervous tissue and there is a distinct lack of alpha gene expression as latency is established (Roizman, Knipe et al. 2007). Furthermore, once the latent state is established, less is known about the mechanism of maintenance of this state. During latency, the LAT expression is maintained and HSV specific T cells are preferentially maintained within neural tissues (Verjans, Hintzen et al. 2007).

Once latency has occurred, the final characteristic of the virus is to reactivate out of the latent state and cause a recurrent infection at the site of mucosal innervation via retrograde transport. The mechanism of reactivation is not clearly defined. The conditions that can cause reactivation have been described and include: any source of local or systemic stress, such as UV radiation, trauma, surgery, excessive heat, another infection, emotional stress, and hormonal imbalance (Roizman, Knipe et al. 2007). Once this stress occurs, the lytic genes are moved from areas of heterochromatin to acetylated histones, become active and begin a recurrent infection (Amelio, Giordani et al. 2006). Viral proteins are created, viral DNA is replicated, and virions are assembled which can then be transported to the site of initial infection (Roizman, Knipe et al. 2007). These progeny virions can also move in the anterograde direction to second and third order neurons progressing deeper into the central nervous system, although this is less common (Roizman, Knipe et al. 2007). The final fate of the reactivated neuron is not clear. Guaranteed destruction seems unlikely since areas of mucosal infection do not always become anesthetized after many reactivations.

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CHAPTER II

THE HERPES SIMPLEX VIRUS TYPE-1 (HSV-1) GLYCOPROTEIN K(gK) IS ESSENTIAL FOR VIRAL CORNEAL SPREAD AND NEUROINVASIVENESS*

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is an enveloped, double-stranded DNA virus that is a ubiquitous pathogen of humans. When symptomatic, HSV-1 commonly causes mucocutaneous lesions with acute encephalitis and ocular pathology occurring less frequently (Whitley 2001). Although a less frequent manifestation of disease, ocular HSV is one of the most common infectious causes of blindness in developed countries with estimates of the incidence of herpes ocular disease episodes ranging from 4.1 to 20.7 cases per 100,000 in developed countries (Liesegang, Melton et al. 1989; Liesegang 2001). An estimated 400,000 people in the United States have had herpes simplex ocular disease with about 50,000 new or recurrent cases occurring yearly (NoAuthor 1998). Herpesvirus infection usually begins at a mucosal surface and from this site the virus invades the ganglionic sensory neurons and can establish a latent infection for the life of the host (Whitley, Kimberlin et al. 1998).

HSV-1 specifies at least 11 glycoproteins that are expressed in the infected cells (Roizman and Knipe 2001). Glycoproteins B and C are primarily involved in initial attachment of the virion to cell surface glycosaminoglycans by binding to heparan sulfate (Laquerre, Argnani et al. 1998). Attachment is followed by fusion of the viral envelope

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with cellular membrane receptors and viral glycoproteins B (gB), D (gD), H (gH) and L(gL) (Furth, Whitbeck et al. 1997; Whitbeck, Peng et al. 1997; Warner, Geraghty et al. 1998; Krummenacher, Rux et al. 1999; Whitbeck, Muggeridge et al. 1999). These glycoproteins have also been shown to be essential for viral entry and infectious virus production (Wanas, Efler et al. 1999). Virus spread, a priori, occurs by either infectious virions produced by cells that can then infect adjacent cells, or via inter-cellular transmission of fully infectious virions facilitated by virus-induced cell fusion (syncytia formation). Virus-induced cell fusion is mediated by viral glycoproteins expressed on cell-surfaces, while gB, gD, gH and gL are necessary and sufficient to cause cell fusion in the absence of virus replication (Davis-Poynter, Bell et al. 1994; Turner, Bruun et al. 1998; Muggeridge 2000; Pertel, Fridberg et al. 2001). Glycoprotein E (gE) may also function to transport virions between cellular membranes by other than cell fusion mechanisms (Dingwell and Johnson 1998; Wisner, Brunetti et al. 2000; Johnson, Webb et al. 2001).

HSV-1 gK is essential for cytoplasmic virion envelopment, virion egress and virus-induced cell fusion (Hutchinson and Johnson 1995). Recently, we have demonstrated that gK physically binds to the UL20 membrane protein (Foster and Kousoulas 2008). Furthermore, UL20/gK interactions are essential for their intracellular transport, cell-surface expression and functions in cytoplasmic virion envelopment and virus-induced cell fusion (Foster, Alvarez et al. 2003; Foster, Melancon et al. 2004a; Foster, Melancon et al. 2004b; Melancon, Fulmer et al. 2007; Foster and Kousoulas 2008). HSV-1 gK is of particular importance to eye infections, because it has been shown that it is involved in the exacerbation of eye disease, most likely, mediated by

anti-gK humoral and cellular immune responses (Osorio, Cai et al. 2004; Mott, Osorio et al. 2007; Mott, Perng et al. 2007; Osorio, Mott et al. 2007). Deletion of the gK gene encoded by the pseudorabies virus (PRV) appeared to attenuate the virus, but did not alter the ability of the gK-null virus to invade the central nervous system and cause clinical eye disease (Flamand, Bennardo et al. 2001; Schwartz, Brittle et al. 2006; Ch'ng, Spear et al. 2007). These results prompted the present work to assess the role of HSV-1 gK in corneal spread and neuroinvasiveness. In contrast to previous findings with PRV gK, work presented here, shows that HSV-1gK is essential for virus spread in the cornea of mice and neuroinvasiveness.

MATERIALS AND METHODS

Cells and Viruses

The ocular and neural virulent McKrae strain of HSV-1 was obtained from Dr. J.M. Hill (Louisiana State University Health Sciences Center, New Orleans, La). The UL 53 (gK) gene of HSV-1 McKrae was replaced with an EGFP gene cassette from plasmid pEGFP-1 (Clontech, Palo Alto, Ca) under control of the human cytomegalovirus immediate early gene promoter as described previously for the KOS Δ gK/GFP (Foster, Rybachuk et al. 1998). Briefly, plasmid PTF9201 contains an EGFP gene cassette under the human cytomegalovirus immediate early promoter control (HCMV-IE) within a DNA fragment spanning the UL52-UL54 genomic region that lacks the UL53 gene (Foster, Rybachuk et al. 1998). Plasmid PTF9201 was used in transfection followed by infection with HSV-1 (McKrae) to isolate the McKrae- Δ gK/EGFP (MK Δ gK) virus appearing as

small fluorescent viral plaques under the fluorescence microscope. Individual viral plaques were plaque-purified six-times before viral stocks were prepared in Vero or VK302 cells. The resulting HSV-1 McKrae UL53 null virus is referred to as MK Δ gK. A rescued virus that was positive for both gK and GFP (MKgK) was constructed from the MK Δ gK, as described previously (Foster and Kousoulas 1999). See map of viral mutations in Fig 2.1. African green monkey kidney (Vero) cells were obtained from ATCC (Rockville, MD) and grown in Dulbecco's modified Eagle Medium (DMEM) supplemented with 7% fetal bovine serum (FBS) and antibiotics. The gK complementing VK302 cell line (generously provided by Dr. David Johnson, Oregon Health Sciences University) was maintained in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin). This cell line was used for generation of high titer MK Δ gK viral stocks. The properties of all virus recombinants were validated as described previously (Foster, Rybachuk et al. 1998; Foster and Kousoulas 1999).

Mice

6-10 week old, female, Balb/c mice were used for all experiments and were handled in accordance with the ARVO statement for the use of animals in ophthalmic and vision research and Louisiana State University-School of Veterinary Medicine IACUC regulations.

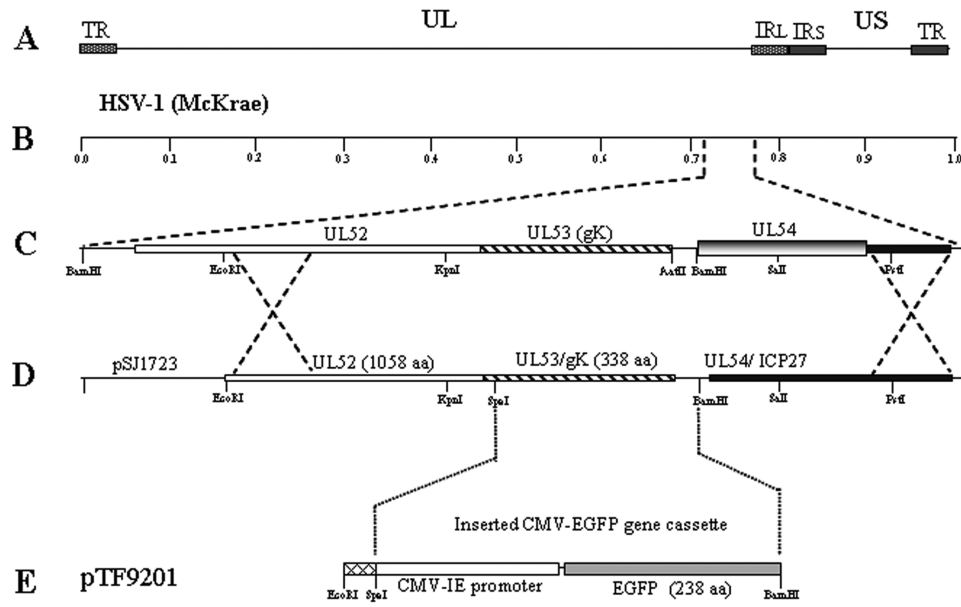


Figure 2.1: Schematic of recombinant virus construction. (A) The top line represents the prototypic arrangement of the HSV-1 (McKrae) genome with the unique long (UL) and unique short (US) regions flanked by the terminal repeat (TR) and internal repeat (IR) regions. (B) HSV-1 genomic map in relative map units. (C) An expanded genomic region between map units 0.7 and 0.8 containing the UL52, UL53, and UL54 open reading frames. (D) Diagram of the pSJ1723 plasmid (Jayachandra, Baghian et al. 1997), which contains the UL52, UL53 and UL54 region of HSV-1 (KOS). (E) Plasmid pTF901 (Foster, Rybachuk et al. 1998) containing the CMV-IE-EGFP gene cassette in-place of the gK gene, which was used to delete the gK gene and insert the EGFP gene cassette within the HSV-1 (McKrae) genome.

Plaque Morphology and Virus Replication Characteristics

For plaque morphology, confluent monolayers of Vero and VK302 cells were infected at multiplicity of infection (MOI) of 0.001 with the MKgK and MKΔgK viruses and were visualized by immunohistochemistry at 48 and 72 hours post infection (hpi). At 48 or 72 hpi, cells were fixed with ice-cold methanol. Immunohistochemistry was performed with horseradish peroxidase-conjugated anti-HSV antibodies (Dako) and the reactions were developed with NovaRed substrate (VectorLabs; Burlingame, CA).

To determine the kinetics of viral replication, cells were infected with the MKΔgK, MKgK or the McKrae parental strain at MOI's of 0.1 and 5. For McKrae parental strain and the rescue strain, plates of Vero cells were used and for the MKΔgK virus Vero and VK302 cells were used. Plates were prepared in triplicate and infections were halted by freezing at 0, 3, 6, 9, 12, 24, 36, 48 and 72 hours post infection (hpi). Plates were frozen, thawed and viral titers were determined for each time point by standard plaque assay on VK302 cells (for the MKΔgK virus) or Vero cells (for the parental and MKgK strains). Plaques were stained with crystal violet and visualized by dissecting microscope. Growth curves show the result of the average of the triplicate plates.

Mouse Eye Infections

Ocular infection with the rescued virus (MKgK) and wild type virus produced similar results in terms of clinical and laboratory findings (data not shown); therefore, the rescued strain data is presented since it serves as a better comparison to the gK null mutant (MKΔgK). In studies in which mouse eyes are scarified, after anesthetizing the

mice each cornea was scarified by four strokes vertically and four strokes horizontally of a 25 gauge needle in a grid pattern. Then the designated viral inoculum, in 5 μ l of DMEM, was applied to the eyes with a micropipetter and the eyelids were gently held shut for 30 seconds. For all studies, either 1.0×10^6 PFU of MK Δ gK was inoculated into each cornea or 2.5×10^4 PFU of MKgK was inoculated into each cornea. If not scarified, mouse eyes were gently abraded by rubbing with sterile cotton swabs 10 times in different directions.

For clinical lesion studies, mice were monitored daily for survival and the degree of blepharitis, hyperemia and ocular discharge were recorded. These symptoms were scored on the scale of 0-3, with 0 representing no symptoms and using the most severe lesions in the group infected with the rescued McKrae strain as the scale of 3. For direct observation of viral infection and spread in the eye, 2 groups of mice were similarly inoculated (5 mice/group/time point). Mice were sacrificed at 12, 24, 48 and 72 hpi as well as 5, 7, 10 and 14 days post inoculation (dpi). The eyes of the mice were excised and viral spread was tracked by direct observation under a Zeiss Stereo Lumar fluorescence microscope and digitally photographed with an Axiocam color camera (Carl Zeiss Inc, USA.). The extent of fluorescent plaques on the eye were scored on the following 0-3 scale: 0 (No fluorescent foci observed), 1 (One or few (<12) pinpoint plaques covering less than 5% of total visual surface or peripheral rim of connective tissue), 2 (One or more plaques larger than pinpoint, or numerous pinpoint plaques, covering 5-20% of the total visual surface or peripheral rim of connective tissue), 3 (greater than 20% of the visual surface covered with fluorescent plaques and/or iris

involvement). Iris involvement was determined by fluorescent highlighting of iris tissue structure.

Detection of Latent Virus in Trigeminal Ganglia Using PCR and In Vitro Tissue Explants Cultures

Mice that survived the initial infection were sacrificed at 30-35 days post-infection. Mice were sacrificed via CO₂ asphyxiation followed by thoracotomy. Then trigeminal ganglia (TG) of the mice were excised. For determination of culture reactivation, excised trigeminal ganglia were cultured as a whole for 5 days in six-well plates in DMEM containing 10% bovine serum and antibiotics in a humidified incubator at 37 degrees Celsius with 5% CO₂ and 95% filtered room air. After 5 days, the TG were triturated with a glass pipet and ganglion cells and media were placed on the Vero or VK302 cell monolayers. Wells were monitored to study evidence of cytopathic effects (CPE) indicative of virus reactivation. Every other day, half the media in each well was passed to new plates with fresh monolayers and these were also monitored for CPE. This process was repeated until a well had demonstrated CPE or for 12 days following plating of the initial ganglion cells.

For viral DNA detection, a nested PCR methodology was used targeting the UL30 gene. Mice surviving the clinical infection to 30 dpi were euthanized and the trigeminal ganglia (TG) were harvested and frozen at -80C. TG's were thawed and total DNA from the TG was extracted using the Qiagen DNeasy blood and tissue kit (Qiagen, CA, USA) per the manufacturer's instructions. For non-scarified mice, TG DNA from the right and left TG was harvested separately and any mouse positive by PCR in either

or both ganglia was considered positive. For scarified mice, the right and left TG were pooled for extraction.

The individual primers for the primary PCR reaction were : A) 5'-CCTGGACAAG CAGCAGGCCGCCATC-3'; and B) 5'-GCGCACCAGATCCACGCCCTTG-3'. The second step PCR utilized primers C) 5'-CTCGGTGTACGGGTTCACGGGAGT-3'; and D)5'-GATGGAGTCCGTGTCCCCGTAGATG-3'. The PCR product of A, B primers was 452bp, while the secondary PCR with primers C, D produced a 207bp DNA fragment. These primers are HSV-1-specific diagnostic primers based on previously published degenerate diagnostic UL30 primers (VanDevanter, Warren et al. 1996). The authenticity of individual PCR fragments was confirmed by DNA sequencing. The FailSafe enzyme system with PCR premix E was used for all PCR reactions (Epicentre Biotechnologies, Madison, WI, USA). Specifically, 1-10µl of extracted DNA was used for the first step reaction and 1-10µl of the first step product was used in the second step. The PCR products were then subjected to electrophoresis and stained with ethidium bromide.

The positive control consisted of DNA extracted from McKrae virus stock. Negative controls were TG DNA from mock infected mice as well as no template controls. To determine the lower limit of sensitivity, serial dilutions of extracted viral DNA mixed with equal volumes of negative mouse TG DNA were used. The lower limit of detection occurred between 10 and 100 copies of viral DNA.

Statistical Analyses

All following statistical analyses in this chapter were performed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California USA.

RESULTS

Recombinant Virus Construction and Characterization

The construction of a HSV (KOS) recombinant virus in which the gK gene was replaced with an EGFP gene cassette was described previously (Foster, Rybachuk et al. 1998). A similar strategy was utilized to construct the recombinant virus MK Δ gK with the gK gene deleted and replaced with a EGFP gene cassette (Fig. 2.1). In addition, the rescued virus MKgK was constructed, in which the missing gK gene was replaced with the wild-type gK gene, without affecting the adjacent CMV-IE-EGFP gene cassette. Both viruses were extensively characterized by PCR diagnostics to ensure the absence of any wild-type gK sequences from the MK Δ gK virus and the restoration of the gK gene in the MKgK virus (not shown). As expected, the MK Δ gK virus formed extremely small plaques on Vero cells composed of a maximum of 5-10 cells, while the MKgK virus formed large plaques, which were indistinguishable from the prototypic McKrae virus. MK Δ gK virus plaques on the gK complementing cell line VK302 were rescued to nearly the same size produced by the MKgK virus (Fig. 2.2: A-F).

The replication kinetics of the MK Δ gK and MKgK viruses was investigated in Vero cells, as well as in VK302 cells that constitutively express gK at different MOI. The MKgK virus appeared to replicate slightly slower than the wild-type McKrae virus, however, both approached similar titers at late times post infection. The MK Δ gK virus exhibited approximately two logs (one hundred times) lower replication in comparison to the McKrae and MKgK viruses. Replication of MK Δ gK virus in the VK302 complementing cell line produced virus titers similar to those observed with the McKrae and MKgK viruses (Fig. 2.3).

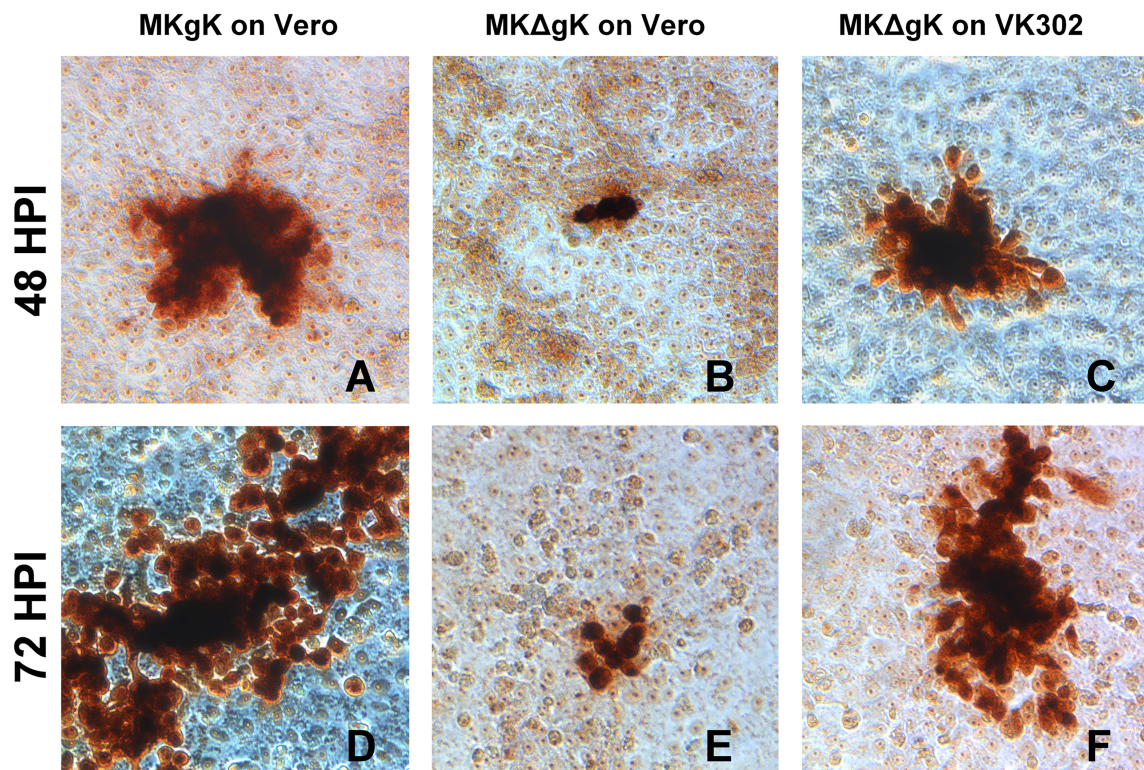


Figure 2.2: MK Δ gK and MKgK plaque morphology. Confluent Vero (A, B, D, E) or gK complementing VK302 cells monolayers (C, F) were infected with either the MKgK-rescued virus, or the MK Δ gK (gK-null) virus at an MOI of 0.001, and viral plaques were visualized by immunohistochemistry at 48 (A, B, C) or 72 (D, E, F) hpi. All images are taken at 200X magnification.

Virus replication kinetics at MOI 0.1

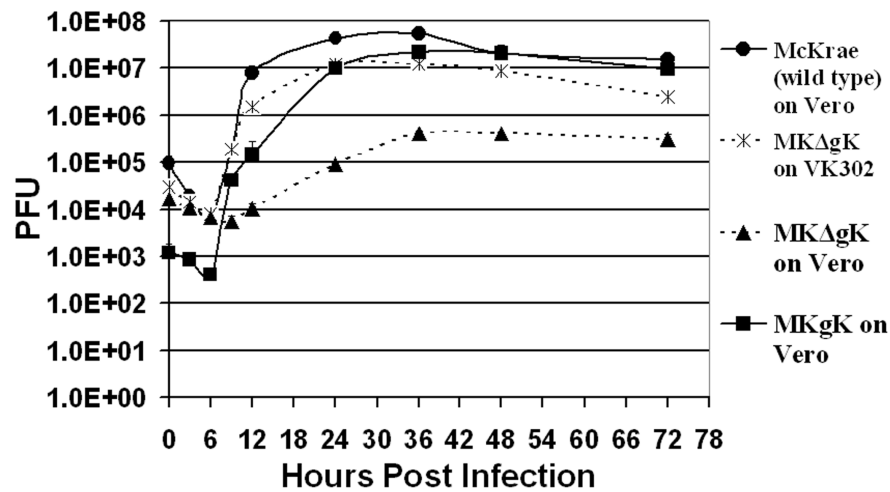


Figure 2.3: Virus replication kinetics. Vero or VK302 cell monolayers were infected with either MKΔgK, MKgK or McKrae viruses at MOI of 0.1 and virus titers were obtained at different times post infection on either Vero or VK302 cells.

In Vivo Phenotypes and Clinical Disease Patterns of MKgK and MKΔgK Viruses in the Mouse Eye Model System

Corneas are composed principally of a fairly thin epithelial layer resting on a much thicker stromal layer composed primarily of collagen fibers. Corneas are typically highly innervated tissues. To ensure that viral infections could access nerve endings within the cornea, infections were performed with mild or more extensive scarification (see Materials and Methods). MKgK viral infections produced viral plaques, which were first visible by stereoscopic fluorescence microscopy at 12 hpi appearing as streaks of multiple viral plaques. By 24 hpi viral plaques were spread substantially, and by 72 hours viruses appeared to have spread to the iris. In mice infected after swab abrasion of their corneas, fluorescent viral plaques were less numerous on the visual surface, but qualitatively appeared to be overrepresented at the limbus in comparison to mouse eyes

infected after needle scarification. In addition, at late times post infection, the iris of mice infected after cotton swab scarification appeared to have less pronounced infection, as evidenced by the substantial reduction in the number of fluorescent viral plaques (data not shown).

Infected mice were monitored daily for clinical indicators of eye infection, such as blepharitis, hyperemia and ocular discharge for 30 days post infection and the intensity of the infection indicators were recorded on scale of 0-3. Typically, mouse eyes infected with viruses after scarification were scored 0 when there were no symptoms of clinical disease (Fig. 2.4: A). Eyes scored with a score of 1 exhibited minimum blepharitis, hyperemia, and ocular discharges at the medial canthus (Fig. 2.4: B). Eyes scored 2-3 exhibited progressively more severe clinical symptoms outlined above producing ultimately crusting and complete occlusion of the eye (Fig. 2.4: C-E). The MKgK virus produced severe disease in most mouse eyes (score 2-3), while few eyes were scored at lower scores (score 1). The MKΔgK virus produced no significant clinical disease (score 0) in infection experiments even though approximately two logs higher virus titers of MΔgK (1.0×10^6 PFU) were used than the MKgK virus (2.5×10^4 PFU) (Fig. 2.5: A). Similar experiments were performed with the exception that mice eyes were abraded with cotton swabs as opposed to needle scarified. Overall, peak clinical disease observed in the unscarified mouse experiments was similar to that of the scarified experiments with disease peaking within 7-9 dpi. Again, clinical disease was pronounced with the MKgK virus, while the MKΔgK virus produced no disease symptoms. The only apparent differences between the abrasion versus the more severe scarification results, were that in the mildly abraded eyes, the resolution of clinical lesions was faster with an average

return to normal conditions at approximately 14-16 dpi. In the more extensively scarified eyes, many mice exhibited grade 1 lesions at much later times post infection (greater than 20 dpi) (Fig. 2.5: A, B).

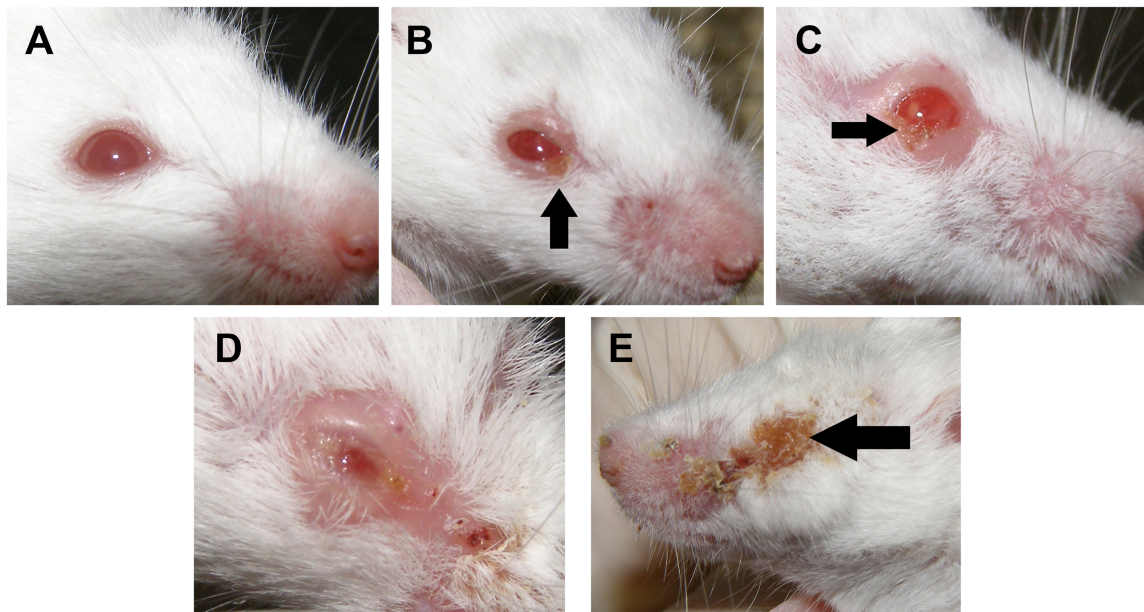


Figure 2.4: Clinical disease progression. Mouse eyes were monitored for clinical signs of disease as detailed in Materials and Methods. (A) Clinically normal mouse. (B) Lesion with a score of 1 characterized by mild hyperemia, blepharitis and ocular discharge. (C) Lesion with a score of 2 characterized by moderate hyperemia, blepharitis and ocular discharge. (D and E) Lesion with a score of 3. (D) Picture shows severe hyperemia and blepharitis. (E) The arrow indicates the position of the eye, which is completely covered by a crust of ocular discharge. In figures B and C, arrows indicate accumulation of ocular discharge near the medial canthus and lateral canthus respectively.

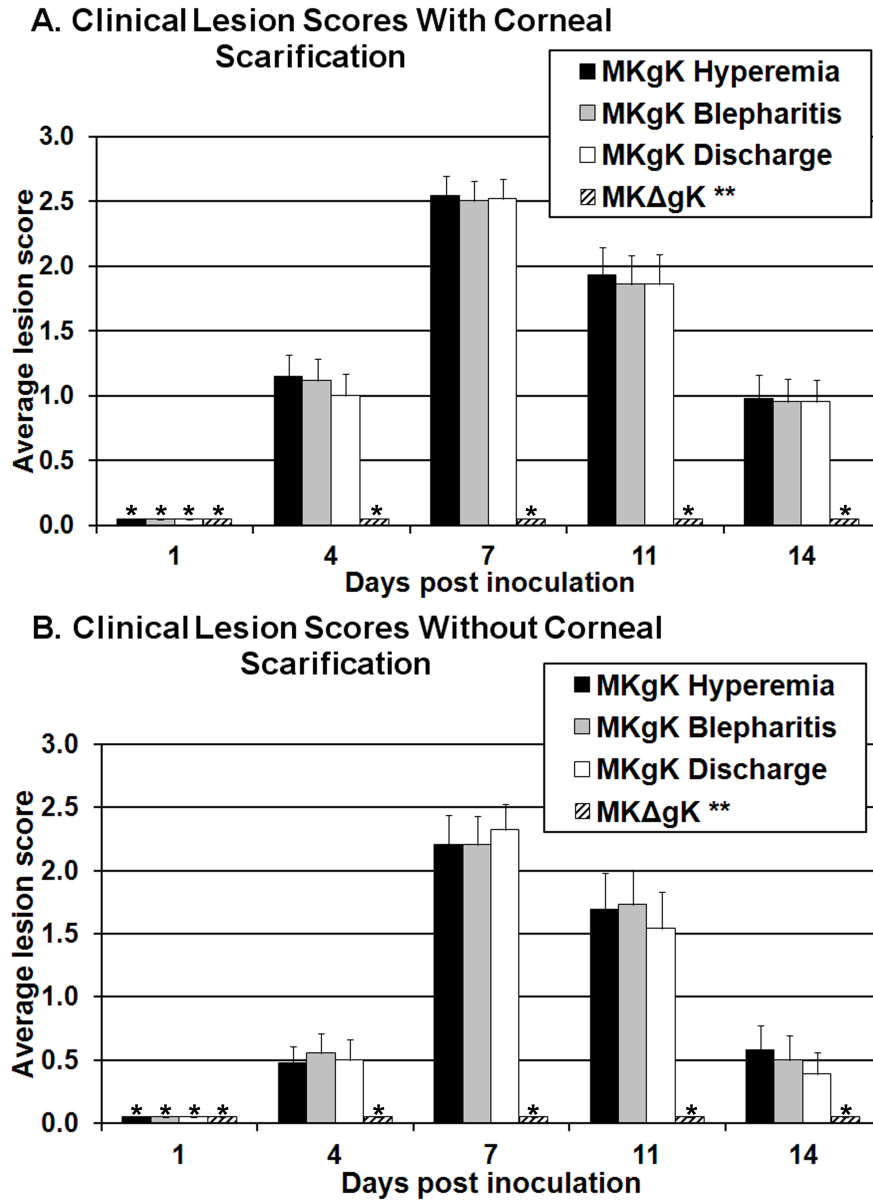
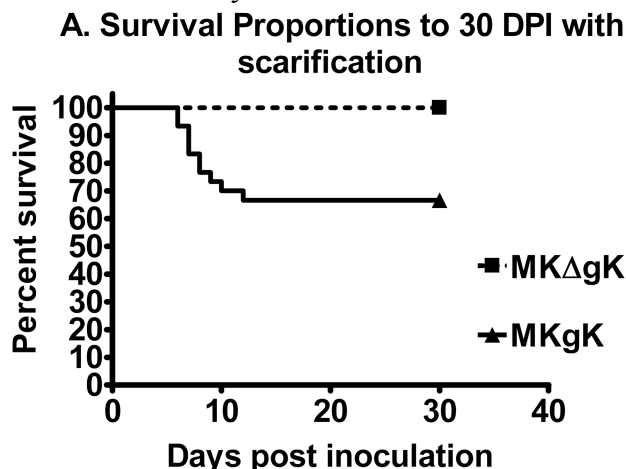


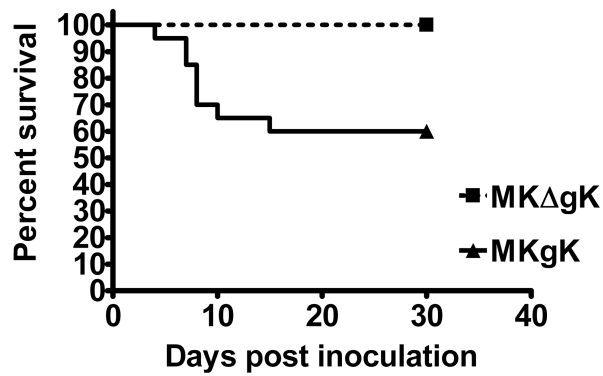
Figure 2.5: Clinical lesion scores. Mice were infected ocularly with MKgK or MKΔgK viruses. Ocular disease was based on a 0 to 3 scale for the parameters blepharitis, hyperemia, and ocular discharge as described in the Material and Methods section. (A) Graph shows the average clinical lesion scores in scarified mice on representative days 1, 2, 4, 7, 9, 11 and 14 following infection. (B) Graph shows the average clinical lesion scores in non-scarified mice on the same days. Data are presented as the group mean of at least 12 mice per group with error bars indicating the standard error. Bars denoted by an * represent an average lesion score of 0 (The small positive bar is presented for visual reference only). Error bars are not present on these bars since standard error is also 0.

Mice were also monitored for survival for 30 days following inoculation. Regardless of scarification status, all mice inoculated with the MK Δ gK strain survived to 30 dpi (Fig 2.6: A, B). In scarified mice, 10 of the 30 mice inoculated with the rescued virus died during the study with deaths occurring between 6 and 12 dpi (Fig 2.6 A). In non-scarified mice, 8 of the 20 mice inoculated with the rescued virus died during the study with deaths occurring between 4 and 15 dpi (Fig 2.6: B). Kaplan-Meier survival curve analysis revealed a statistically significant difference in death rate for both scarified and unscarified groups at a P=.001 (Fig 2.6: A, B). However there was no statistical significance in the survival when comparing the mildly scarified with the more extensively scarified groups that were inoculated with the rescue strain (Fig 2.6: C).

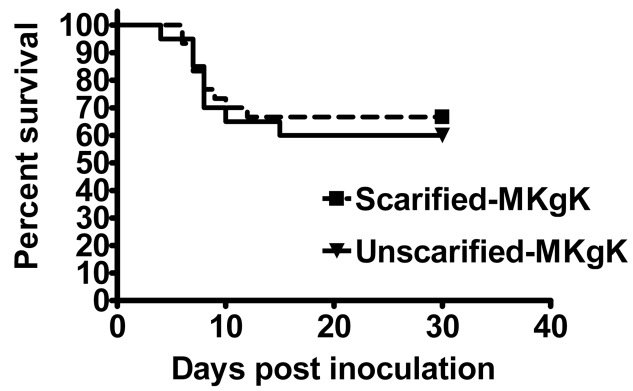
Figure 2.6: Mouse survival data after infection with recombinant viruses. (A-C) In clinical lesion studies, mice were also monitored daily for survival to 30 days post infection. (A) In scarified mice, 10 of the 30 mice inoculated with the rescued virus died during the study with deaths occurring between days 6 and 12 post inoculation. All mice inoculated with the gK-null strain survived. (B): In non-scarified mice, 8 of the 20 mice inoculated with the rescued virus died during the study with deaths occurring between days 4 and 15 post inoculation. All mice inoculated with the gK-null strain survived. (C) There is no statistical difference between scarified or non-scarified groups when inoculated with MKgK. (A, B) This shows a statistically significant difference in death rate for both scarified and unscarified groups at a P=.001 (Fig 2.6: A, B) by Kaplan-Meier survival curve analysis. B and C continued on next page.



B. Survival Proportions to 30 DPI Without Scarification



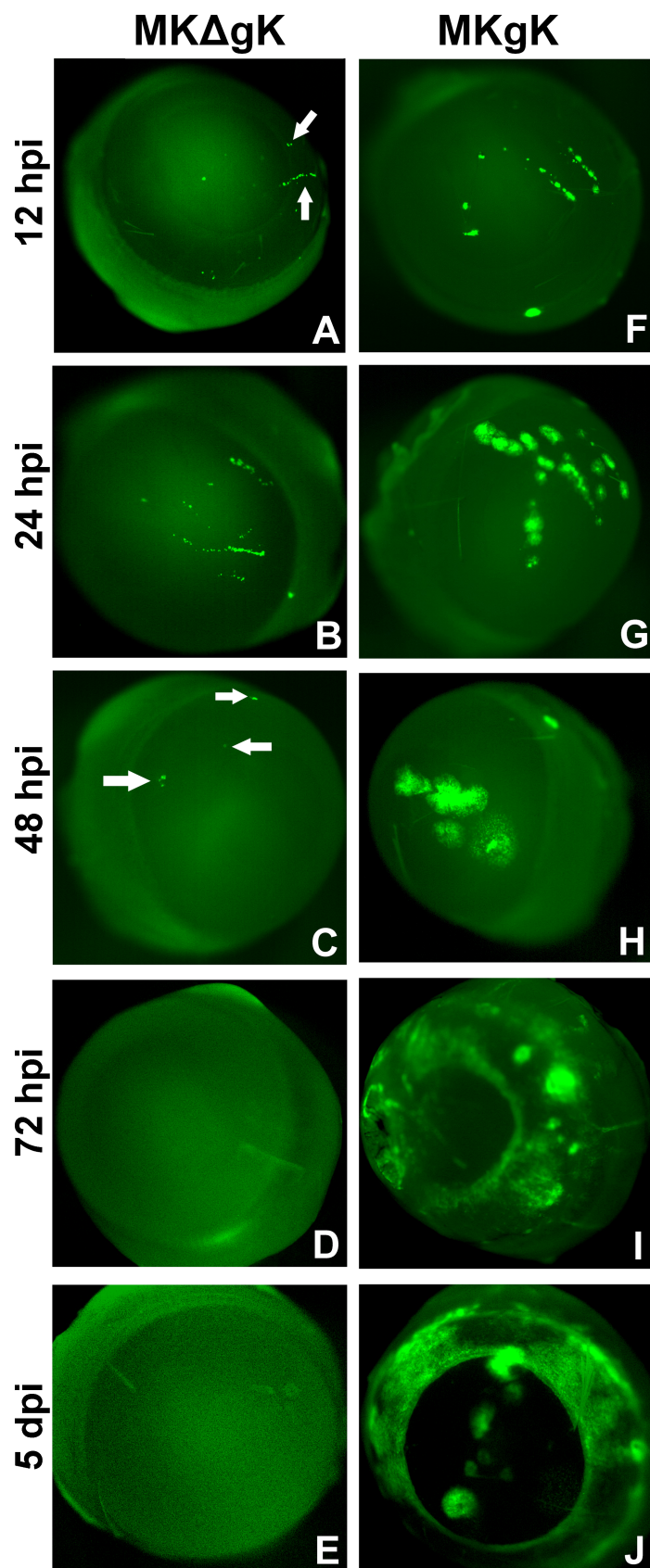
C. MKgK Survival Scarification vs. Non-scarification



Visualization of Viral Plaque Formation in Mouse Eyes

To study the degree of invasiveness and spread of viruses from the site of initial ocular infection, mice were infected, following corneal scarification, with the MK Δ gK or MKgK viruses. All mice in each group were sacrificed at 12, 24, 48 and 72 hpi and 5, 7, 10 and 14 dpi. The eyes were excised intact and observed directly under an inverted fluorescence microscope. 5 mice (10 eyes) /group/time point were examined and representative pictures of the spread of fluorescent viral plaques at various time points are shown (Fig. 2.7). MKgK produced viral plaques in mouse corneas that were easily visible through direct observation of tissues using a fluorescent microscope as early as 12 hpi. These viral plaques increased substantially over time and appeared to spread to mouse iris by 3-4 dpi (Fig. 2.7: F-J). In contrast, the MK Δ gK virus, produced very small plaques at 12 hours post infection, with an increasing number of viral plaques appearing at 24 hpi. However, these viral plaques were substantially less visible at 48 hpi and all mice in this group were devoid of viral plaques by four dpi (Fig. 2.7: A-E). These results were consistent with virus isolation from eye swabs that revealed less than 10 viruses per MK Δ gK-infected eye, while MKgK-infected eyes yielded more than 1,000 plaque forming units (PFU) per eye (not shown). The fluorescent plaques produced in mouse eyes were also scored on a 0-3 point scale, average scores by day are shown (Fig. 2.8). This graph and the previous pictures show that virus spread was markedly impaired in the MK Δ gK virus, with minimal plaque formation, smaller plaques and more rapid resolution of plaques.

Figure 2.7: Virus spread in mouse corneas. Spread and replication of MKgK versus MKΔgK was studied in ocular infection of mice. The infected eyes in each group were directly examined under an inverted fluorescence microscope. (A-E) Mouse eyes infected with MKΔgK at 12hpi (A), 24hpi (B), 48hpi (C), 72hpi (D) and 5dpi (E). (A, C) White arrows point to small plaques in these eyes. (F-J) Mouse eyes infected with MKgK at 12hpi (F), 24hpi (G), 48hpi (H), 72hpi (I) and 5dpi (J). Figures are representative of 5 mice (10 eyes)/group/time point. All images were taken at 17-22X magnification.



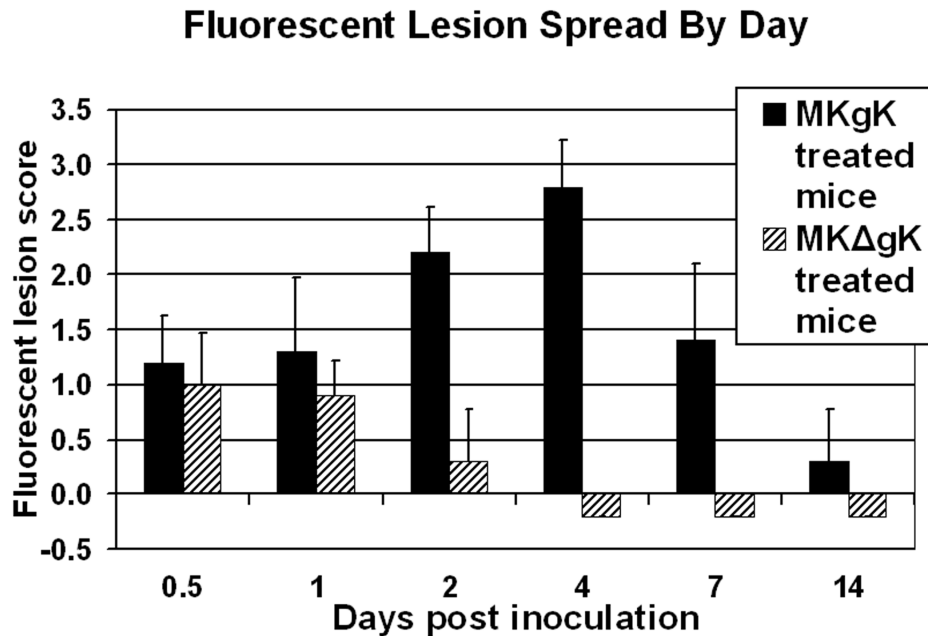


Figure 2.8: Graphical representation of virus spread. Mice were infected after corneal scarification with the MKΔgK or MKgK viruses. Then the eyes of the mice were excised and viral spread was tracked by direct observation under a fluorescence microscope. Fluorescent plaque lesions were scored using a 0-3 scale described in materials and methods. Bars represent the mean of 5 mice (10eyes)/group/time point. Mice were sacrificed at 12, 24, 48 and 72 hpi and 5, 7, 10 and 14 dpi. Bars denoted by an * represent an average lesion score of 0 (The small positive bar is presented for visual reference only). Error bars are not present on these bars since standard error is also 0.

Determination of Virus Spread to the Trigeminal Ganglia

Typically, infection of mouse corneas with wild-type HSV-1 leads to transport of virions in a retrograde fashion within neuronal axons and establishment of latency in trigeminal ganglia. To determine the relative efficiency by which MKgK and MKΔgK viruses reached the trigeminal ganglia, extracted ganglia were tested for the presence of infectious virus by co-culture in Vero or VK302 cells. In these studies, mice were used at least 10 days post complete resolution of clinical symptoms. In the scarified groups, 19 of 20 mice in the MKgK virus infected group had trigeminal ganglia that produced infectious virus after coculture with Vero cells, while 0 of 20 mice in the MKΔgK group

produced infectious virus (Table 2.1). In the non-scarified mice inoculated with the MK Δ gK virus, 0/12 trigeminal ganglia produced infectious virus after co-culture with VK302 cells (Table 2.1). In contrast, 5/12 non-scarified mice that were inoculated with MKgK virus produced infectious virus after co-culture with Vero cells (Table 2.1).

Additional testing was performed to detect the presence of viral DNA within trigeminal ganglia of infected mice. In mice inoculated with MKgK without scarification, HSV DNA was detected by PCR in 14/24 (58.3%) TG's, corresponding to 9/12 (75%) mice (Fig 2.9, Table 2.1), while MK Δ gK infections identified 0/24 TG ganglia as PCR-positive corresponding to 0/12 mice (Table 2.1). In mice inoculated with MKgK after scarification, HSV DNA was detected in the pooled TG by PCR in 19 out of 20 mice. In mice inoculated ocularly after scarification with MK Δ gK, 3 of 20 mice were PCR-positive for HSV-1 viral DNA (Table 2.1).

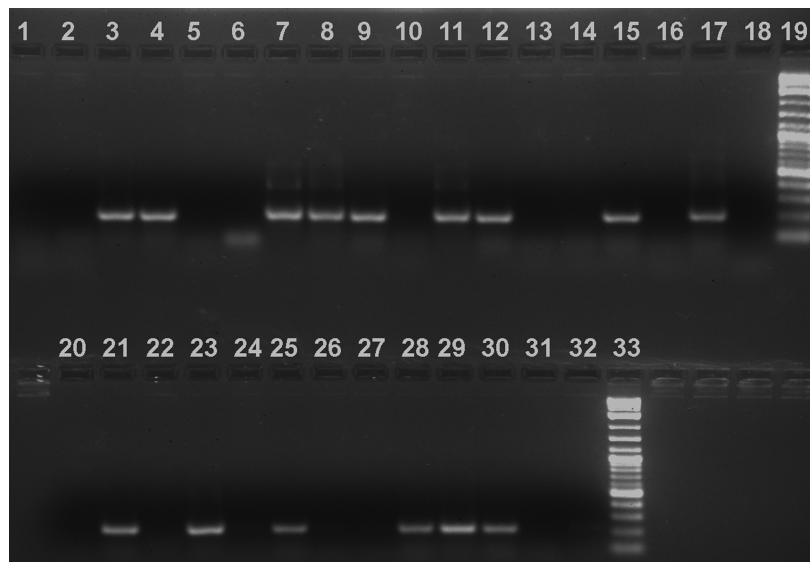


Figure 2.9: Nested PCR detection of viral DNA within mouse ganglia. Total DNA was extracted from mouse ganglia and viral DNA was detected using a nested PCR protocol (see Materials and Methods). Lanes 1-18 and 20-25 correspond to the right and left TG, respectively, of 12 unscarified and MKgK-infected mice (24 lanes). Lanes 19 and 33 contain 2-Log DNA molecular mass ladder. Lanes 26, 27, 31 and 32 contain negative controls. Lanes 28-30 contain positive controls.

Table 2.1: Rates of Viral Latency, Reactivation, and Mouse Survival Data

Virus	Scarified or unscarified	Inoculum Dose	Survival Rate – No. alive at 30 dpi/total mice	No. of mice that had TG reactivation/total	No. of mice that had HSV PCR positive TG/total
MKgK	Unscar	2.5 x 10 ⁴ pfu/eye	12/20*	5/12+	9/12#
MKΔgK	Unscar	1.0 x 10 ⁶ pfu/eye	12/12*	0/12+	0/12#
MKgK	Scar	2.5 x 10 ⁴ pfu/eye	20/30**	19/20++	19/20##
MKΔgK	Scar	1.0 x 10 ⁶ pfu/eye	30/30**	0/20++	3/20##

Table 2.1: (* and +) There is a statistically significant difference between the two groups at P<0.05. (#, **, ++, ##) There is a statistically significant difference between the two identified groups at P<0.001. Fishers exact test for each pairing was performed using GraphPad Prism version 4.0 for Windows.

DISCUSSION

We have shown previously that HSV-1 glycoprotein K (gK) is not essential for viral entry into cells, but is essential for virus spread in cell culture, inasmuch as gK-null viruses form very small viral plaques typically consisting of less than 10 infected cells per plaque (Jayachandra, Baghian et al. 1997; Melancon, Luna et al. 2005). This viral phenotype is produced because lack of gK drastically inhibits cytoplasmic virion envelopment presumably occurring at trans-Golgi network (TGN) membranes (Foster, Melancon et al. 2004a; Foster, Melancon et al. 2004b; Foster and Kousoulas 2008). A similar viral phenotype has been also described for pseudorabies virus (PRV) indicating that HSV-1 and PRV gK function in a similar fashion in viral morphogenesis and egress in tissue culture (Klupp, Baumeister et al. 1998). In vivo work with PRV indicated that although gK was essential for viral spread in cell culture, it was not essential for

neuroinvasiveness (Flamand, Bennardo et al. 2001). Therefore, we undertook this study to investigate whether HSV-1 gK was essential for neuronal infection and disease causation in the mouse model system. We found that HSV-1 gK is essential for corneal spread in the mouse eye, subsequent transmission and infection of the central nervous system, and disease causation.

HSV-1 McKrae was isolated from a human ocular herpes infection (Kaufman, Ellison et al. 1969) and it is highly virulent in mice and rabbits (Perng, Thompson et al. 1995; Perng, Chokephaibulkit et al. 1996; Halford, Balliet et al. 2004). The recombinant virus MK Δ gK was constructed to have a deletion of the gK gene, while at the same time carrying a HCMV-IE driven EGFP gene cassette that enabled tracking of viral infection using a fluorescence microscope. The rescued virus, MKgK was produced by rescuing the gK gene, while leaving the EGFP gene cassette intact. The MKgK virus titers at 48 hours post infection were similar to the wild-type McKrae virus indicating that the presence of the EGFP gene cassette constitutively expressing EGFP did not adversely affect virus replication. However, it was noted that the MKgK virus appeared to replicate slightly slower than the MKgK virus at earlier times post infection suggesting that EGFP expression may had a mild negative effect on virus replication at early times post infection.

HSV-1 virulent strains including HSV-1 McKrae can readily infect the mouse eye via the cornea leading to efficient transmission to the central nervous system and establishment of viral latency in the trigeminal ganglia. Following infection of murine eyes with HSV-1, viral replication occurs initially in the corneal epithelium, and subsequently the virus is transmitted via epithelial-neuronal interfaces to neurons.

Virions are thought to move in the retrograde direction within neuronal axons to neuronal bodies where they establish latency (Cunningham, Diefenbach et al. 2006; Diefenbach, Miranda-Saksena et al. 2008). Infection of the CNS via the ocular route is very efficient because the cornea is a highly innervated tissue with a high density of nociceptors per square millimeter. Specifically, the human cornea is estimated to have more than 7,000 nociceptors per square millimeter (Muller, Marfurt et al. 2003). Thus, direct inoculation of HSV-1 in the eyes of mice was predicted to have ample access to sensory neuronal fiber endings innervating the cornea. Mild abrasion of the cornea with cotton swabs was expected to expose neuronal endings innervating the cornea, while more aggressive scarification of the cornea would be expected to give direct access of the virus to nerve bundles in the sub-basal plexes underlying the epithelium of the cornea.

Apparently, constitutive expression of the EGFP gene did not significantly affect viral replication in tissue culture. Similarly, viral plaques in mouse corneas were similar to those produced by the wild-type McKrae strain (not shown). Infection of the mouse eyes either with the cotton-swab or needle scarification protocols revealed that, in comparison to the MKgK rescued virus, the MK Δ gK virus was not able to efficiently replicate and spread in mouse corneas despite the fact that a two log higher virus inoculum of the MK Δ gK virus was used and prepared in the complementing cell line VK302. Specifically, MK Δ gK was able to infect cells, as evidenced by the production of small viral plaques that appeared to enlarge by 24 hpi; however those plaques were not any more visible at later times post infection presumably because infected cells had been eliminated. Clinical disease symptoms absolutely paralleled the observed infection patterns with the MK Δ gK infected eyes exhibiting no clinical signs at any time point

following viral infection, while the MKgK rescued virus produced significant eye disease by 4 dpi. This disease was characterized by severe hyperemia, blepharitis (thickening of the eyelid), inflammation and ocular discharge. In addition, the MKΔgK virus did not cause any mortality in the infected mice, while the MKgK virus produced significant mortality, as well as clear signs of neurological damage characterized by visual observation of hunched posture, ataxia, circling and seizures.

A priori, neuronal infection via the eye route should lead to efficient establishment of viral latency in mouse trigeminal ganglia. Nineteen-of-twenty mice infected with the MKgK-rescued virus released infectious virus after cocultivation of mouse ganglia with Vero cells, and 19/20 mice contained viral DNA in their ganglia detectable by PCR. Mice infected after cotton swab eye scarification appeared to have a lower incidence of viral latency reduced by approximately 25% -50% taking into account either PCR detection of viral DNA, or virus isolation, respectively. Infection of murine eyes following either cotton swab or needle scarification revealed that the MKΔgK virus was not able to efficiently establish latency as evidenced by the fact that only 3/20 ganglia were positive for viral DNA by PCR in the scarification model, while none of the ganglia were positive in the cotton swab scarification model. The fact that no virus was isolated from any ganglia in coculture experiments may be partly due to the inability of gK-null viruses to efficiently egress out of infected cells.

Overall, these results strongly show that gK is essential for corneal spread, neuroinvasion, and infection and establishment of latency in mice infected via the ocular route. In this regard, this result is substantially different from those obtained with the PRV system suggesting that HSV-1 and PRV may exhibit substantial differences in their

requirements for neuroinvasion. In the PRV gK-null virus experiments, mice were infected via the intranasal route. It is possible that infection via this route may access the central nervous system more efficiently than the ocular route. However, additional experiments in mice have revealed that the MK Δ gK virus is also avirulent when administered at relatively high titers (greater than 10^6 PFU) either via the intranasal or intramuscular routes (not shown) suggesting that PRV and HSV-1 gK-null viruses use substantially different mechanisms for neuroinvasion.

HSV-1 gK may be particularly important for the causation of ocular disease in humans because it has been reported to exacerbate ocular disease mediated by anti-gK humoral and cellular immune responses (Ghiasi, Cai et al. 1996; Ghiasi, Cai et al. 1997; Mott, Perng et al. 2007; Osorio, Mott et al. 2007). Thus, gK is an important determinant of both viral infectivity and ocular disease. The high probability that neuronal endings were readily accessible in the mouse eyes after extensive scarification and the relative lack of viral DNA in mouse ganglia argues for a role of gK in direct neuronal infection as well as indirect transmission of virus from corneal epithelial cells to nerve endings. It is also possible that the lack of gK may lead to the formation of defective tegumented capsids, which are unable to move in a retrograde fashion. Additional in vitro and in vivo experiments are needed to resolve the potential role(s) of gK in neuronal infectivity and retrograde transport.

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CHAPTER III

A MUTANT HERPES SIMPLEX VIRUS TYPE-1 (HSV-1) MCKRAE LACKING THE GLYCOPROTEIN K (GK) GENE IS UNABLE TO BE TRANSPORTED IN AXONS IN EITHER THE ANTEROGRADE OR RETROGRADE MANNER

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is an important human pathogen that typically causes mucocutaneous lesions in facial and genital epithelial/mucosal surfaces. The hallmark of HSV-lifecycle is infection of sensory neurons where the virus establishes a latent infection for the life of the host (Whitley, Kimberlin et al. 1998). HSV-1 neuronal infection can occasionally cause acute encephalitis (Kimberlin, Lin et al. 2001), while ocular HSV-associated disease is one of the most common infectious causes of blindness in developed countries (Liesegang, Melton et al. 1989; Liesegang 2001).

HSV-1 enters into neuronal cells via a pH-independent fusion of the viral envelope with neuronal plasma membranes (Qie, Marcellino et al. 1999; Nicola, Hou et al. 2005), while it can enter into a wide range of non-neuronal cells via either pH-independent or pH-dependent endocytosis (Milne, Nicola et al. 2005). Virus entry into all cells involves the coordinated functions of glycoproteins gD, gB, gH, gL and gC (Cai, Gu et al. 1988; Desai, Schaffer et al. 1988; Ligas and Johnson 1988; Hutchinson, Browne et al. 1992). Viral glycoprotein gD binds to different cellular receptors including the herpesvirus entry mediator (HVEM, or HveA), nectin-1 (HveC), or 3-O-sulfated heparan sulfate (Montgomery, Warner et al. 1996; Geraghty, Krummenacher et al. 1998; Shukla, Liu et al. 1999). Apparently, gB can also bind to additional receptors including paired immunoglobulin-like type 2 receptor alpha (PILR-a), non-muscle myosin heavy chain

IIA (NMHC-IIA), and myelin-associated glycoprotein (MAG) that function in virion attachment and virus entry (Satoh, Arai et al. 2008; Arai, Goto et al. 2010; Suenaga, Satoh et al. 2010). Binding of gD and gB to their cognate receptors is thought to trigger sequential conformational changes in gH/gL and gB causing fusion of the viral envelope with cellular membranes during virus entry, as well as fusion among cellular membranes (Heldwein, Lou et al. 2006; Hannah, Heldwein et al. 2007; Connolly, Jackson et al. 2011).

Fusion of the viral envelope with cellular membranes causes deposition of the viral capsid into the cytoplasm, which is subsequently transported to the cell nucleus (Morrison, Stevenson et al. 1998; Morrison, Wang et al. 1998; Ojala, Sodeik et al. 2000). This retrograde transport of capsids to the neurons is highly dependent on the cellular microtubule network and mediated via, most likely, direct interactions by one or more tegument and capsid proteins with the dynein motor and the dynein cofactor dynactin (Kristensson, Lycke et al. 1986; Topp, Meade et al. 1994; Sodeik, Ebersold et al. 1997; Dohner, Wolfstein et al. 2002), reviewed in (Diefenbach, Miranda-Saksena et al. 2008).

Infectious virus production is initiated in the nucleus of cells where capsids assemble. The virus acquires an initial viral envelope by budding of capsids into perinuclear spaces (Diefenbach, Miranda-Saksena et al. 2008; Mettenleiter, Klupp et al. 2009). Fusion of the viral envelope with the outer nuclear membrane is thought to deliver partially tegumented capsids into the cytoplasm. These capsids acquire additional tegument proteins before acquiring their final envelopes by budding into intracellular vesicles, most likely, originating from the trans-Golgi network (TGN) (Mettenleiter 2002; Mettenleiter 2004). Fully-matured virions are finally released through an exocytosis

process that appears to involve the kinesin family of microtubular network-associated motors reviewed in (Diefenbach, Miranda-Saksena et al. 2008; Mettenleiter, Klupp et al. 2009).

The mechanism of anterograde transport of alphaherpesvirus in neuronal cells is highly controversial to a large extent because of disparate results obtained with pseudorabies virus (PRV) and HSV-1. Two principle mechanisms of axonal transport have been proposed: 1) independent axonal transport of viral glycoproteins and capsids and assembly of the enveloped virions in the plasma membrane; 2) transport of fully enveloped virions that have formed via budding of capsids into TGN-derived membranes (Diefenbach, Miranda-Saksena et al. 2008; Liu, Goodhouse et al. 2008; Mettenleiter 2008; Mettenleiter, Klupp et al. 2009; Antinone and Smith 2010; Antinone, Zaichick et al. 2010; Maresch, Granzow et al. 2010; Osakada and Cui 2011b).

A number of viral glycoproteins have been implicated in efficient egress of virions from infected non-neuronal cells (Mettenleiter, Klupp et al. 2009). However, the role of viral glycoproteins in both retrograde and anterograde neuronal transport has not been extensively studied. PRV glycoproteins gE and gI are required for anterograde transport, but not retrograde transport (Card, Whealy et al. 1992; Jacobs, Mulder et al. 1993; Babic, Klupp et al. 1996; Johnson, Webb et al. 2001; Brittle, Reynolds et al. 2004; Ch'ng and Enquist 2005). HSV-1 gE has been reported to function in both retrograde and virus spread from epithelial cells to neurites (Balan, Davis-Poynter et al. 1994; Saldanha, Lubinski et al. 2000; Wang, Tang et al. 2005; Brittle, Wang et al. 2008). Experiments with neuronal cultures in microfluidic chambers revealed that HSV-1 (gE) was not required for direct infection of neurites, however, it enhanced transmission of virus from

epithelial cells to neurites. In addition, gE-null virions that entered into neurites did not exhibit any defect in retrograde axonal transport (McGraw and Friedman 2009).

HSV-1 gK is a structural component of the virion particle and functions in virus entry into epithelial cells (Foster, Rybachuk et al. 2001; Jambunathan, Chowdhury et al. 2011), cytoplasmic virion envelopment, virion egress and virus-induced cell fusion (Hutchinson, Roop-Beauchamp et al. 1995). In infected cells, gK is found in a functional complex with the membrane protein UL20 (Foster and Kousoulas 2008). UL20/gK interactions are essential for their intracellular transport, cell-surface expression, and functions in cytoplasmic virion envelopment and virus-induced cell fusion (Foster, Alvarez et al. 2003; Foster, Melancon et al. 2004a; Foster, Melancon et al. 2004b; Melancon, Fulmer et al. 2007; Foster and Kousoulas 2008). Recently, we showed that HSV-1 gK and UL20 physically bind to gB and gH and modulate gB-mediated membrane fusion (Chouljenko, Iyer et al. 2009; Chouljenko, Iyer et al. 2010). Also, we reported that gK was essential for virus spread in the cornea of mice, neuroinvasiveness and establishment of latency into ganglionic neurons (David, Baghian et al. 2008). In this report, we utilize a microfluidic chamber to show that gK is required for infection of neurites and retrograde and anterograde transport in neurons.

MATERIALS AND METHODS

Cells and Viruses

African green monkey kidney (Vero) cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and grown and propagated in Dulbecco's modified Eagle Medium (DMEM) supplemented with 7% fetal bovine serum (FBS) and

antibiotics. The gK complementing VK302 cell line (generously provided by Dr. David Johnson, Oregon Health Sciences University, Portland, OR, USA) was maintained in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. This cell line was used for generation and maintenance of McKrae Δ gK viral stocks. Vero and VK302 cells were used to determine the replication characteristics (plaque morphology) for parental McKrae virus and all recombinant viruses used in these experiments. The clinical ocular isolate and neuroinvasive strain of HSV-1 (the parental wild-type), McKrae strain, was obtained from Dr. J. M. Hill (Louisiana State University Health Sciences Center, New Orleans, LA, USA) (Fig 3.1:A).

Construction of Recombinant Viruses

The construction methodology of mutant viruses for this experiment is essentially similar to the techniques that were previously described to create KOS strain mutants (Jayachandra, Baghian et al. 1997; Foster, Rybachuk et al. 1998; Foster and Kousoulas 1999; David, Baghian et al. 2008). McKrae virus with deleted UL53 (gK) gene (Δ gK; (Fig 3.1: B)) was constructed using the ICP27-null (d27-1) virus and plasmid pSJ1724, as described previously for the HSV-1 (KOS) Δ gK virus (Jayachandra, Baghian et al. 1997). The ICP27-null virus has 1627 base pair (bp) of UL54 gene deleted, but contains a complete UL53 gene. Plasmid pSJ1724 has a 1068 bp deletion in UL53, but has an intact UL54. Recombination between homologous regions of d27-1 virus and plasmid pSJ1724 resulted in the Δ gK virus. This virus does not contain an EGFP cassette and is designated in this paper as Δ gK/GFP-. This Δ gK virus was rescued (R/GFP-) (Fig 3.1: C) by transfecting Vero cells with PCR amplified fragments of UL53 gene from wild-type

HSV-1 (KOS) strain followed by infection with the Δ gK virus (Jayachandra, Baghian et al. 1997). The UL 53 (gK) gene of HSV-1 McKrae was replaced with an enhanced green fluorescence protein (EGFP) gene cassette under control of the human cytomegalovirus immediate early gene promoter (CMV-EGFP) (Foster, Rybachuk et al. 1998). Briefly, The CMV-EGFP cassette was cloned into the plasmid pSJ1723 to create plasmid pTF9201 as described previously (Foster, Rybachuk et al. 1998; David, Baghian et al. 2008). Plasmid PTF9201 contains an EGFP gene cassette under the human cytomegalovirus immediate early promoter control (HCMV-IE) within a DNA fragment spanning the UL52–UL54 genomic region that lacks the UL53 gene. Plasmid PTF9201 was used in transfection followed by infection with HSV-1 (McKrae) to isolate the McKrae- Δ gK/EGFP virus (Fig. 3.1: D) designated Δ gK/GFP+, appearing as small fluorescent viral plaques under the fluorescence microscope (Foster, Rybachuk et al. 1998; David, Baghian et al. 2008). The McK Δ gK/EGFP virus was rescued using plasmid pTF9105, which contains a complete UL53 gene from the HSV-1 (KOS) strain creating the virus designated R/GFP+ (Fig. 3.1: E) (Foster and Kousoulas 1999).

Animals

Adult female Sprague-Dawley rats, 15-18 days pregnant, were used (Charles River, Wilmington, MA). All procedures were approved by the Louisiana State University-School of Veterinary Medicine Institutional Animal Care and Use Committee.

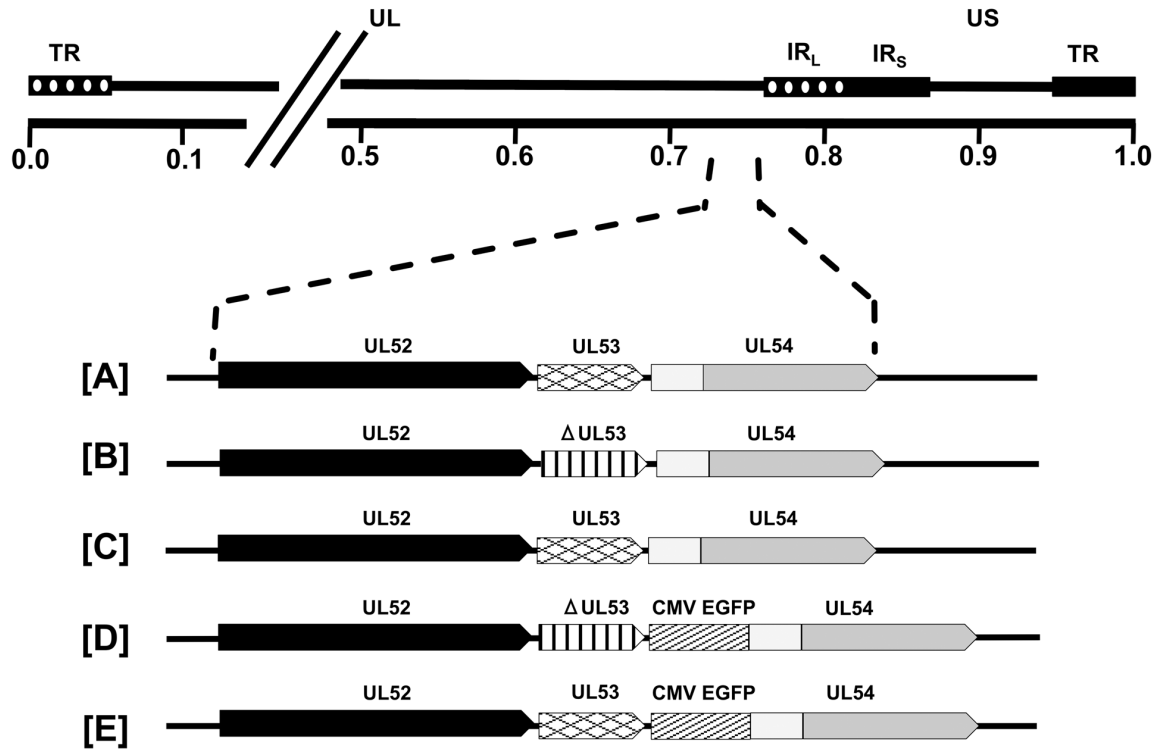


Figure 3.1: Schematic representation of mutant viruses. The top line represents the prototypic arrangement of the HSV-1 genome with the unique long (UL) and unique short (US) regions flanked by the terminal repeat (TR) and internal repeat (IR) regions. (A) McKrae wild type. (B) McKrae parental strain with deletion mutation of gK and without the EGFP cassette (Δ gK/GFP-). (C) Rescue of the gK deletion in the virus (B) without the EGFP cassette (R/GFP-). (D) McKrae parental strain with deletion of the gK gene by replacement with an EGFP cassette (Δ gK/GFP+). (E) Rescue of the virus in (D) with preservation of the EGFP (R/GFP+).

Dorsal Root Ganglion Primary Neuron Culture

Dorsal root ganglia (DRG) neurons were cultured under conditions that were adapted from those described previously for culturing sympathetic superior cervical ganglia-derived neurons (Liu, Goodhouse et al. 2008). Additional modifications were adapted from previously described culture conditions for dorsal root ganglia (Osakada and Cui 2011b; Osakada and Cui 2011a). Specifically, ganglia were dissected from fetal Sprague-Dawley rats at embryonic day 15-18. DRG were incubated at 37° C for 30 min in 250 mg/ml of trypsin (Worthington Biochemical Corp., Lakewood, NJ). Trypsin was neutralized by incubating ganglia in neuron culture media with 10% fetal bovine serum (FBS) and 1 mg/ml trypsin inhibitor (Sigma Aldrich, St. Louis, MO) for 2 min. Ganglia were rinsed with fresh neuron culture media and ganglionic neurons were gently triturated into a single-cell suspension using a pulled-glass Pasteur pipette. Then cells were plated into one side of the microfluidic neuron devices (Cat. number: SND450; Xona Microfluidics, LLC, Temecula, CA) adhered to glass coverslips or the surface of 35mm plastic petri dishes. Glass coverslips or 35mm plastic petri dishes were prepared for neurons by coating with Poly-D-Lysine, molecular weight 75,000 to 150,000 Daltons, (Sigma Aldrich) dissolved in borate buffer at 0.2mg/ml, incubated at 37° C for 48 hours prior to plating of neurons. Neuronal devices were assembled by placing a standard neuronal microfluidic device firmly onto the coverslip or petri dish to ensure a sealed barrier. Maintenance neuron culture media consists of Neural Basal Media with B-27 supplement, at manufacturer's recommended concentration, (Invitrogen, Grand Island, NY). Media was supplemented with 50 ng/ml

neural growth factor 2.5s (Invitrogen), 2% normal rat serum (Invitrogen), 1X Glutamax (Invitrogen) and 0.2% Primocin (Invivogen, San Diego, CA). Once plated, neurons were maintained in a humidified incubator at 37° C and 5% CO₂ for the duration of all experiments. To eliminate non-neuronal cells, 1 and 3 days post-plating, neuronal cultures were treated with 4 mM cytosine β -d-arabinofuranoside (CAB) (Sigma-Aldrich) in neuronal media for 24 hours. Neuronal culture media was changed every 48-72 hours during the course of the experiment.

Infection of Neuron Devices

Neurons were allowed to grow for 10-14 days post plating until robust axonal processes with abundant branching were observed on the axon side of neuron devices. Once present, the neuronal devices were prepared for infection. For retrograde infections, media was completely depleted from the wells of the axonal side of microfluidic devices. Then on the neuron-soma-side of the devices, wells were filled to maximum fluid capacity until a small meniscus formed in the neuronal culture media. After establishment of the fluid pressure gradient, axonal side wells would be inoculated with the virus at the concentration indicated in the results. Then, approximately 100 microliters of neuron culture media was placed in the infected wells to ensure mixing of the virus into the central chamber containing the axons. Once the fluid pressure gradient had been established media was only added to the soma side if necessary to combat dehydration and maintain an obvious fluid level difference. In the case of anterograde neuronal infection studies, the axonal side was co-plated with a monolayer of VK302 or Vero cells. Briefly, these cells were trypsinized from a cell-culture flask, the trypsin was

neutralized using 100% FBS, and an abundance of cells were plated on the axon side of the microfluidic neuron devices using a media consisting of a 1:1 mixture of DMEM with 10% FBS and neuronal culture media. VK302 or Vero cells were seeded in devices and allowed to grow for 24-48 hours to ensure a nearly confluent monolayer for experiments. For anterograde infections, media was completely depleted from the wells of the neuron soma side of microfluidic devices. Then on the axon/reporter cell side of the devices, wells were filled to maximum fluid capacity. After establishment of the fluid pressure gradient, soma side wells were inoculated with the virus at the concentration indicated in the results. Then approximately 100 ml of neuron culture media was placed in the infected wells to ensure mixing of the virus into the central chamber containing the axons. Again, after establishment of the gradient, media was only added to the high side (axon/reporter cell side in this case) to replace any loss due to dehydration.

Growth Curves on Neurons

To determine the kinetics of viral replication on ganglionic neurons, 24-well plastic plates were plated with 175,000 ganglionic cells using the above described harvesting protocol. Ganglionic neurons were infected with approximately a multiplicity of infection (MOI) of 0.1 with each of the viruses. Plates were prepared in triplicate and infections were halted by freezing at 0, 12, 24, 48 and 72 hpi. Plates were frozen, thawed and viral titers were determined for each time point by standard plaque assay on VK302 cells (for the Δ gK viruses) or Vero cells (for the parental and rescue strains). Plaques were stained with crystal violet and visualized with a dissecting microscope. Growth curves show the result of the mean of the triplicate plates.

Plaque Morphology

For fluorescent plaque morphology, confluent monolayers of Vero and VK302 cells were infected at MOI of 0.001 with the indicated viruses. EGFP fluorescence of representative plaques was visualized and photographed at 72 hpi with a fluorescence microscope (Olympus, Tokyo, Japan). For immunohistochemical plaque morphology, cells were fixed with 10% formalin at 72 hpi (Melancon, Foster et al. 2004; David, Baghian et al. 2008). Immunohistochemistry was performed with primary rabbit anti-HSV antibodies (1:1000) (Dako, Carpinteria, CA) and the reactions were developed with NovaRed substrate (VectorLabs; Burlingame, CA). Images were taken with an inverted light microscope (Olympus) using relief contrast.

Visualization of Retrograde and Anterograde Infections

For direct observation by phase contrast of the viral infection, neuronal soma sides and axon/reporter cell sides of devices were observed and imaged using an inverted microscope (Olympus) and relief contrast. EGFP fluorescence was observed using the same inverted microscope set for fluorescence. For immunofluorescent antibody analysis of anterograde infections, microfluidic devices were fixed with cold 10% formalin. Then the devices were incubated with blocking buffer (PBS containing 4% BSA and 2% goat serum) for 1 hour at room temperature. Next the primary antibodies were added and devices were incubated overnight at 4°C. Primary antibodies used were mouse monoclonal anti-HSV glycoprotein C IgG2a (Virusys Corp., Taneytown, MD) diluted 1:3000 and mouse monoclonal anti-neurofilament IgG1 (Invitrogen) diluted 1:1000. Next secondary antibodies, goat anti-mouse IgG1 conjugated to Alexafluor 488 and goat

anti-mouse IgG2a conjugated to Alexafluor 594 (Invitrogen), both diluted to 1:1000 were applied. All antibodies were diluted in PBS containing 2% BSA and 1% goat serum. After 30 minutes devices were washed 5 times with PBS. The microfluidic neuron devices were placed on ice and removed from the glass coverslips (per the manufacturer's instructions). Prolong Gold with DAPI (Invitrogen) was added to the coverslips and cells were photographed with an inverted fluorescence microscope.

Sample Collection for PFU and Quantitative PCR (qPCR) Studies

For studies of viral DNA transmission and virus/viral DNA production neuronal devices were sampled in the following manner. The low side (axonal side in retrograde studies and somal side in anterograde studies) was always aspirated first. At the appropriate time points, the media present in the low side wells was aspirated and collected. Then, the sampled side was flushed with a small amount of media. Following that, media from the higher side was collected and that side was flushed with a small amount of media. Subsequently, the high side wells were filled with media and the devices were frozen overnight. Devices were then thawed and the low side was flushed with a small amount of media and this media was collected. Finally, the high side was flushed and collected. This procedure allowed for maintenance of the fluid pressure gradient by always keeping the gradient and allowed for collection of cell-associated virus as well as any free virus in the media. At this point all samples were frozen. Later these samples were thawed and used for analysis of PFU or viral DNA.

Detection of PFU from Anterograde and Retrograde Infections

Samples of media collected from the neuronal devices were thawed and plated in serial dilutions. The number of infectious virions in the media was determined by plaque assay on appropriate (Vero or VK302) monolayers visualized with anti-HSV-1 rabbit polyclonal antibody (Dako) developed with Nova Red (Vector Labs).

QPCR

For quantitative PCR, once thawed, samples were processed using the Qiagen DNeasy blood and tissue kit (Qiagen, Valencia, CA) per the manufacturer's instructions to isolate DNA. qPCR was performed as described previously for Kaposi's sarcoma herpesvirus (KSHV) (Subramanian, D'Auvergne et al. 2008; Subramanian, Sehgal et al. 2010). Specifically, the primers and probe (6-carboxytetramethylrhodamine (TAMRA)) for the real-time PCR were designed to detect HSV-1 US6 (gD). Equal volumes of viral DNA were used for TaqMan PCR 196 analysis. Purified plasmid containing the gD gene was initially used to generate the standard curve. Samples were also tested and genome numbers were determined using validated standards provided by the Path-HSV1-genesig real-time PCR detection kit for Human Herpes Virus 1 (Herpes simplex type 1) (PrimerDesign, LTD, South Hampton, UK).

RESULTS

Replication Characteristics of Recombinant Viruses on Rat Dorsal Root Ganglionic Neurons

We have previously described the construction of recombinant HSV-1 (McKrae) viruses constitutively expressing the enhanced green fluorescence protein (EGFP) under the human cytomegalovirus immediate early promoter (HCMV-IE) (David, Baghian et al. 2008). To determine the role of glycoprotein K (gK) in neuronal replication, a complete set of recombinant viruses with intact or deleted gK genes were constructed and studied. This set of viruses include: 1) the wild-type McKrae strain compared to a mutant virus lacking the gK gene (Δ gK/GFP-) and a virus in which the deleted gK gene was rescued to wild-type (R/GFP-) (Fig. 3.1: A, B, C, respectively); 2) a set of McKrae derived viruses that have a HCMV-IE-EGFP gene cassette replacing the gK gene sequence (Δ gK/GFP+) and a derivative virus that replaced the missing gK gene and retained the HCMV-IE-GFP gene cassette adjacent to the gK gene (R/GFP+) (Fig. 3.1: D, E, respectively).

Infection of dorsal root ganglionic neurons with wild-type McKrae virus resulted in rapid cytopathic effects exhibited as early as 12 hpi. Typically, neurons appeared rounded up, swollen, while the overall number and length of axons present before infection, appeared to be markedly reduced at early times after infection (not shown). Wild-type-like McKrae virus as well, as wild-type-like virus produced after rescue of the deleted gK gene, replicated with approximately equal efficiencies in the presence or absence of the EGFP gene (Fig. 3.2: A, B). However, mutant viruses lacking

the gK gene grown on either Vero, or the gK complementing cell line VK302 replicated 1-2 logs less efficiently than the parental McKrae viruses at late times post infection (Fig. 3.2: A, B).

All wild-type-like viruses (wild-type and gK-rescued virus) produced on average similar size plaques on both Vero and VK302 cells (Fig. 3.3) (David, Baghian et al. 2008). In contrast, gK-null viruses prepared in either Vero or VK302 cells produced on average drastically smaller viral plaques on Vero cells. The gK-null defects were efficiently complemented on VK302 cells, where gK-null viruses produced viral plaques similar in size to the wild-type like viruses (Fig. 3.3). Examination of individual viral plaques by fluorescence microscopy revealed that all viruses containing the GFP gene cassette readily expressed EGFP (Fig. 3.3).

Microfluidic Multi-Chamber Device for Neuronal Transport Studies

A commercially available microfluidic device was used for neuronal transport studies (see materials and methods). This device enables the physical separation of neuronal somas and axon termini under virion impermeable conditions (Fig. 3.4: A). To ensure that virions could not be passively transmitted from one chamber to the other, control experiments were performed as follows: Vero cells were plated in wells on both sides of the microfluidic chamber. Wells of one side were infected with R/GFP+ virus at an MOI of 10. A hydrostatic pressure differential between the infected and uninfected chambers was maintained by ensuring that the uninfected fluid levels were substantially higher than those in the infected wells. The infected cells exhibited extensive cytopathicity and expression of EGFP at 48 hpi. In contrast, uninfected cells did not

exhibit any cytopathic effects, or fluorescence at 5 days post infection suggesting that virions were unable to passively leak from the infected into the uninfected wells (Fig. 3.4:B)

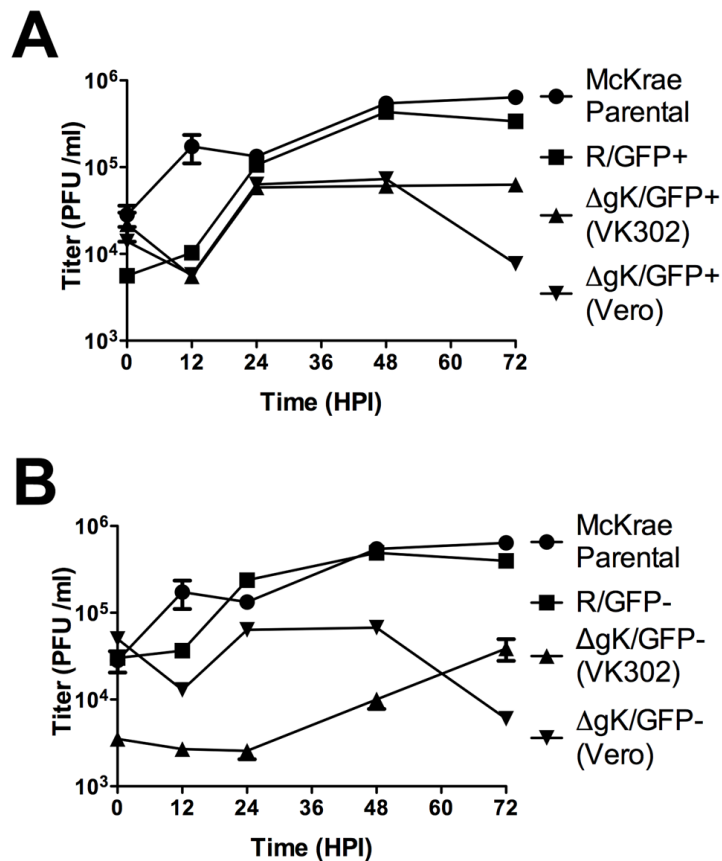


Figure 3.2: Growth curves of HSV-1 (McKrae) and mutants on DRG neurons.

Approximately 175,000 ganglionic cells plated in 24 well plates were infected with the parental McKrae strain, R/GFP+, ΔgK/GFP+ (Vero), ΔgK/GFP+ (VK302), R/GFP-, ΔgK/GFP- (Vero), or ΔgK/GFP- at an MOI of approximately 0.1. (A) Curves for the three GFP+ viruses with the parental McKrae strain for comparison. (B) Curves for the three GFP- viruses with the parental McKrae strain for comparison. Viral titers were obtained at indicated time points post infection by standard titration plaque assay on Vero or VK302 cells. PFU totals represent the mean of triplicate experiments and error bars represent the standard error.

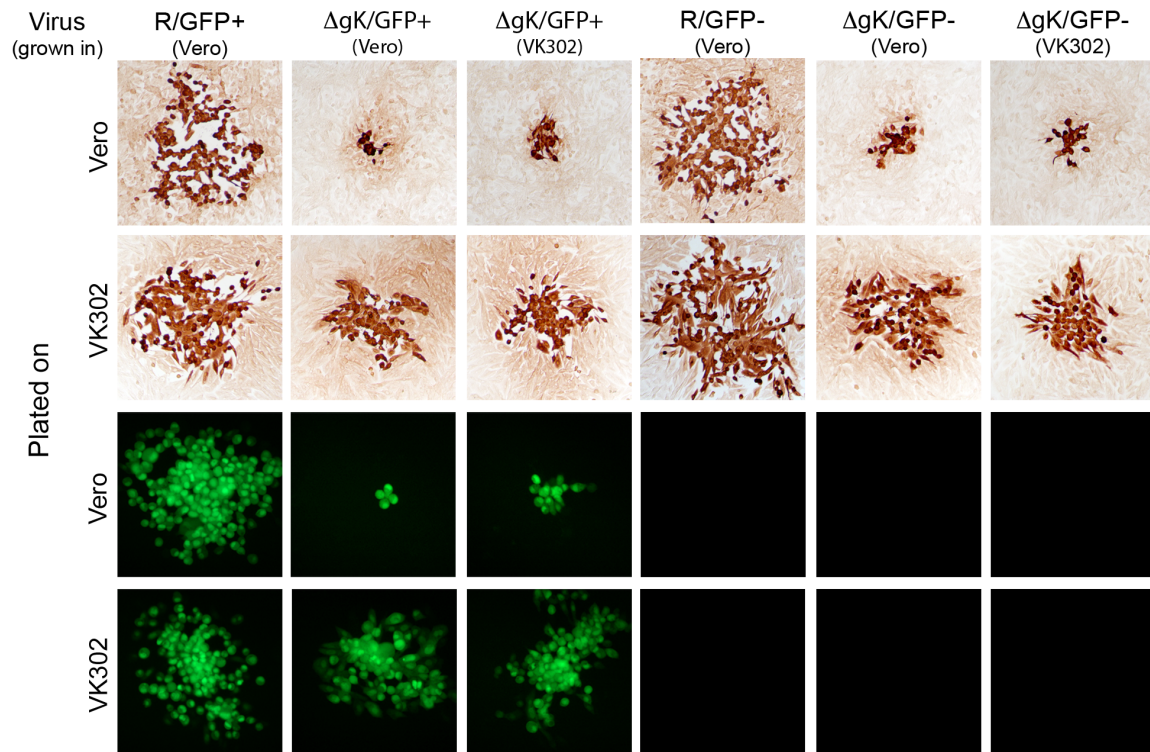


Figure 3.3: Representative plaque morphologies of HSV-1 (McKrae) strain and mutants on Vero and VK302 cells. Confluent Vero or gK complementing VK302 cells monolayers were infected with the R/GFP+, Δ gK/GFP+ (Vero), Δ gK/GFP+ (VK302), R/GFP-, Δ gK/GFP- (Vero), or Δ gK/GFP- at an MOI of approximately 0.001. Viral plaques were visualized by immunohistochemistry at 72 hpi using rabbit anti-HSV-1 developed with Nova Red (Vector Labs) with contrast microscopy. Fluorescent plaques from viruses with EGFP were observed using a fluorescence microscope at 72 hpi. Plaques shown are representative of average plaque size observed. All images are taken at 100X magnification.

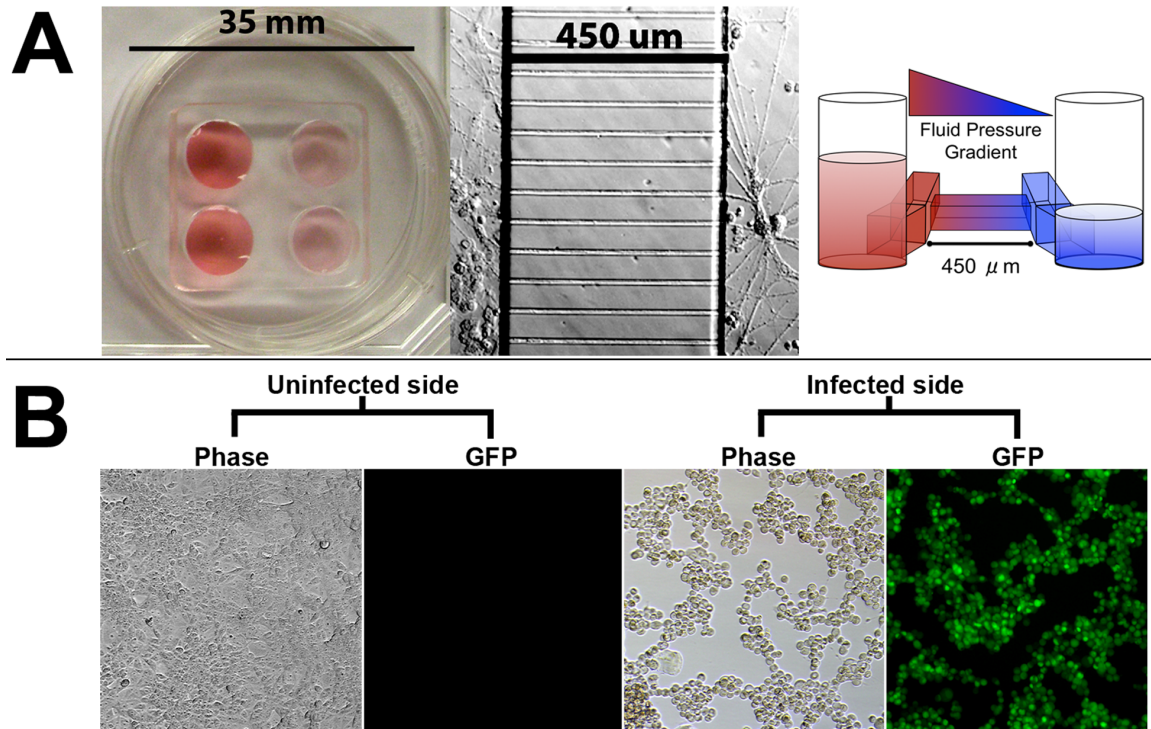


Figure 3.4: Microfluidic Neuron Device System. (A) Photograph of the neuron device (Xona Microfluidics) that maintains a fluid pressure (left panel). Virus impermeable barrier containing axons (middle panel). Side view schematic showing wells, main central channel and microgroove barrier showing the maintenance of a fluid pressure gradient (right panel). (B) Control experiment to test for system integrity. Phase contrast and GFP fluorescence of infected Vero cells at 3 days post infection, and uninfected Vero cells at 5 days post infection. During all 5 days the uninfected side wells were maintained at a higher fluid level than the infected side wells.

Retrograde Transport of Virions

Retrograde transport of virions from infected neuronal endings to neuronal somata was investigated. For retrograde studies, microfluidic chambers were seeded with rat dorsal root ganglionic neurons on one side of the chamber. Neurons were allowed to propagate axons across the microgroove barrier for 10-15 days, or until axons and neuronal endings were visibly present across the barrier and the apposed chamber, respectively (see Materials and Methods). The neuronal endings chambers were infected with R/GFP+ and ΔgK/GFP+ viruses at an approximate MOI of 5. Progression of

infection on the neuronal soma side of the microfluidic device was monitored daily using a fluorescence microscope to detect EGFP expression. EGFP expression was readily detected as early as 24 hpi in the R/GFP+, while infection with the Δ gK/GFP+ virus failed to produce any EGFP expression, as late as 5 days post infection (Fig. 3.5). Surprisingly, Δ gK/GFP+ virus grown on the VK302, gK complementing, cells failed to be transmitted to the neuronal soma side of the microfluidic device, although an occasional fluorescent cell was infrequently detected (approximately 100-1000-fold less than the R/GFP+) (Fig. 3.5 F).

To determine whether infectious virions and/or viral DNA were transported in a retrograde manner, neuronal termini chambers were infected with an MOI of approximately 5. The number of viral genomes was obtained by quantitative PCR, while the presence of infectious virus was assessed by determining viral titers on VK302 cells as detailed in Materials and Methods. Generally, all virus stocks appeared to have a genome to PFU ratio of less than 10 suggesting that most viral genomes were contained within infectious virion particles (Fig. 3.6). The number of viral genomes detected on the neuronal termini side of the device decreased by approximately 35% at 48 hpi and 45% at 72 hpi, respectively, while it did not decrease in wells infected with either the Δ gK/EGFP+ or Δ gK/EGFP+ produced in VK302 cells suggesting that the gK-null viruses failed to enter into neuronal endings, unlike the wild-type-like virus. Comparison of total PFU numbers revealed a time-dependent decrease of PFU remaining within the neuronal endings chambers for all viruses. This PFU reduction was similar to decreases in PFU obtained when viral samples were incubated for similar time periods at 37C suggesting that it reflected a natural decay of viral infectivity over time (not shown).

Examination of the number of viral genomes and PFU obtained from samples derived from the soma side of the devices revealed that the R/GFP+ virus efficiently transported and replicated in soma chambers producing maximum viral genomes and PFU at 72 hpi. In contrast, both the $\Delta gK/EGFP+$ and $\Delta gK/EGFP+$ grown on VK302 cells failed to be transported and replicate in soma chambers at 72 hpi (Fig 3.6.).

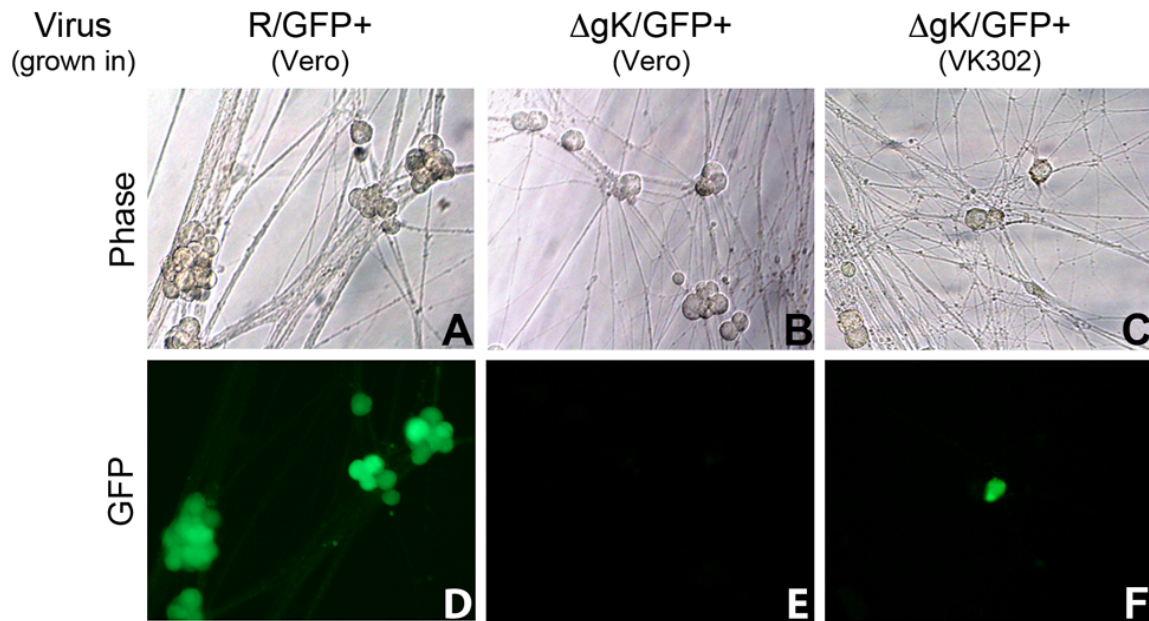


Figure 3.5: Infection of neuron cell bodies (Soma) via retrograde transport. Phase contrast (A-C) and GFP fluorescence (D-F) of neurons. Axonal side of chambers (not shown) were infected with $\Delta gK/GFP+$ (VK302), $\Delta gK/GFP+$ (Vero) and R/GFP+. Neurons potentially infected with $\Delta gK/GFP+$ (VK302 or Vero) were photographed, using phase and fluorescence microscopy, at 5 dpi to allow ample time for retrograde transport. Neurons infected with R/GFP+ were photographed, using phase and fluorescence microscopy, at 2 dpi due to the presence of ample infected neurons. All images were taken at 100X magnification.

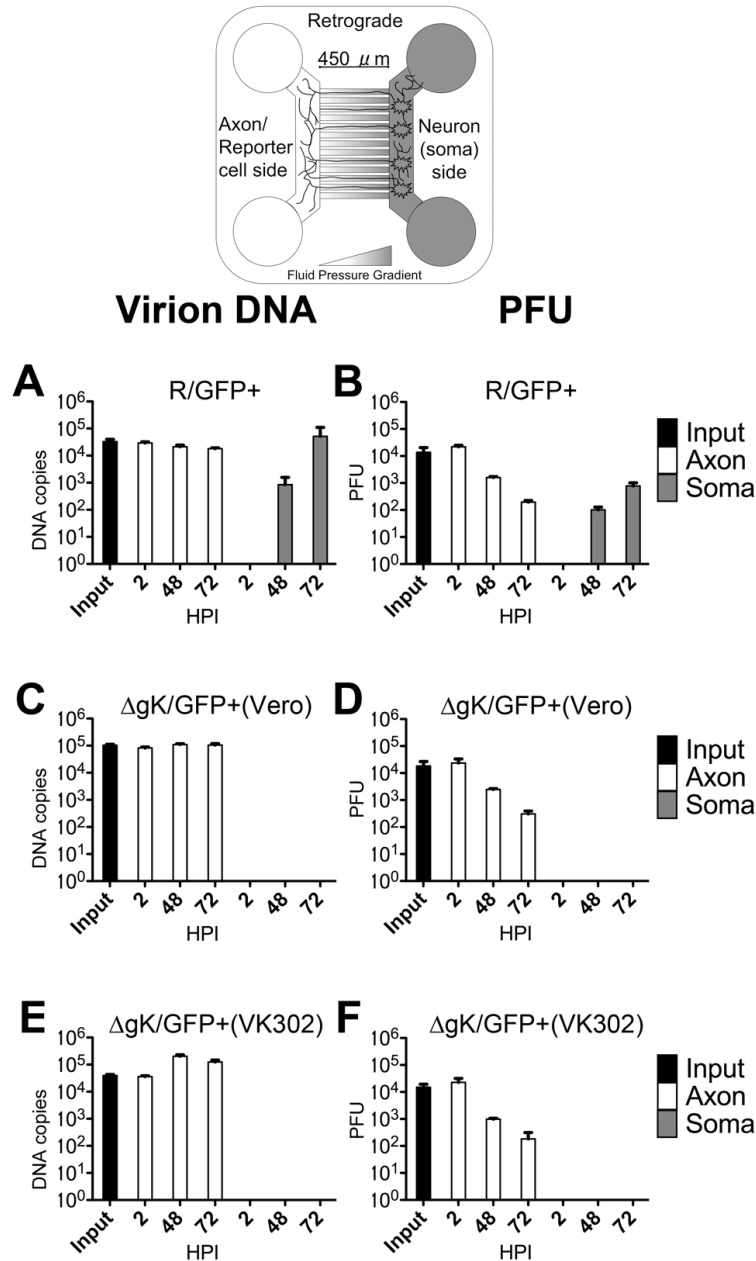


Figure 3.6: Determination of PFU and viral genomes recovered in retrograde virus transport experiments. Viral DNA copy number determined by QPCR, and PFU, determined by serial titration plaque assay are shown for samples recovered from the axon and soma sides of devices infected in retrograde manner. Bars represent the mean of 6 (DNA) or 4 (PFU) samples taken from devices at 2, 48, and 72 hpi as well as corresponding volume samples of inocula taken at the time of infection (input). (A, B) R/GFP+ infected. (C, D) $\Delta\text{gK}/\text{GFP}+(\text{Vero})$ infected. (E, F) $\Delta\text{gK}/\text{GFP}+(\text{VK302})$ infected. Input bars are black. Bars representing the axon side (white) and soma side (gray) are shaded to correspond to the sides in the schematic (top).

Anterograde Transport of Virions

For anterograde studies, after seeding of microfluidic chambers and propagation of axons, axonal side wells were co-plated with a monolayer of VK302 cells. Then, neuronal somata were infected as described in the retrograde studies. Infection of the neuronal side of the microfluidic chambers was also monitored daily by visualizing EGFP fluorescence. All three viruses, R/EGFP+, Δ gK/GFP+(Vero) and Δ gK/GFP+(VK302) infected neuronal somas efficiently, as evidenced by the strong EGFP fluorescence (Fig. 3.7). However, only the R/GFP+ virus was transported in an anterograde manner to the Vero reporter cells on the microfluidic chambers (Fig. 3.7). Similar virus axonal transport experiments were performed, but this time the cells within the soma and reporter sides of the microfluidic chambers were fixed and immune-stained with antibodies specific for viral glycoprotein C (gC), nuclei, and neurofilament (neuron-specific stain) and viruses not expressing GFP were utilized (to allow multicolor immunofluorescence). Cells in both the soma and reporter sides of the chambers expressed relatively high levels of gC only when infected R/GFP- virus, while both Δ gK/GFP- (Vero) and Δ gK/GFP- (VK302) viruses expressed in gC in cells in the soma side of the chambers, while no gC expression was detected on the reporter side of the chambers (Fig. 3.8).

Anterograde transport of virions was also monitored by determining the number of infectious viruses produced on the reporter side of the microfluidic chamber (Vero cells) after infection of the neuronal soma sides, as well as determining the number of viral genomes transmitted axonally to the reporter sides using QPCR. Infectious virions were retrieved only in infections with the R/GFP+ virus. The relative number of infectious

virions present at 72 hpi in somas appeared to decrease in comparison to input virus, while the total number of viral genomes appeared to increase reflecting continued viral replication within neuronal somata (Fig 3.9).

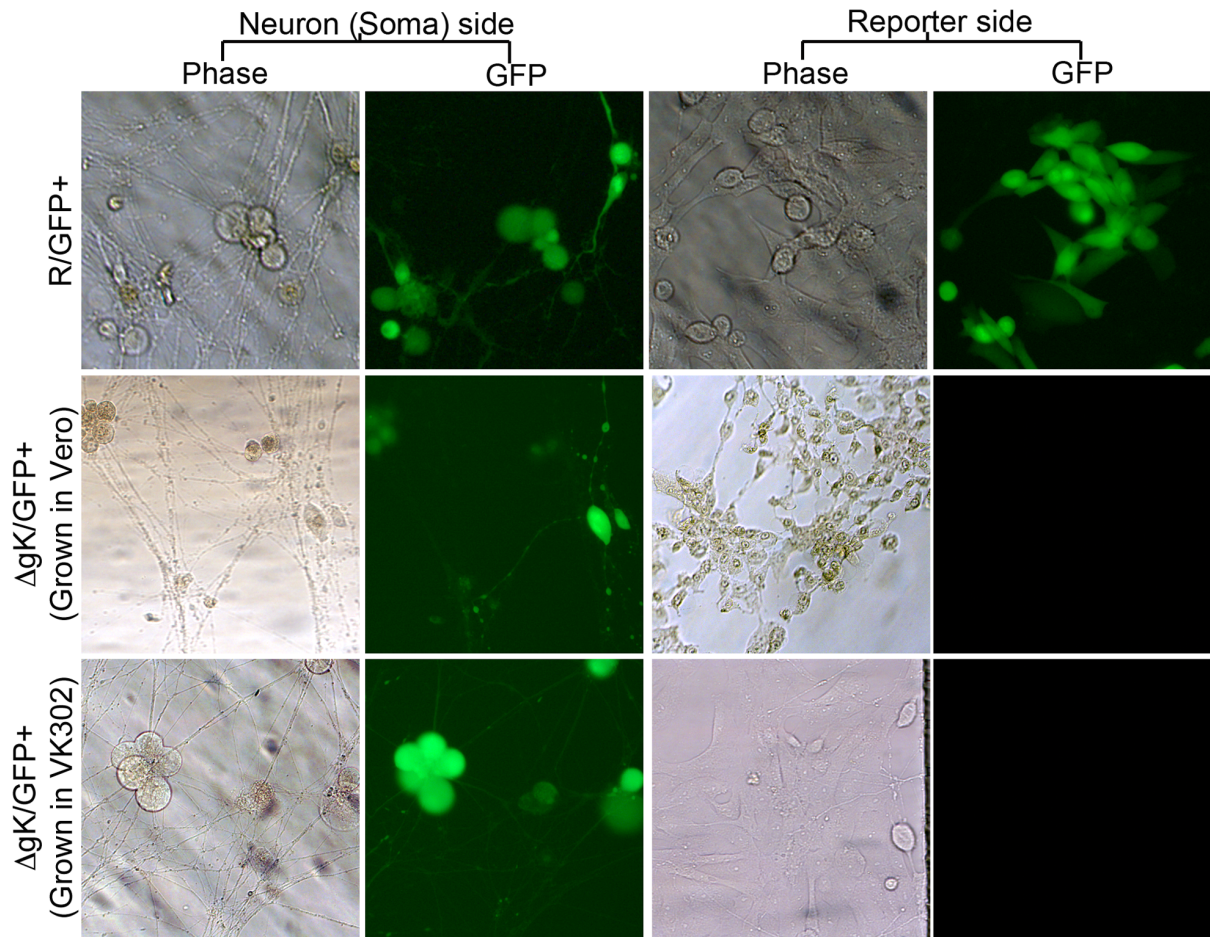


Figure 3.7: Anterograde infection and transmission. Anterograde infection and transmission demonstrated by phase and fluorescence microscopy of the infected neuronal side and uninfected reporter cell/axon side. Only the rescued virus was observed spreading the virus in the anterograde direction to reporter cells.

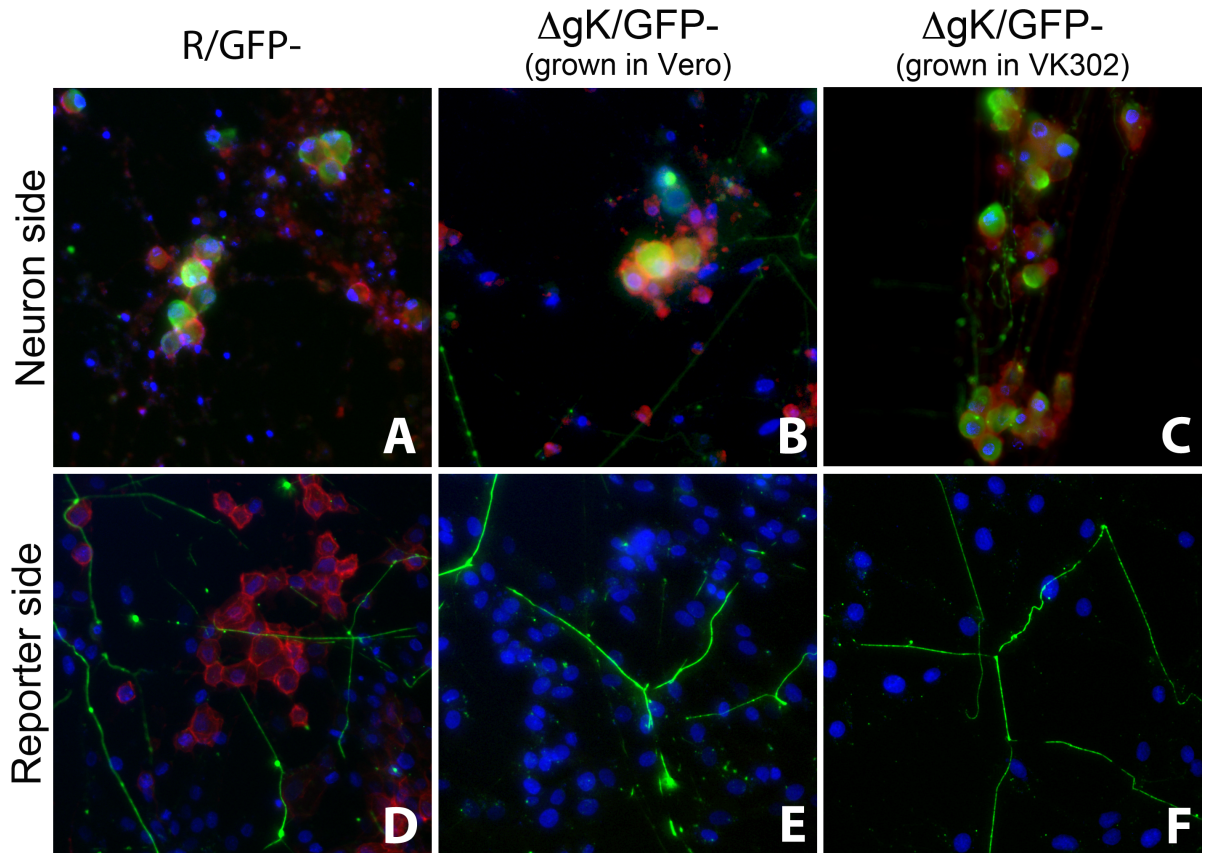


Figure 3.8: Anterograde infection and transmission via immunofluorescence of viral proteins. Anterograde infection and transmission demonstrated by fluorescent antibodies. Images are stained with anti-neurofilament antibody with Alexafluor 488 secondary (green), anti-HSV-1 gC with Alexafluor 594 secondary (red) and DAPI (blue). Neuron sides of the microfluidic devices were infected with R/GFP-, Δ gK/GFP- (vero) and Δ gK/GFP- (VK302). Only the rescued virus was observed spreading in the anterograde direction to reporter cells as demonstrated by red staining indicating the production of viral glycoprotein C in reporter cells.

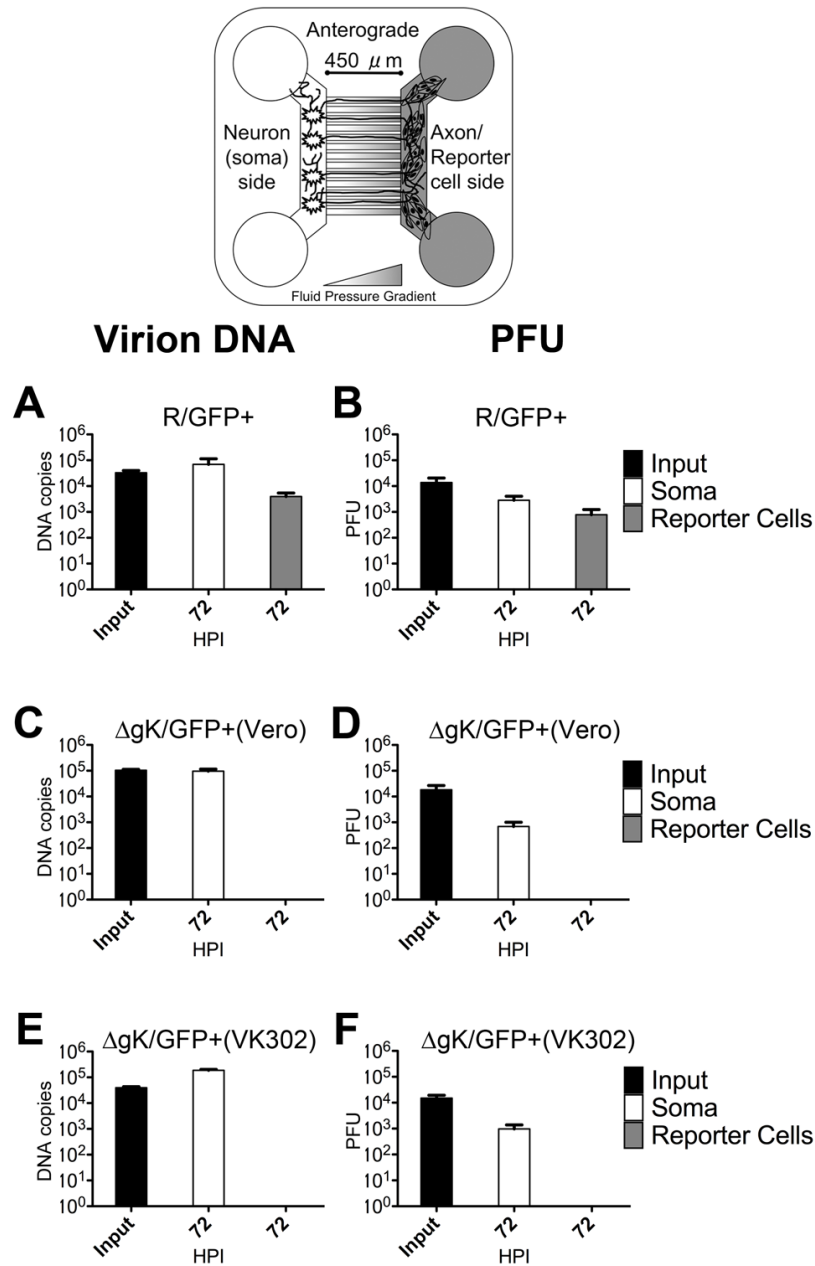


Figure 3.9: Determination of PFU and viral genomes recovered in anterograde virus transport experiments. Viral genome and PFU numbers from the axon/reporter cell and soma sides of devices infected in the anterograde manner. Bars represent the mean of 6 (DNA) or 4 (PFU) samples taken from devices at 72 hpi as well as corresponding volume samples of inocula taken at the time of infection (input). (A, B) R/GFP+ infected. (C, D) $\Delta\text{gK}/\text{GFP}+(\text{Vero})$ infected. (E, F) $\Delta\text{gK}/\text{GFP}+(\text{VK302})$ infected. Input bars are black. Bars representing the soma side (white) and reporter cell side (gray) are shaded to correspond to the sides in the schematic (top).

DISCUSSION

HSV-1 gK functions as a protein complex with the membrane protein UL20 in membrane fusion phenomena during virus entry and virus-induced cell fusion enabling virions to enter into cells and spread into adjacent uninfected cells, respectively (Jayachandra, Baghian et al. 1997; Melancon, Luna et al. 2005; Foster and Kousoulas 2008; Chouljenko, Iyer et al. 2009; Chouljenko, Iyer et al. 2010; Jambunathan, Chowdhury et al. 2011). Previously, we showed that lack of gK prevented corneal spread in mice, transmission of the gK-null virions to the central nervous system, and establishment of latency. Here, we show that gK is essential for infection of free neurites, and retrograde and anterograde transport in rat dorsal root ganglionic neurons in culture, in agreement with the previous *in vivo* studies (David, Baghian et al. 2008).

Herein, we have chosen to work with the HSV-1 McKrae strain because this virus was isolated from a human ocular herpes infection (Kaufman, Ellison et al. 1969) and shown to be highly virulent in mice and rabbits (Perng, Thompson et al. 1995; Perng, Chokephaibulkit et al. 1996; Halford, Balliet et al. 2004). In these studies we utilized a set of recombinant viruses lacking gK gene expression in the presence or absence of EGFP expression for comparative purposes, because constitutive expression of the EGFP gene may alter viral replication characteristics (DeWire, Money et al. 2003). Wild-type-like viruses replicated to similar levels in the presence or absence of GFP expression in ganglionic neurons. In contrast, gK-null virions reached maximum viral titers that were 1-2 logs lower than their corresponding wild-type-like viruses, while EGFP expression did not adversely affect viral growth. These experiments indicated that EGFP expression did not alter viral replication characteristics of these viruses.

The relative size of viral plaques is an indication of the ability of the virus to spread from infected to uninfected cells. Consistent with previous findings, the lack of gK caused the production of substantially smaller viral plaques indicating a severe defect in virus spread. EGFP expression did not have any effect on the gK-null defect in virus spread.

Microfluidic devices separating axonal termini from neuronal somata have been extensively used in herpes viral experiments to investigate the contribution of individual genes in retrograde and anterograde axonal transport (Ch'ng, Flood et al. 2005; Feierbach, Bisher et al. 2007; Liu, Goodhouse et al. 2008; Curanovic and Enquist 2009). We exclusively utilized a commercially available microfluidic device (Xona Microfluidics, LLC) originally developed from a collaboration between the Enquist laboratory (Princeton Univ.) and the Jeon laboratory (Univ. of CA Irvine) (Liu, Goodhouse et al. 2008). It is particularly important in all studies using these devices that control experiments are performed carefully to ensure that there is no leakage of fluids between the device and the cover slip on which the device is placed. Therefore, methods utilized in these studies were validated for obtaining appropriate sealed chambers prior to the onset of each experiment. Furthermore, chambers were inspected to ensure that axons were exclusively appearing within the device microgrooves.

Infections of chambers containing axonal termini showed that the wild-type-like virus (R/GFP+) was readily transported to the somata containing chamber, while the gK-null virus Δ gK/GFP+ failed to be transported. Surprisingly, Δ gK/GFP+ virus grown on the complementing cell line VK302 was also largely unable to be transported to the somata containing chamber. This result suggests that expression of the HSV-1 (KOS)

gK gene in VK302 cells may complement for infectious virus production in Vero, however virions produced in VK302 cells may not be able to infect neuronal axons. We have shown that the amino terminus of gK interacts with the amino terminus of gB, most likely affecting the ability of gB to mediate fusion of the viral envelope with cellular membranes during virus entry (Chouljenko, Iyer et al. 2009; Chouljenko, Iyer et al. 2010; Jambunathan, Chowdhury et al. 2011). Comparison of the predicted amino acid sequences of KOS and McKrae gB and gK reveal a number of amino acid differences including amino acid changes within the amino termini of gB and gK. Furthermore, lack of gK prevented entry via fusion of the viral envelope with cellular membranes (Chowdhury, Naderi et al. 2012). Thus, it is conceivable that KOS gK cannot efficiently complement the McKrae gK-null virus for entry into neuronal axons via fusion of the viral envelope with neurites, because of altered interactions between KOS gK and McKrae gB. It is important to note these experiments cannot differentiate whether virions entered into neurons but were subsequently unable to be either transported or replicated within somatas, since only GFP expression was monitored. In an attempt to address this issue, the total viral genomes and infectious virions remaining within the axonal termini-containing chambers were determined. In the inoculated chambers, viral genome numbers appeared to decrease by up to 45% in the wild-type-like infections with no comparable decrease observed with gK-null infections, possibly suggesting that the gK-null virions were unable to enter into axons and be removed from the inoculated side. This result was not corroborated by the PFU counts, most likely, because of the natural decay of infectious virions at prolonged incubation at 37C. Despite the consistent detection of few cells expressing GFP in infections with the Δ gK/GFP⁺ virus within

somata, there were no infectious virions recovered from these chambers even at 72 hpi. Collectively these results indicate that EGFP expression may be due to transport of DNA fragments containing the EGFP gene cassette. These gene fragments may exist in the original virus stocks used for infections, or produced after degradation of endocytosed virions into neurons.

UL20 and gK are required for cytoplasmic virion envelopment and egress in epithelial cells (Hutchinson, Roop-Beauchamp et al. 1995; Jayachandra, Baghian et al. 1997; Melancon, Luna et al. 2005). Furthermore, direct comparison of the role of gK and UL20 in cytoplasmic envelopment, virion egress and infectious virus production with similar functions provided by the carboxyl terminus of gD, gE, UL11, or gM have revealed that gK and UL20 are more important than each of these proteins alone or in combination (Chouljenko, Kim et al. 2012). In agreement with these known functions of gK, lack of gK drastically inhibited transmission of virus from somata-containing chambers to axonal termini embedded within VK302 cell monolayers. Neurons, unlike epithelial or fibroblasts have an intricate distribution of cytoplasmic organelles within somata and axons. Specifically, TGN derived-membrane vesicles migrate to synapses and play important roles in synapse formation among neurons (Sytnyk, Leshchyn'ska et al. 2004). Therefore, it is conceivable that cytoplasmic envelopment of virions occur within both somata and via budding into TGN-derived vesicles proximal to synaptic membranes.

Apparently, both Δ gK virions grown on Vero or VK302 cells appeared to infect somata with approximately similar efficiencies indicating that lack of gK does not substantially alter entry of the gK-null virions via infection of neuronal cell bodies.

Neurons are known to express nectin-1, which binds gD and functions in virus entry (Richart, Simpson et al. 2003; Simpson, Manchak et al. 2005). In agreement with these published results, we readily detected nectin-1 expression in neuronal termini, axons and somata (not shown). Thus, an altered distribution of nectin-1 between neurites and somas are probably not responsible for the lack of gK-null virus infection of neuronal termini. Nectin-1 is known to primarily mediate endocytosis of virions rather than fusion of the viral envelope with cellular membranes (Milne, Nicola et al. 2005). Thus, it is possible that virions enter into neurites primarily via membrane fusion suggesting that gK functions in virus-cell membrane fusion utilizing cellular receptors other than nectin-1.

Herpes simplex virions have evolved a highly complex system for optimum infection of neurons, the ultimate destination of the virus in the human host. It is conceivable that virus entry into neuronal synapses via transmission of virus through epithelial neuronal synapses, or via infection of axonal termini requires the presence of gK. We hypothesize that gK, known to interact with the amino terminus of gB, may modulate fusogenic activity of gB via specific gB cellular receptors. Lack of gK may result in loss of critical regulatory functions provided by gK on gB to mediate fusion of the viral envelope with synaptic membranes resulting in inability to enter into axons, or entry and subsequent degradation of virions within lysosomal compartments. Direct visualization of fluorescent virions via specially equipped fluorescent microscopes may resolve these issues that will be addressed in future experiments.

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CHAPTER IV

CONCLUDING REMARKS

SUMMARY

Earlier work in our laboratory has shown that HSV-1 glycoprotein K (gK) is not essential for virus entry into cells, but is essential for virus spread in cell culture.

Glycoprotein K-null mutant viruses form very small viral plaques typically consisting of less than 10 infected cells per plaque (Jayachandra, Baghian et al. 1997; Melancon, Luna et al. 2005). This phenotype is the result of gK's critical role in cytoplasmic virion envelopment presumably occurring at trans-Golgi network (TGN) membranes (Foster, Melancon et al. 2004a; Foster, Melancon et al. 2004b; Foster, Chouljenko et al. 2008). A similar viral phenotype had been described for pseudorabies virus (PRV) indicating that HSV-1 and PRV gK function in a similar fashion in viral morphogenesis and egress in tissue culture (Klupp, Baumeister et al. 1998).

In vivo work with PRV indicated that gK was essential for virus spread in cell culture, but it was not essential for neuroinvasiveness (Flamand, Bennardo et al. 2001). To investigate whether HSV-1 gK was essential for neuroinvasiveness and disease causation, we performed the experiments detailed in chapter 2 of this dissertation. Using a mouse eye model, we established that HSV-1 gK is essential for spread in the cornea during primary infection. No clinical symptoms and very low levels of virus were found in gK-null virus infected mouse eyes, which correlated well with data from cell culture, in that HSV-1 can infect without gK but the infection is scant at best with no ability to spread. For our studies, we used the McKrae strain of HSV-1. This strain was initially isolated from a human ocular infection (Kaufman, Ellison et al. 1969). This strain is

highly virulent in the eyes and neural tissues of mice and rabbits and, therefore, served as an excellent standard to judge manipulations that resulted in reduced pathology (Perng, Thompson et al. 1995; Perng, Chokephaibulkit et al. 1996; Halford, Balliet et al. 2004).

Following infection of mouse eyes with HSV-1, virus replication occurs initially in the corneal epithelium, and subsequently the virus is transmitted via epithelial-neuronal interfaces to neurons. Virions are thought to move in the retrograde direction within axons to neuronal soma where they establish latency (Cunningham, Diefenbach et al. 2006; Diefenbach, Miranda-Saksena et al. 2008). To determine the neuroinvasiveness of the gK-null mutant virus, mice that were infected in the corneas were monitored for survival and symptoms of neurologic disease. The eye is a good model for neural transmission because the eye cornea is a highly innervated tissue with a high density of nociceptors per square millimeter and thus, efficient, ready access to the nervous system (Muller, Marfurt et al. 2003). Clinical disease symptoms absolutely paralleled the observed infection patterns with the MK Δ gK infected eyes exhibiting no clinical signs at any time point following viral infection, while the rescued virus produced significant eye disease by day 4 post infection. Furthermore, neuroinvasion was confirmed due to the development of severe neurologic symptoms and death in rescue inoculated mice. Significantly no clinical eye disease or neurologic symptoms were observed in the gK-null inoculated mice.

The final test of virulence and transmission was to determine whether the gK-null viruses could cause retrograde spread to the sensory ganglia and establish latent infection in the peripheral nervous system. With the efficiency of neuronal infection and transmission in a scarification and direct inoculation system, any level of neuronal

infection should result in latent infection. Thirty days after infection of the eyes, trigeminal ganglia from infected mice were cultured to isolate reactivated HSV-1. No reactivation could be found in mice infected with the gK-null virus, but mice infected with the rescue strain had abundant reactivation. To provide greater certainty, the ganglionic tissues were also examined by PCR for HSV-1 DNA. HSV-1 DNA was very rarely present in the ganglia of gK-null infected mice.

These results taken together, provide strong evidence for gK as a critical determinant of spread, pathogenesis, neuroinvasion, and the establishment of latency. These results are substantially different from the results reported for PRV. To better characterize these disparate results, it was decided that this infection process should be evaluated in a primary neuron cell culture system to eliminate confounding factors that are present in animal models. To that end, a primary neuronal culture system was developed. This system was developed using dorsal root ganglionic neurons from rats. These ganglia provide sensory neurons, which are the primary neuronal target of HSV-1. Use of neurons, allowed us to separate the phenomena of retrograde and anterograde transport in a more definite way than is possible in a live animal model. This was important because PRV glycoproteins gE and gI are required for anterograde transport, but not retrograde transport (Card, Whealy et al. 1992; Jacobs, Mulder et al. 1993; Babic, Klupp et al. 1996; Johnson, Webb et al. 2001; Brittle, Reynolds et al. 2004; Ch'ng and Enquist 2005). And HSV-1 gE has been reported to function in both retrograde and virus spread from epithelial cells to neurites but was not required for direct infection of neurites or retrograde transport (Balan, Davis-Poynter et al. 1994; Saldanha, Lubinski et al. 2000;

Wang, Tang et al. 2005; Brittle, Wang et al. 2008; McGraw and Friedman 2009). Clearly these processes have different interacting proteins.

The experiments contained in chapter 3 use the primary neuronal culture system and our gK-null mutant viruses to demonstrate that gK is a critical determinant in infection at the level of the neurite/axonal process. Viruses without gK could infect and apparently replicate in neurons that were infected at the level of the cell body, however, this virus could not be transported in the anterograde direction or be released to infect adjacent cells. Although a number of viral glycoproteins have been implicated in efficient egress of virions from non-neuronal cells, their role in neuronal transport has not been studied extensively (Mettenleiter, Klupp et al. 2009). The findings of this dissertation suggest a possible similarity between the role of gK in neurons and non-neuronal cells. The infection phenotype observed in neurons recapitulates in many ways the infection observed in non-neuronal cultures. Herpes simplex virions have evolved a highly complex system for optimum infection of neurons, the ultimate destination of the virus. It is conceivable that virus entry via transmission of virus through epithelial neuronal synapses, or direct infection of axonal termini requires the presence of gK.

UL20 and gK are required for cytoplasmic virion envelopment and egress in epithelial cells (Hutchinson, Roop-Beauchamp et al. 1995; Jayachandra, Baghian et al. 1997; Melancon, Luna et al. 2005). Lack of gK drastically inhibited transmission of virus from somata-containing chambers to axonal termini embedded within VK302 cell monolayers. Neurons, unlike epithelial or fibroblasts have an intricate distribution of cytoplasmic organelles within somata and axons. Specifically, TGN derived-membrane vesicles migrate to synapses and play important roles in synapse formation among neurons (Sytnyk, Leshchyn'ska et al. 2004). Therefore, it is conceivable that cytoplasmic

envelopment of virions occur within both somata and via budding into TGN-derived vesicles proximal to synaptic membranes.

CURRENT AND FUTURE WORK

Neurons are known to express nectin-1, which binds gD and functions in virus entry (Richart, Simpson et al. 2003; Simpson, Manchak et al. 2005). In agreement with these published results, we have readily detected nectin-1 expression in neuronal termini, axons and somata. In particular, nectin-1 appears to be expressed at or near the synapse of sensory neurons (Salinas, Schiavo et al. 2010). Nectin-1 is known to primarily mediate endocytosis of virions rather than fusion of the viral envelope with cellular membranes (Milne, Nicola et al. 2005). However, HSV-1 is thought to enter the neuron via viral membrane fusion with the neuron plasma membrane (Salinas, Schiavo et al. 2010). Therefore one area of interest is determining the receptor constituents used by HSV-1 as it enters the neuron and the exact entry mechanism; e.g. fusion versus endocytosis. The entry mechanism may have a significant impact on what viral proteins accompany the capsid as it enters the neuron cytoplasm and eventually axon.

Following other work in this lab, this may require extensive investigation in the gB, gK, UL20 interactions. Another area of interest for further investigation is a further refinement of the exact domains that influence the virus' ability to infect and be transported in neurons. These domains may provide additional insights to the complex interactions of the envelope proteins the domains responsible for neuroinvasiveness, morphogenesis, egress, and fusion are examined for simultaneous functions and higher order interactions with other proteins.

As these properties of the virus are determined, this research may bring great advances in the potential development of vaccine strains and safety mechanisms in viruses used for viral

tumor therapy. The identification of the exact determinants of neuronal infection, transport and eventual latency, if combined with the determinant for a robust immune reaction would enhance the efficacy and safety of herpes viruses used for therapies or preventative vaccines.

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APPENDIX

DEVELOPMENT OF PRIMARY NEURON CULTURE

CELL SOURCES

I attempted to use a variety of different cells as sources for the primary neuronal culture system. These included harvested cerebral cortical cells from mice, commercial frozen cortical and ganglionic cells from mice and rats, harvested cortical cells from rats, and harvested dorsal root ganglionic neurons from rats. In addition to the primary cells, redifferentiation of neuroblastoma cell lines to a primary cell was attempted. The greatest and most easily reproduced success was with the dorsal root ganglionic neurons from the rat.

When evaluating these potential systems, three main factors were necessary for a usable system for our experiments. The first factor was survival of the neurons in sufficient quantity and density to provide a reasonable population for experimentation and microscopic visualization. The second factor was the growth of axons of sufficient length to allow the use of a culture system that could physically separate the neuron cell body (soma) from the termini of axons (neurites) so we could separate anterograde and retrograde transport. In practice this required an axon to grow to a length of at least 450 microns before the neuron would die from senescence in prolonged cell culture. The third factor was the development of a robust network of axons and neurites on the axonal side of the culture system that would provide a reasonable substrate for infection and reporter cell co-plating.

Redifferentiation of neuroblastoma cell lines is accomplished using retinoic acid in varying doses to force the cells to go from an undifferentiated, tumor state to a differentiated neuron. Using this system, we could get the neuroblastoma cells to achieve a morphology more similar to a primary neuron rather than a tumor cell-line; however, these were unsuitable for our experiments because the axons produced were not consistently long enough to be placed in a chamber system that could isolate the axonal processes from the cell bodies. In fact, the morphology of these neurons usually more closely resembled the morphology of a cortical neuron with many short dendrites surrounding the soma and very rare, short axons when they were present. Cultures of cortical neurons, in all cases, were unsuitable for the same reasons. Although cortical neurons consistently produce dense, hearty neurons the processes are uniformly of insufficient length for the purposes of our proposed experiments. This was found with all cortical neuron types, including: commercial, primary harvested, frozen and shipped rat or mouse neurons as well as on-site harvested rat and mouse cortical neurons.

Ganglionic neurons harvested from mice in our lab were also unsatisfactory. This is likely a technical problem that may be overcome; however, in my experience yields were insufficient for our experiments. This is likely due to the extreme difficulty of harvesting ganglionic neurons from the neonatal mouse, as the ganglia are very small and difficult to visualize. Using the commercial, primary, frozen rat and mouse ganglionic neurons was initially promising with decent density of neurons at plating. These neurons seemed to lack viability as the duration of cultures progressed. Axonal growth was slow and by the time axonal processes were nearing sufficient length, the density of neurons was extremely poor.

By far the most suitable cultures came from dorsal root ganglia of rats. These ganglia are challenging to dissect for primary culture, but not prohibitively difficult like mouse ganglia. One particular challenge to the harvest of a primary cell such as a neuron; is that there is significant time lapse from euthanasia of the animal to removal of the neural tissue and placement of this tissue in a media that will prevent cell death. In these systems, animals are generally euthanized and then dissection proceeds to the ganglia. After dissection, the ganglia must be enzymatically treated to achieve a single cell suspension before addition to a growth medium for plating in a culture system. I found two things to significantly enhance neuron survival, and therefore plating density, during this stage. The use of a dissecting solution, composed of phosphate buffered saline supplemented with 3.5 grams per liter of sucrose and 7 grams per liter of glucose, for all stages of dissection and enzymatic dissociation greatly influenced neuron survival. Also, whenever possible keeping the tissues to be dissected and the dissected ganglia cold, as situated in a Petri dish over ice, was helpful. The method used to produce consistent, robust cultures of rat dorsal root ganglia will be covered in more detail next.

PRIMARY RAT DORSAL ROOT GANGLIONIC NEURON HARVEST

Rats

Harvesting the ganglia of varying ages of rats was attempted. All attempts were made ranging from embryonic to 7 days after birth. Neurons harvested from rats between 3 and 7 days after birth may give initial robust plating densities; however, these neurons usually die during the second week of culture leading to insufficient axon growth and

very poor neuron densities by the time neurons are ready for experimentation. For our experiments we used Sprague-Dawley rats that had a gestation of 21-22 days. Dorsal root ganglia (DRG) neurons from rats ranging from embryonic day 17 (E17) to 48 hours, 2 days, after birth (P2) provide suitable neurons for abundant plating densities and sufficient survival times to allow for axon growth and experimentation. The only difficulty with this age for harvest is that the spinal column has mineralized to a more significant degree making removal of the ganglia more time consuming, which can contribute to loss of cells if the dissector is not proficient. For my harvests, the ideal age was day 15-16 of gestation (identified as E15-E16 pups). Any earlier and the tissues were too soft to handle without major structural disruption. At E15-16, once the vertebral column is dissected from the spinal cord, the spinal cord can be removed with the DRG maintaining their attachment. Later than E16, the dissection of the vertebrae is easier due to greater size and structure of the tissues but the DRG will separate from the spinal cord unless careful and time-consuming dissection is performed. The DRG must be individually separated from connections that hold them against the remaining spine.

Dissection and Processing

My preferred technique is as follows. First a pregnant rat is euthanized at day 15-16 of gestation. The rat is rapidly dissected along the abdominal midline and the uterine horns containing the E15-16 pups are placed in conical tubes containing dissecting solution that are situated in an ice bath. The rat pups are dissected from the uterus as needed and are otherwise left in the solution in the ice bath. Using a dissecting microscope, individual rat pups are laid on their back and the ventral midline is incised

with sharp-tipped micro-dissection forceps. These forceps are used to remove all the thoracic and abdominal viscera from the pup. Then micro-dissection, spring scissors are used to remove the tail at the pelvis. Then from the caudal to the cranial end of the pup, one tip of the scissors is inserted into the spinal column and the lateral wall of all vertebral bodies is cut. Care must be taken to avoid traumatizing the spinal cord excessively. The best cut is made with the flat cutting axis of the scissors running parallel to ribs and the scissors as low as possible to the level of the rib attachments, without actually cutting the ribs. This will usually take several snips to travel the full length of the spine. After making this cut from the tail to the cervical spine near the base of the skull, the ventral floor of the vertebral bodies can be removed. During this process rib attachments have usually separated from vertebrae. At this point, the spinal cord can be visualized and the DRG should be visible as small, oval, lumps lying next to the spinal cord between and alternating with the remnants of the vertebral bodies or ribs (Fig A.1). For the next cut, the cutting axis of the scissors is directed perpendicularly to the direction of the ribs. From the tail to the head, a cut is made longitudinally just lateral to the spinal cord on both sides. The longitudinal cuts should run from the caudal to the cranial extremity of the pup, cutting soft tissues and rib attachments just slightly lateral to the vertebrae, where the DRG are situated, but leaving the DRG with the spinal cord. Then the head is cut from the cervical spine with the scissors. Using two sets of micro-dissection forceps, the dorsal remnant of the spine is stabilized against the dissecting surface with one hand while the spinal cord is gently pulled up using the other hand. Using E15-16 pups should allow all DRG to be removed still attached to the spinal cord.

At this point, the spinal cord with the attached DRG, is placed in a Petri dish containing dissection solution that is on ice (Fig A.1). The dissection process can be repeated to increase the number of ganglia. Care must be taken to balance a large harvest with the time taken to remove the ganglia, as the percentage of neurons that will not survive increases with increasing time to placement in media. I usually harvest between 10 and 15 pups, trying not to take longer than 90 minutes for the harvest step. Once spinal cords with attached ganglia are collected in a petri dish, the individual ganglia are dissected from the spinal cords using either micro-dissection scissors or a small scalpel blade. Spinal cords are discarded. Using a glass pipette, ganglia are suctioned and placed in a 15 ml conical tube with approximately 2 ml of dissection solution. Care must be taken to prevent excessive adherence of ganglia to the sides of the glass pipette. Some dissection protocols recommend pre-coating the inside of the glass pipette with serum prior to suctioning a tissue that may adhere. I avoid this because serum is also used to inactivate the trypsin that will be used to enzymatically digest the tissues in the next step. I have found a quick pre-pipette of dissection solution can help. Also pipetting quickly in multiple batches is preferable to allowing the ganglia to sit in the barrel of the pipette for any length of time.

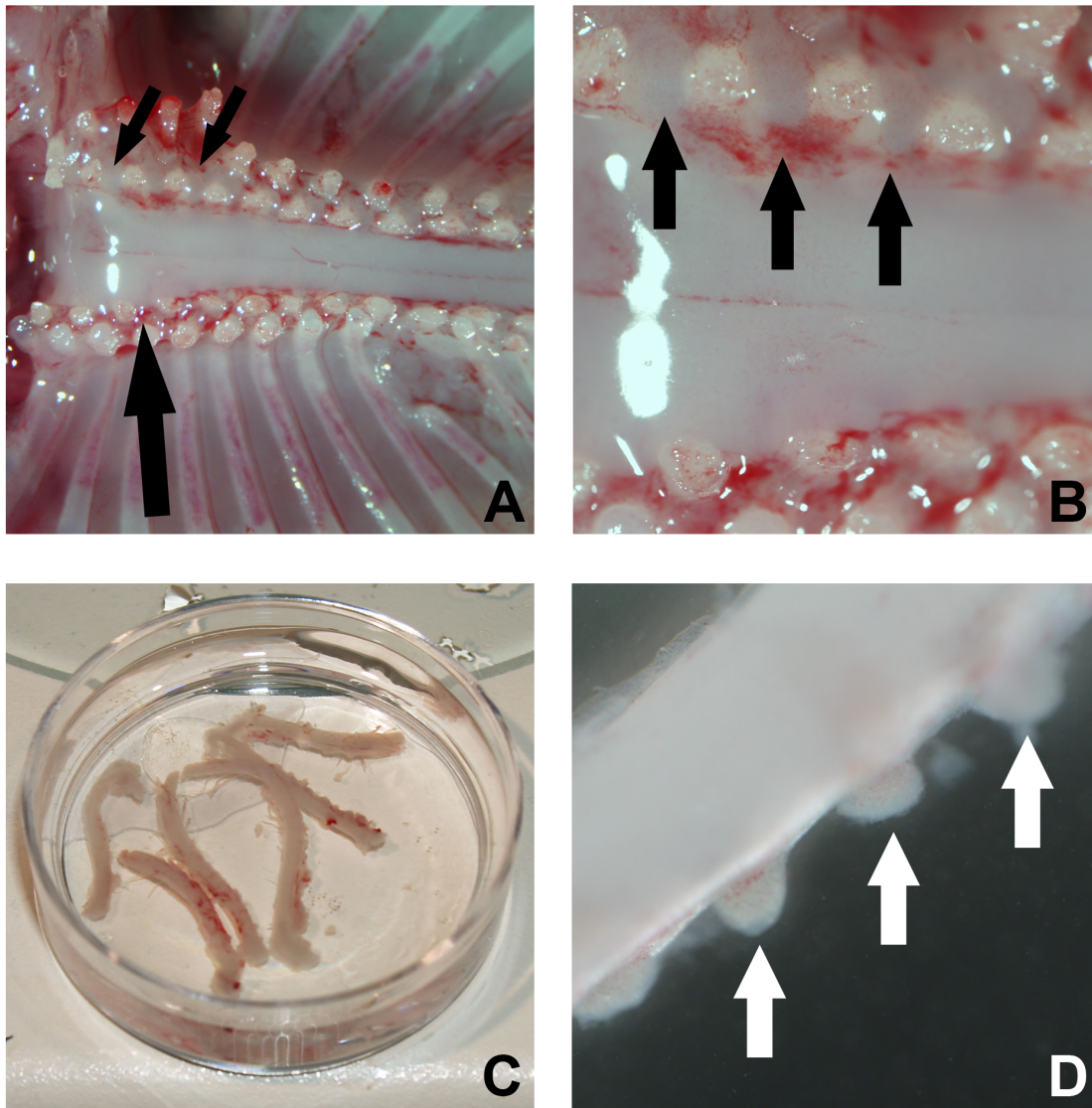


Figure A.1: Pictures of primary neuron harvest from embryonic rat dorsal root ganglia. For all pictures, rats at 4 days post birth were used to enhance visualization of pertinent tissues. A: The spinal cord can be observed following the removal of the ventral segments of the vertebral bodies. Black arrows designate the positions of the DRG interspersed between the remnants of the attachments of the ribs and the remaining vertebral body tissue. B: Higher magnification of the spinal cord, DRG, and vertebrae. The position of the DRG are denoted by black arrows. Note DRG are slightly less shiny and more translucent appearing than the adjacent bone and cartilage. C: A 35 mm petri dish filled with dissection solution, containing several spinal cords that have been removed with the DRG attached. At this magnification, DRG are just visible as small nodules slightly protruding from the sides of the spinal cords. D: Higher magnification of a segment of a spinal cord with DRG attached. White arrows demonstrate the DRG as small nodules extending from the spinal cord.

Once ganglia are in the conical tube, trypsin is added at a final concentration of 0.25 mg/ml. Ganglia are placed in a 37 degree Celsius water bath for 45 minutes, swirling every 5 minutes to agitate. Trypsin may be diluted to stock concentrations using dissection solution and stored for at least 6 months at -80 degrees Celsius. Once ganglia have incubated for 45 minutes, allow to sink to the bottom of the tube by gravity for 1-2 minutes. Pipette off excess enzyme solution. The next step neutralizes trypsin, therefore, leaving some is preferable to removing more than a few ganglia when removing the enzyme solution. For neutralization of trypsin and plating of neurons, neuronal media (recipe will be included later) supplemented with 10% fetal bovine serum (FBS) is used. This will be referred to as plating media. Add approximately 2 ml of plating media to the tube, swirl, and allow ganglia to settle by gravity for 1-2 minutes. Remove media by pipetting and repeat this neutralization step twice more to ensure full rinsing and neutralization of trypsin. Add the plating media to a final volume of 2 ml on the ganglia. Using a glass Pasteur pipette, pipette up and down to agitate the ganglia to make a single cell suspension. A fired, pulled pipette may be used for a more narrow diameter that will provide greater turbulence and agitation but these are usually not necessary. The opening of the pipette can be placed against the bottom of the tube to restrict flow and give greater turbulence as well. The solution should rapidly become cloudy. At this stage the tissues are very soft and should dissociate fairly easily with 10 or less pipettings. Neurons are also fragile at this point and exceedingly vigorous pipetting can cause cell death.

Once a single cell suspension is achieved, a sample should be taken to count the cell number. Use of a vital dye, such as Trypan Blue, can be used to count living cells in

the 2 mls of plating media. While counting cells, centrifuge the neuron suspension to form a pellet. Centrifuge at 400 times gravity or less for 5 minutes. Remove supernatant taking care to not disturb the cell pellet. Resuspend the cells in a small amount of residual media. Using the cell count obtained earlier, calculate the total number of live cells and dilute to the desired plating concentration using plating media. I usually plate between 60,000 and 100,000 cells, with 75,000 cells being a good average to achieve the desired plating for our neuronal devices. It is important to note that not all of these are neurons. Although I have not performed specific counts, I suspect half or less of the total cell amount is neurons. From a harvest of the ganglia from 12 pups, I can usually achieve approximately 4 million total live cells.

After dilution to the desired concentration, neurons may be plated. For our neuron devices, the SND450 from Xona Microfluidics (more information in Materials and Methods section of second data chapter), a pipetting volume of 12-15 microliters is necessary for use with the device. The central chamber of interest only holds approximately 1 microliter but it is impossible to pipette a single microliter in a manner that fills the chamber without significant and disruptive air bubbles. Therefore, I plate at a concentration of 5,000 cells per microliter. Allow the neurons to adhere to prepared surface (instructions on preparing a substrate to follow) for 15 min to 1 hour, then fill chambers of the neuron device with plating media.

PRIMARY CULTURE

Neuron Medias and Maintenance

Unless otherwise specified, the neuronal media used is as follows (suppliers and more information included in Materials and Methods of the second data chapter): Neural basal media supplemented with 1 X B27 supplement, 1% Glutamax, 2% normal rat serum, 2% Primocin and 50 nanograms per milliliter neural growth factor 2.5s. For the plating and neutralizing media used during dissection, this media is further supplemented with 10% FBS. I have found that plating with this media enhances neuron attachment, survivability, and axon growth. Generally neurons grow best using a system of removing half the current media and replacing with fresh, not full media changes. Therefore, the only full media changes occur when adding or removing anti-mitotic agents. Twenty-four hours after plating, an anti-mitotic agent must be added to the culture. These agents enrich the culture for neurons. All other cultured cell types will attempt to divide in culture. The addition of these agents inhibits division causing the other cells (namely astrocytes and glial cells with some fibroblasts) to die and be removed from the culture with successive media changes. At 24 hours post plating, neuronal media, supplemented with 5 micromolar cytosine- β -d-arabinofuranoside (CAB), is added to the culture. At 48 hours post plating, this media is removed and replaced with normal neuronal maintenance media. At 72 hours post plating, neuronal media, supplemented with 2 micromolar CAB, is added to the culture. At 4 days post plating, this media is removed and replaced with normal neuronal maintenance media. Successive media changes all occur with maintenance media, every 2-3 days depending on pH indicator color to determine

metabolic state and needs. Unless necessary to maintain proper pH, only half of the media should be changed.

With this plating strategy, large numbers of neurons are placed into the central chamber (the area of interest) of the neuron devices. Primary neurons in cell culture tend to aggregate especially when densely cultured. Neurons also tend to grow processes in an attempt to build a significant network of interconnections with neighboring cells. A serendipitous side effect of the anti-mitotic agents is that they will help prevent this somewhat. Large numbers of neurons are also present in the wells. These neurons are very dense and are more likely to cause aggregation of chamber neurons to the well than contribute to the area of interest. To prevent this, the neurons in the device chambers can be vacuumed out during the early media changes as long as care is taken to avoid disrupting the central chambers. In practice, this seems to also stimulate additional growth across the microgrooves to the axonal side of the chamber; likely as the neurons attempt to find connections that would have been preferentially diverted toward the wells if many neuron somata were in the wells. Once established, neuron cultures are rather stable and will grow robust interconnected axons within 1-2 weeks post plating. At this point they are ready for use in an experiment.

During the course of developing the culture system, maintenance media and plating media went through many permutations. There are reports of various neurotrophic factors enhancing axonal development, particularly in enhancing directional growth. In my experience, the use of brain derived neurotrophic factor, glial cell derived neurotrophic factor, and ciliary neurotrophic factor did not provide a substantial benefit whether used individually or in combination. Anecdotally, I believe they may have

enhanced directional axonal growth to some degree, however, this was not sufficient to overcome the negative effects. It appeared that the use of these factors in concentrations sufficient to stimulate some growth, also stimulated increased migration of cells through the microgrooves. This is highly problematic, since it must be definitively determined that these are not neurons or the integrity of the anterograde versus retrograde state of the device is suspect. Some of these devices were tested and I was able to confirm neuron migration post plating through the microgrooves (or possibly under the device as a whole) as a consequence of this treatment. We also attempted to use varying concentrations of neural growth factor to stimulate growth, as axons will tend to grow toward the high side of a gradient. In the devices we were using this was similarly dissapointing. Once again, there seemed to be minimal increase in directional axonal growth with increased cellular migration to the non-plated side.

Reporter Cell Co-Culture

If a reporter cell culture is desired, to simulate an epithelial-axonal interface as might be found in an animal, cell-line cultures may be co-plated over the axons. Once the axon density on the axon side of the chamber is at the desired level, cells such as Vero or VK302 cells may be co-plated. To plate these over the axons, cells grown to a confluent monolayer are trypsinized as normal for splitting of the cells. Once trypsinized and disrupted to a single cell suspension, trypsin is neutralized with 100% FBS. Then the cells in the FBS are centrifuged to produce a cell pellet. Cells are resuspended, diluted in media and plated by filling the wells of the axon side with this media. For the Vero and VK302 cells that we use, one T-75 flask can be resuspended in 4 mls of media with about

150 microliters of that used for plating per well (so 300 microliters for a device with two wells on the axon side). The media used for this purpose is a one-to-one ratio of our standard Vero cell media and neuronal media supplemented with a final concentration of 10% FBS. Our standard Vero or VK302 cell media consists of Dulbecco's modified eagle media supplemented with 20 micromolar HEPES buffer. Plated cells in the axon chamber often do not grow well beyond the initial plating. To provide more access to nutrients, cells in the wells may be suctioned out the day after plating, similar to neurons.

Setting up Neuronal Devices and Substrates for Plating Neurons

Neuron devices are commercially available polydimethylsiloxane chips that have been shaped using laser lithography. These devices were described previously in the neuronal transport chapter. Briefly, when placed on a coverslip or Petri dish, they can create a chamber with that allows culture of neurons and growth of axons to a second chamber that is functionally impermeable to virus by diffusion. This type of chamber allows the study of retrograde and anterograde transport separate from one another. Neurons may be plated on glass coverslips or directly on plastic Petri dishes that will act as the floor of these chambers. In either case neurons grow best with the least clumping using a dual coating of poly-D-lysine and laminin. However, with a laminin coated substrate, axonal growth seems more aggressive and neurons are much more likely to penetrate under a neuron device. The virus impermeable barrier can only be guaranteed if axons only grow through the device microgrooves and growth under the device as a whole disrupts this barrier. Lysine only coated surfaces seem to have a failure rate of about 10% when well prepared while laminin coated devices can reach 20-25% failure.

To prepare a substrate, whether glass or plastic, poly-D-lysine, at a concentration of 0.1-0.25 milligrams per milliliter in borate buffer, is placed on the substrate and incubated at 37 degrees Celsius. For a lysine only coating, this is best done for 48 hours prior to use. For a dual coating, lysine is allowed to coat the substrate for 24 hours. Then the substrate is washed 5 times with sterile water and laminin, at a concentration of 0.01 milligrams per milliliter in borate buffer, is placed on the substrate for 24 hours at 37 degrees Celsius. After coating with either method, the substrate is washed 5 times with sterile water and allowed to dry completely but not excessively. Ideally the substrate should be assembled with the neuron device within 30 minutes of fully drying. Neuron devices are prepared by treating with an ethanol soak followed by rinsing 5 times with sterile water and fully drying. Once substrate and neuron device are dry, a device is pressed very firmly onto the substrate to ensure a complete seal. It is recommended that when using glass coverslips a very flat, sterile, surface is used, otherwise coverslips may crack from unequal pressure during this step. Very aggressive pressure can help to make laminin coated substrated less likely to fail; and in a recent experiment the failure rate with extensive pressure was below 20%. Once the device and substrate are assembled, they should be placed in a humidified incubator to prevent excess dehydration of the laminin. Prepared devices should be plated with neurons the same day as they are prepared but lysine coated devices can be used with some success after 1-2 additional days if necessary. When devices are ready for an infection, as determined by sufficient axonal growth and branching and reporter cell culture if desired, media levels must be adjusted to maintain barrier. The side being infected should always have a lower fluid level in the wells than the uninfected side. Removal of media with a media change for

the “high” side is recommended at this time, since following the addition of virus, only addition of media, not removal and change, is recommended to avoid accidental loss of fluid pressure gradient. Media may be added to either side to combat dehydration as long as gradient is maintained. See schematics of neuron devices with fluid pressure gradients demonstrated (Fig A.2). A scale model of a cross section of a single microgroove with representations of the sizes for the HSV virion particle, a typical dorsal root ganglionic neuronal axon, and a typical dorsal root ganglionic neuronal soma are provided for comparison (Fig A.3).

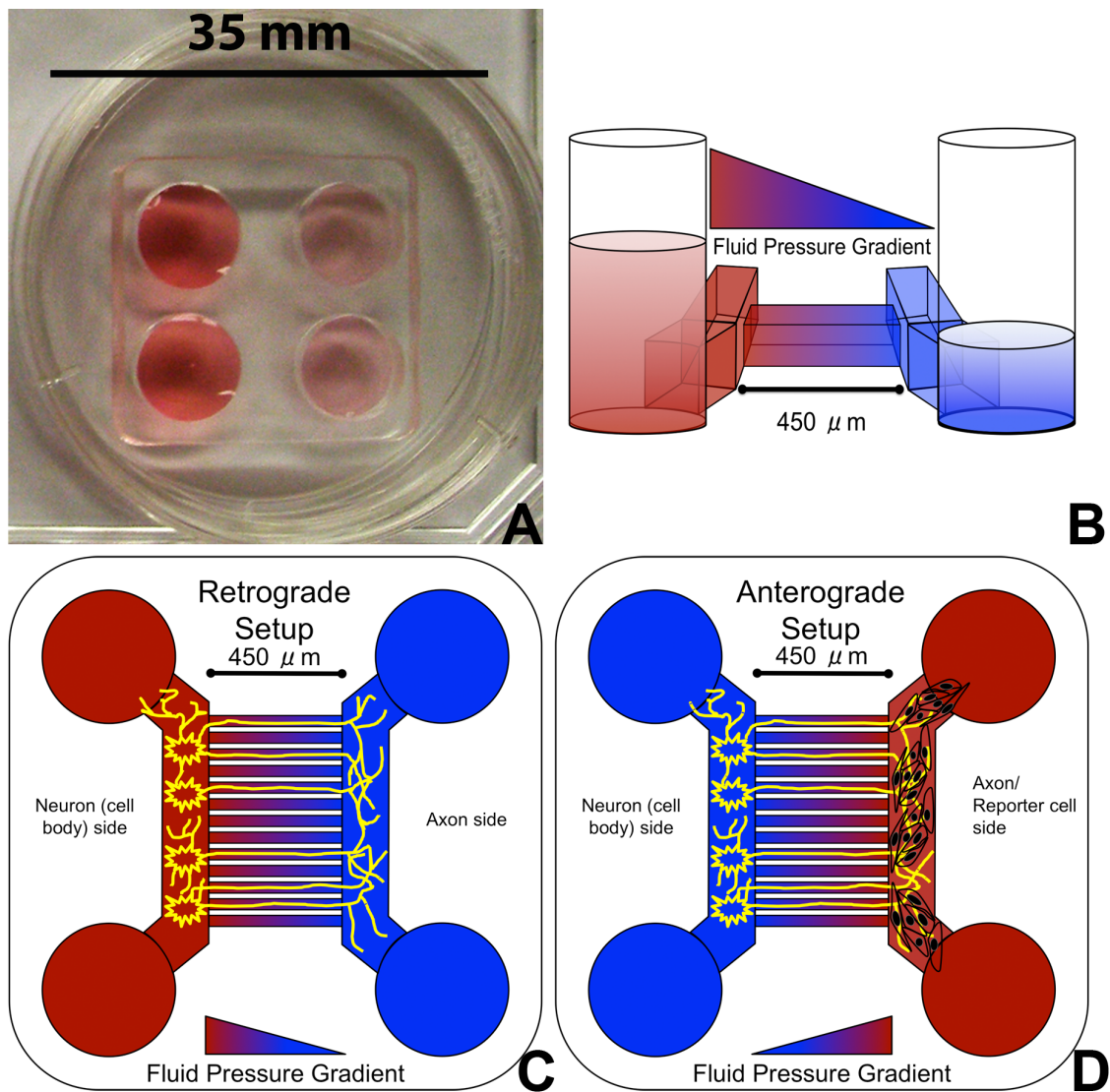


Figure A.2: Microfluidic Neuron Device. A: Picture of a microfluidic neuron device containing media in a 35mm Petri dish. B: Three dimensional wire-frame, side view schematic of wells, central chamber and microgrooves of microfluidic device. The microgrooves are 3 microns tall by 10 microns wide and 450 microns long. The central chambers that connect same side wells (only one well from each side shown in this schematic) are 100 microns high by 1.5 mm wide and 7 mm long. Wells are 8 mm in diameter. C: Color schematic, top view, of a retrograde setup. D: Color schematic, top view, of an anterograde setup with reporter cells on axon side of device.

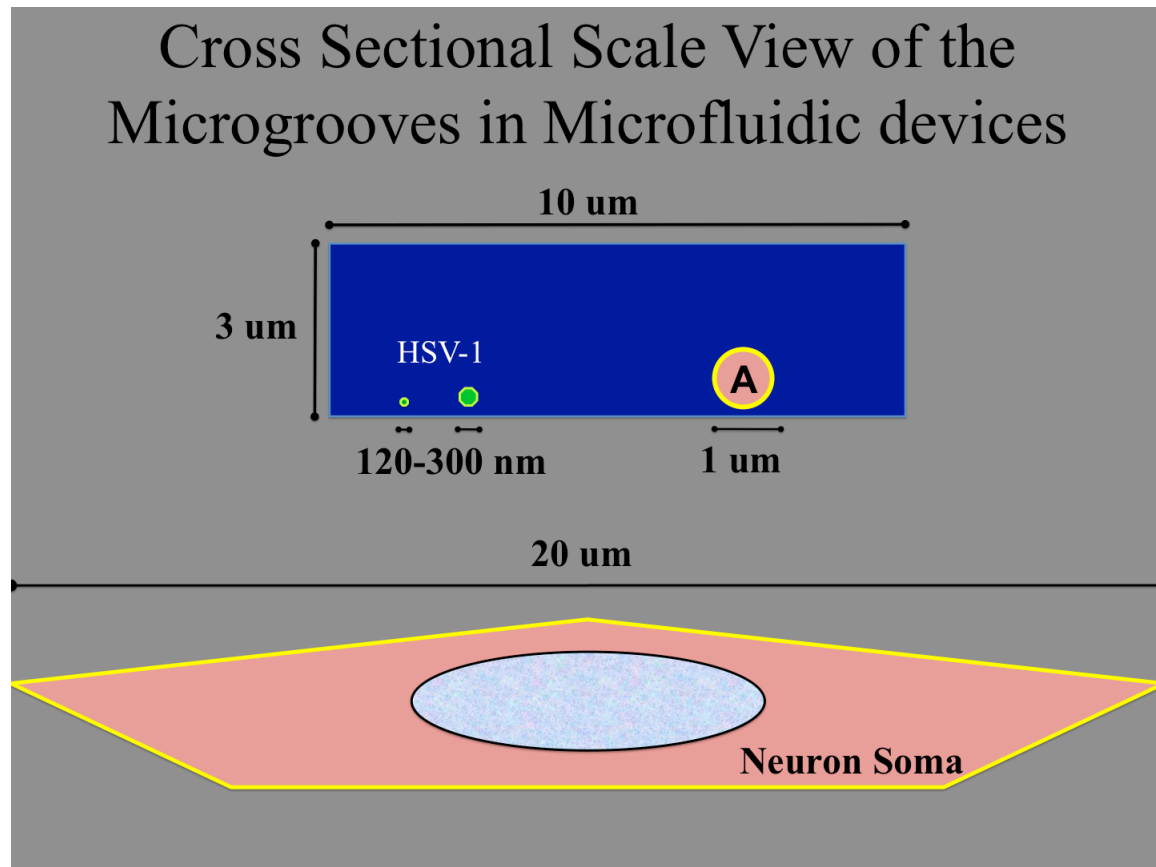


Figure A.3: Scale model of the cross section of a microgroove in a microfluidic neuron device. Scale side view schematic of the cross section of a microgroove from a microfluidic neuron device. The microgrooves are 10 x 3 microns (width x height) and 450 microns long. There are scale appropriate size examples of an average dorsal root ganglion cultured axon with a diameter of about 1 micron and the range of the HSV virus particle with a reported 120-300 micron diameter. For comparison, on the bottom: In one study, that characterized cultured DRG's, neuronal cell bodies ranged from 20-50 microns for the cell body diameter. The smallest of these soma diameters is represented in this schematic.

APPENDIX

LETTER OF PERMISSION

To: Informa Healthcare Customer Operations
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VITA

Andrew Timothy David was born in 1976 to Karl and Marcia David in St. Louis, Missouri. He grew up in St. Louis and graduated from Hazelwood Central High School in 1994. Andrew attended the University of Missouri-Columbia, receiving a Bachelor of Science in 1998. He was accepted to the veterinary school at the University of Missouri-Columbia in 1998. He received his DVM in 2002 and entered a residency in anatomic veterinary pathology at the University of Missouri-Columbia. In 2004, Andrew transferred to the Louisiana State University-School of Veterinary Medicine to complete additional residency training. He also enrolled at the Louisiana State University graduate school at that time. Under the mentorship of Dr. Konstantin G. Kousoulas, he pursued a Doctor of Philosophy degree in pathobiological sciences. Following graduation, he plans to prepare for the board examination to become a diplomate of the American College of Veterinary Pathologists and pursue a position in research, service, and education.