Herpes simplex virus type 1 infection in the central nervous system

Experimental and clinical studies

Charlotta Eriksson
Cover illustration – *Immature human cortical neurons infected with green fluorescent protein-labelled herpes simplex virus type 1*

Herpes simplex virus type 1 infection in the central nervous system – *Experimental and clinical studies*  
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Ineko
To my parents for your endless love and support
Abstract

Alphaherpesvirus infections in the central nervous system (CNS) are rare but severe, and many patients show remaining neurological sequelae. While antiviral treatment has reduced the mortality, morbidity has not been diminished to the same degree, and the immune system activation might contribute to the pathogenesis. Clinical symptoms have often been in focus in previous studies of such infections, while the entry and spread of viral agents is less thoroughly elucidated. Therefore, the aim of this thesis was to investigate aspects of the pathogenesis of herpes simplex virus type 1 (HSV-1) infections in the CNS, including viral properties related to virulence, transport and tropism, and to host immune responses in this compartment.

Infection in a rodent model of herpes simplex encephalitis (HSE) revealed that HSV-1 can enter the brain via the trigeminal nerve or the olfactory bulb. Furthermore, HSV-1 was found to utilize the anterior commissure (AC), a bundle of nerve fibres between the two brain hemispheres, for transport to the contralateral hemisphere. In the AC, HSV-1 targeted cells morphologically resembling oligodendrocytes, which could suggest that virus may utilize additional cells to neurons for rapid transport.

Cerebrospinal fluid (CSF) samples from HSE patients and controls were analysed for concentrations of CNS aquaporins (water channels) and complement components participating in the innate immune response. Increased concentrations were found in HSE patients for aquaporin 9 (AQP9) and complement components C3a, C3b, C5 and C5a as compared with healthy controls, indicative of an increased intrathecal immune activity in HSE. For C3a and C5a, the activity was increased both in acute and convalescent stages of HSE, further contributing to previous observations of increased immune activity in convalescence.

In a cell culture model for differentiation of induced pluripotent stem cells into cortical neurons, reflecting neuronal development, the susceptibility of differentiating cells to infection with HSV-1 or herpes simplex virus type 2 (HSV-2) was investigated. Despite production of high viral titres and high viral DNA quantities both early and late in differentiation, the cell viability of cells in late differentiation was higher than for cells in early differentiation. Thus, neuronal progenitor cells were more vulnerable to infection than mature cortical neurons.

The role of the mucin-like region of glycoprotein C of HSV-1 was studied in cell culture and surface binding resonance experiments. Here it was found that the mucin-like region facilitated both viral attachment to cell
surface glycosaminoglycans upon infection and, more importantly, to egress and release of newly produced virions from infected cells.

Altogether, the findings in this thesis supported previous findings of viral and immunological factors contributing to the CNS infectivity and outcome in HSE. In addition, a novel pathway for HSV-1 transport in the brain in form of AC was discovered. Finally, the importance of the complement system activation in the CNS in HSE patients, and a role for mucin-like region of gC in HSV-1 attachment and egress \textit{in vitro} was demonstrated.

**Keywords:** herpes simplex virus; herpes simplex encephalitis; central nervous system infection; complement system; aquaporin 9; glycoprotein C; differentiating neuronal cells; mucin-like region
Sammanfattning på svenska

Alfaherpesvirus är en grupp virus som vid infektion inte försvinner ur kroppen utan istället stannar kvar hela livet. Till dessa virus hör herpes simplexvirus typ 1 (HSV-1) som oftast ses vid munsår, herpes simplexvirus typ 2 (HSV-2) som vanligen ses vid könsherpes och varicella-zostervirus (VZV) som orsakar vattkoppor och bältros. Även om de synliga symtomen vid aktiv virusinfektion involverar hud och slemhinnor, kan dessa virus infektera även andra celler och vävnader i kroppen. Exempelvis infekterar alfaherpesvirus känselnervernas nervknutor (ganglier) belägna utmed ryggraden där de återfinns i ett viloliknande stadium, så kallad latensfas, under långa perioder. Hos vissa människor kan dessa virus periodvis aktiveras för att åter ge symptom.

Vanligtvis är alfaherpesvirusinfektioner milda, men i ovanliga fall kan de sprida sig till centrala nervsystemet (CNS), upp till hjärnan, där utgången kan bli mycket allvarlig. Beroende på vilken del av hjärnan som infekteras kan infektionen leda till döden, speciellt om den inte behandlas med läkemedel i tid. Även om infektionen behandlas kan dock många få bestående skador i form av epileptiska anfall, minnessvårigheter, och problem med att hantera känslointryck. Medan HSV-1 kan orsaka det svåra tillståndet hjärninflammation (encefalit) hos vuxna, leder HSV-2 infektion ofta till virusorskadad hjärnhinneinflammation (meningit) som är mycket lindrigare. Hos nyfödda barn är däremot HSV-2-infektion i hjärnan allvarligare än HSV-1-infektion. Mycket är känt om de olika symptom och skador man ser i samband med herpesinfektioner i hjärnan, men man vet desto mindre om varför dessa komplikationer uppstår och hur HSV-1 tar sig till och infekterar en specifik del av hjärnan.

Runt området kring näsa och mun finns två stora nerver som kan signalera till hjärnan: luktnerven och trilling- (trigeminus-) nerven. HSV-1 kan utnyttja dessa nerver för att ta sig in i hjärnan och sedan spridas till det område som infekteras. Genom att infektera rättor med HSV-1 i näsborren och sedan följa infektionen, kunde vi se att efter att infektionen etablerat sig i luktnoben i ena hjärnhalvan så sprid sig viruset snabbt till den andra luktnoben genom att utnyttja ett utvecklingsmässigt uråldrigt signaleringsystem, den främre kommissuren, mellan de två hjärnhalvorna.

En orsak till de bestående skador man ser efter infektioner i hjärnan kan vara att det egna immunförsvaret attackerar infekterade celler. För att testa aktiviteten för en komponent av det medfödda immunförsvaret, komplementsystemet, mätte vi flera av dess faktorer i rygmgärsgvätska och
jämförde med nivåerna i blodet. Till skillnad från friska kontroller fann vi
att komplementsystemets aktivitet var hög i CNS hos patienter med
herpesencefalit, även lång tid efter att virusinfektionen läkt ut. Den ökade
komplementaktiviteten bekräftade att immunförsvaret kan påverka förloppet
vid encefalit, och vid sidan om läkemedel mot herpesvirus kan dessa
patienter även tillfälligt behöva läkemedel som dämpar immunförsvaret.

För att studera likheter och skillnader i infektion av nervceller mellan
HSV-1 och HSV-2 använde vi oss av en cellmodell där cellerna undersöktes
under olika utvecklingsgrad. Försöksresultaten visade att när det inte finns
immunceller närvarande är delande nervceller under utveckling mycket
känsligare för virusinfektion än mognas, icke-delande nervceller.

Slutligen studerade vi hur ett specifikt protein som sitter på virushöljet,
glykoprotein C (gC), kan binda till konstgjorda membraner och påverka
HSV-1-infektion av celler. Ett område på proteinet med många
sockermolekyler, en så kallad mucindomän, gynnade bindning både av gC
och av hela viruspartiklar, till en virusreceptor i form av kondroitinsulfat. Ett
viktigt fynd var också att mucindomänen på gC underlättade för nybildade
virus att lämna sin värdcell.

Sammanfattningsvis visar studierna i denna avhandling på att förloppet
av herpesvirusinfektioner i hjärnan inte enbart beror på en enda faktor, utan
att flera egenskaper både hos virus, nervceller och immunförsvar samverkar
och avgör hur allvarlig infektionen blir.
List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals:

I. Jennische E*, Eriksson CE*, Lange S, Trybala E, Bergström T.
The anterior commissure is a pathway for contralateral spread of herpes simplex virus type 1 after olfactory tract infection.

II. Eriksson CE, Studahl M, Bergström T.
Acute and prolonged complement activation in the central nervous system during herpes simplex encephalitis.
_J Neuroimmunol_ 2016; 295-296: 130-138

III. Eriksson CE, Agholme L, Trybala E, Nazir FH, Satir TM, Zetterberg H, Bergström T, Bergström P.
Transient cytopathogenicity despite increasing infectivity of herpes simplex virus types 1 and 2 during neuronal differentiation.
_Manuscript_

IV. Altgärde N, Eriksson C, Peerboom N, Phan-Xuan T, Moeller S, Schnabelrauch M, Svedhem S, Trybala E, Bergström T, Bally M.
Mucin-like Region of Herpes Simplex Virus Type 1 Attachment Protein Glycoprotein C (gC) Modulates the Virus-Glycosaminoglycan Interaction.
_J Biol Chem_ 2015; 290(35): 21473-21485
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<th>Description</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>arbovirus</td>
<td>arthropod-borne virus</td>
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<tr>
<td>AC</td>
<td>anterior commissure</td>
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<tr>
<td>ACA</td>
<td>acute cerebellar ataxia</td>
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<tr>
<td>AF-16</td>
<td>anti-secretory factor peptide 16</td>
</tr>
<tr>
<td>AQP1, AQP4, AQP9</td>
<td>aquaporins 1, 4, 9</td>
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<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
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<tr>
<td>C1q, C3a, C3b, C5, C5a</td>
<td>complement components 1q, 3a, 3b, 5, 5a</td>
</tr>
<tr>
<td>C4b2a</td>
<td>the C3 convertase of the classical and lectin pathways in complement activation</td>
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<tr>
<td>CA</td>
<td>cell-associated</td>
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<tr>
<td>CFB</td>
<td>complement factor B</td>
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<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CS</td>
<td>chondroitin sulphate</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>CT</td>
<td>computed tomography</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
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<tr>
<td>EEG</td>
<td>electroencephalogram</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>EX</td>
<td>extracellular</td>
</tr>
<tr>
<td>Fc</td>
<td>fragment crystallisable</td>
</tr>
<tr>
<td>FoHM</td>
<td>Folkhälsomyndigheten (the Public Health Agency of Sweden)</td>
</tr>
<tr>
<td>GACHE</td>
<td>German trial of acyclovir and corticosteroids in herpes simplex virus encephalitis</td>
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<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
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<tr>
<td>GalNAc</td>
<td>N-acetylgalactosamine</td>
</tr>
<tr>
<td>gB, gC, gD, gE, gG, gH/gL</td>
<td>Envelope glycoproteins B, C, D, E, G, H/L</td>
</tr>
<tr>
<td>gDNA</td>
<td>genomic DNA</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GMK</td>
<td>green monkey kidney</td>
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<tr>
<td>GOS</td>
<td>Glasgow outcome scale</td>
</tr>
<tr>
<td>HA</td>
<td>hyaluronic acid</td>
</tr>
<tr>
<td>HHV-6</td>
<td>human herpesvirus 6</td>
</tr>
<tr>
<td>HS</td>
<td>heparan sulphate</td>
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<tr>
<td>HSE</td>
<td>herpes simplex encephalitis</td>
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<tr>
<td>HSM</td>
<td>herpes simplex meningitis</td>
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<tr>
<td>HSV</td>
<td>herpes simplex virus</td>
</tr>
<tr>
<td>HSV-1</td>
<td>herpes simplex virus type 1</td>
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</table>
HSV-2  herpes simplex virus type 2
HVEM  herpesvirus entry mediator
i.c.  intracranial
i.v.  intravenous
IC50  50% inhibitory concentration
ICP  intracranial pressure
IFN  interferon
IgG, IgM  immunoglobulins G, M
IL  interleukin
iPSC  induced pluripotent stem cell
IR  inverted repeat
LAT  latency-associated transcript
mab  monoclonal antibody
MAC  membrane attack complex
MBL  mannose-binding lectin
MRI  magnetic resonance imaging
NMDAR  N-methyl D-aspartate receptor
NS1  non-structural glycoprotein 1
PCR  polymerase chain reaction
pfu  plaque forming unit
PHN  post-herpetic neuralgia
PILRα  paired immunoglobulin-like receptor alpha
qPCR  quantitative PCR
RNA  ribonucleic acid
SA  sialic acid
Ser  serine
SPR  surface plasmon resonance
TBE  tick-borne encephalitis
Th1 cells  T helper cells type 1
Thr  threonine
TK  thymidine kinase
TLR  toll-like receptor
TMR  transmembrane region
TNF-α  tumour necrosis factor alpha
TNFR  tumour necrosis factor receptor
TR  terminal repeat
UL  unique long
US  unique short
VZV  varicella zoster virus
1. Aims

The overall aim of this thesis was to investigate aspects of the pathogenesis of herpes simplex virus type 1 (HSV-1) infections in the central nervous system (CNS), through studies in cell cultures, animal experiments and clinical studies. The studies included those on viral properties related to virulence, transport and tropism, and of host responses to such viral infection.

Specific aims:

- To investigate the spread of HSV-1 within the CNS after olfactory infection in a rat model, with emphasis on involvement of specific neuroanatomical structures and connections.

- To assess whether the complement system is activated systemically and/or intrathecally in herpes simplex encephalitis (HSE) and, if so, to determine its route of activation and the duration of this immune response in the patients.

To investigate, in a cell culture model differentiating towards human cortical neurons, if neuronal differentiation affects the viral replication and cytopathogenicity during HSV-1 (and HSV-2) infection.

- To define a role for the mucin-like region of HSV-1 glycoprotein C (gC) for attachment to the viral receptor of glycosaminoglycan (GAG) nature during viral entry and release from the same binding during egress, functions that may be decisive for tropism and transport of this virus.
2. Introduction

Viruses are so-called obligate intracellular parasites. Lacking a functioning cell machinery, viruses require a living host cell for replication and protein synthesis. Cells from all living organisms can be infected by viruses; animals, plants, fungi, bacteria, but the families of infecting viruses are usually specific for their respective host organism [1].

In animals, viruses infect by breaking through the existing natural protective barriers of the body. Once inside, viruses normally evade the immune control and thereby cause disease, either by killing cells or by triggering a destructive immune and inflammatory response.

After infection, viruses display a tropism for specific tissues, which means that they preferably infect tissues and cells to which they are adapted. Some viruses show a broad tropism and can infect many different tissues, while other viruses present with narrow tropism and can only infect specific cell types. Once inside the host, the viral infection has three potential outcomes [2]: abortive (failed) infection, lytic infection, with cell death as a result, or persistent infection, where cells are infected but not subjected to cell death. Persistent infections can be subdivided into chronic latent infections or transforming infections. Chronic latent infections are non-lytic with restricted transcription of viral genes; no complete viral particles are synthesized unless the host cell is exposed to stress or other stimuli that may reactivate the infection. Transforming infections result in immortalisation or transformation of the host cell [2].

Susceptibility and severity of an infection may in part be determined by the functional capacity of the immune response [3]. Immunosuppression, and thereby reduced activity or efficacy of the immune response, can certainly be a negative determinant of how the infection is resolved and how severe the outcome of the symptoms will be (reviewed in [4]). However, although immunosuppressed patients are at a greater risk of more severe course of a primary infection and are more prone to experience recurrent infections after reactivation from latency of certain viruses, other factors may also be of importance for the outcome of the infection. Specific age groups, such as young children and the elderly, are more at risk for being infected by certain viruses, and the severity of the infection may also be increased [5]. Moreover, genetic alterations related to the virus and to the host can affect the course of the infection, as can the viral exposure dose, geographic restrictions and seasonal variations. A viral mutation can result in either reduced or increased infectivity, and could also influence the outcome [6-17]. Similarly, a mutation in the host can increase or reduce the risk for viral infection when genes coding for viral receptors are targeted, and alterations in
genes responsible for immune functions can be decisive for the outcome of the infection [18-20]. All viruses have, just as all other organisms, an ecologic niche and prefer a certain climate and temperature for spread and infection (reviewed in [21]). Connected to this are seasonal variations in infectivity, and some viral infections are more prevalent during the winter, when temperature drops, humidity might decrease and people may gather inside in larger groups [5]. Other viruses are more prevalent during the summer when increased temperatures favour water- and food hygiene-related spread and enable spread of viral vectors such as mosquitoes and ticks [5].

Viruses are transmitted between hosts through different mechanisms. Some viruses are airborne and travel via aerosols, others via faecal-oral transmission, some via direct contact (with lesions, saliva, breast milk etc.) or indirect contact (contaminated objects, water, food) [5]. Furthermore, some viruses can be transmitted as zoonoses (animals or insects are vectors or reservoirs), others via blood transfusions, or through sexual contacts, and vertically from mother to child congenitally or during delivery [5].

2.1 Herpesviridae

Herpesviridae, the herpesvirus family, consists of large enveloped DNA viruses that cause latent and/or lytic infection in hosts throughout the animal kingdom [22]. Because of their envelope, herpesviruses are sensitive to acids, detergents and drying and therefore often spread via close contact (although some are airborne). The name herpes is derived from a Greek word, Herpein, meaning “to creep”, referring to the creeping skin eruption of zoster and oral/genital herpetic lesions.

To date, there are three known subfamilies of the mammalian herpesviridae, divided according to the biological properties and site of latency of the viruses. Viruses infecting humans are represented in all subfamilies [23]:

- **Alphaherpesviruses**
  - Herpes simplex virus type 1 (HSV-1)
  - Herpes simplex virus type 2 (HSV-2)
  - Varicella zoster virus (VZV)

- **Betaherpesviruses**
  - Cytomegalovirus (CMV)
  - Human herpesvirus 6A (HHV-6A)
  - Human herpesvirus 6B (HHV-6B)
  - Human herpesvirus 7 (HHV-7)

- **Gammaherpesviruses**
Alphaherpesviruses

Alphaherpesviruses have a wide tropism as they can infect most cells and organs in the body and often cause an initial viraemia during the primary infection. However, thereafter they specifically target mucoepithelial cells (fibroblasts and epithelial cells) where they cause lytic infections, and establish latency in sensory neurons from which they can reactivate to cause recurrent mucoepithelial lesions [23]. Other features of the alphaherpesviruses are relatively short reproductive cycles, fast progression of infection in cultured cells and lytic effect on infected cells where the virus is not harboured latently [22].

As presented above, the human alphaherpesviruses include HSV-1, HSV-2 and VZV. HSV-1 and 2 cause mucoepithelial lesions of the oral area (more common for HSV-1) or the genital area (more common for HSV-2) while VZV causes chickenpox (varicella, lesions all over the body) and shingles (zoster, lesions limited to a dermatome). Other types of infections can also occur, where some are more severe, including eye infection (keratitis) [24], hepatitis [25-27], pancreatitis [26] and pneumonitis [28]. However, these manifestations lie outside the scope of this thesis, as the focus is on alphaherpesvirus infections of the CNS.

Primary infections with HSV-1 and VZV often occur during childhood, when the presentation is usually benign, while such infection later in life can be more severe. HSV-1 is often spread through contact with secretions such as saliva or breast milk, while VZV is airborne causing outbreaks in schools and day-care centres. HSV-2 spreads mainly via sexual contact or in rare cases from mother to child (congenital or post-natal infection) [29]. As the spread of HSV-2 is predominantly via sexual contact, the average age for primary HSV-2 infection is higher than for primary HSV-1 [30]. The seroprevalence of these viruses is high in the population, although HSV-2 has a lower seroprevalence (10-30%) than HSV-1 (50-80%) and VZV (80-100%) [29, 31-34].

As illustrated by the phylogenetic tree (Figure 1), HSV-1 and HSV-2 are more closely related to each other than to VZV [35]. In fact, VZV belongs to a different subgroup of alphaherpesviruses, namely the varicelloviruses, while HSV-1 and HSV-2 are part of the subfamily of simplexviruses. Previous work from our laboratory has shown that HSV-1 strains can be divided into three genetic groups, but frequent homologous recombination events during their evolution has resulted in mosaic patterns in all investigated clinical strains which complicates the genotyping [36].
Herpes simplex virus type 1

HSV-1 virus is highly contagious. In 2012 it was estimated that around 3.6 billion people under the age of 50 (67%) were infected worldwide; seroprevalence was estimated as 87% in Africa and 40-50% in the Americas [37, 38].

As mentioned above, the most common manifestation of HSV-1 infection is oral herpes i.e. mucocutaneous lesions in the orolabial region. However, many infected persons are asymptomatic, and shed the virus unaware of that they are contagious [39]. HSV-1 is mainly transmitted through oral-to-oral contact, but can also be transmitted to the genital area, and in some Western countries, HSV-1

Figure 1. The phylogenetic relationship within alphaherpesviruses. Simplexviruses and varicelloviruses are present in mammals, Mardiviruses and Iltoviruses are found in birds and Scutaviruses are prevalent in reptiles. Please note that not all existing alphaherpesviruses are represented in the figure. The three human viruses HSV-1, HSV-2 and VZV are highlighted in black. Cercopithecine herpesvirus 2 (CeHV-2), Papiine herpesvirus 2 (PaHV-2) and Herpes B virus (McHV-1) as well as Cercopithecine herpesvirus 9 (CeHV-9) all infect primates, but McHV-2 may also in rare instances infect humans resulting in severe brain infections. Pseudorabies virus (SuHV-1) has been of special importance for neuroscientific research. The phylogenetic relatedness is based on aa sequence alignment for 6 genes (unique long region (UL) 15, U1,19, U1,27, U1,28, U1,29, U1,30) performed by Davison [35].

BoHV = bovine herpes virus, EHV = equine herpes virus, GaHV = Gallid herpes virus, MeHV = Meleagrid herpesvirus, ChHV5 = Chelonid herpesvirus 5.
infection has been reported to be almost equal or superior to HSV-2 as the primary cause of genital herpes in younger women [31, 38, 40-42]. In some circumstances, HSV-1 can be transmitted from mother to child during delivery (if the mother has a genital HSV-1 infection) [43, 44]. HSV-1 is also associated with the skin infections commonly known as herpetic whitlow (infection on fingers) and herpes gladiatorum or wrestler’s herpes (infection on the chest or face) [39]. Although HSV-1 is normally regarded as a “mild” virus, on rare occasions it can cause herpes keratitis [24] or severe CNS-infections such as herpes simplex encephalitis (HSE) that can be fatal. This manifestation will be described in detail later.

HSV-1 can be transmitted through asymptomatic shedding from the mucosal surfaces, saliva and breast milk, but transmission from active lesions is more common, and constitutes a greater risk. As for all herpesviruses, latent infection is established after active infection with HSV-1, and the trigeminal nerve is the preferred site of latency. HSV-1 infection can be asymptomatic, but it can also be recurrent. On reactivation, HSV-1 can be transported anterogradely via the neuron’s axon to the skin where virus is shed and new lesions may reappear in the affected area. These recurrences can be triggered by various stimuli, including UV radiation, radiotherapy, trauma, upper respiratory tract infection, stress and menstruation [39]. Reactivation will be discussed in detail in a later section.

In adults, the primary infection can be more painful and more extensive than recurrent episodes [45], while primary infection in childhood usually is mild or goes unnoticed [46]. However, primary gingivostomatitis, where extensive and painful blistering appears on the lips and on the tongue and mucosal surfaces inside the mouth, can occur also in children [46, 47].

In 2012, 140 million of the world’s population aged 15-49 years was estimated to have a genital HSV-1 infection [37], but the prevalence varied between different regions, where Europe, the Americas and the Western Pacific had the highest prevalence. This is probably related to the fact that HSV-1 infection is acquired well into the adolescent years in Western countries, while in Africa HSV-1 infections mostly are acquired during childhood. In the Western world, better hygiene and awareness of that herpetic lesions can spread virus might have reduced the transmission of HSV-1 in childhood, when the infection is milder, and increased the spread of HSV-1 upon sexual debut [48].

*Herpes simplex virus type 2*

HSV-2 is the human alphaherpesvirus with the lowest seroprevalence worldwide, with 417 million people (11%) estimated to be infected in 2012 [29]. The infection is rare in children, and most primary infections are found among adolescents and young adults. As for HSV-1, the seroprevalence is not evenly
distributed between the continents. HSV-2 infection shows the highest prevalence in Africa (31.5%) followed by the Americas (14.4%) [29].

Almost exclusively transmitted sexually [49], and mainly associated with genital herpes [31], HSV-2 is more prevalent in women [32], since sexual transmission of HSV-2 from man to woman may be more efficient than from woman to man. Like HSV-1, HSV-2 infection is often asymptomatic and can be transmitted through shedding from areas that lack signs of infection [50].

The preferred site of latency for HSV-2 is the sacral ganglia, which innervate the genital area. While mainly being transmitted sexually, HSV-2 can also be transferred vertically in utero as a congenital infection, or during delivery from infected mother to child. Such transmission can cause a neonatal herpes infection, which can be fatal. Besides the severe neonatal herpes infection, HSV-2 can also cause meningitis in adults. The CNS infections caused by alphaherpesviruses will be described in more detail later in this thesis.

Having an HSV-2 infection increases the risk for acquiring HIV (up to three-fold increase [51]), and individuals co-infected with HIV and HSV-2 have increased risk for spreading HIV to others (reviewed in [52]). These patients, as is the case for other immunocompromised individuals, often have more frequent severe and painful recurrences than do immunocompetent individuals.

**Varicella Zoster virus**

VZV, which causes chickenpox (varicella) as primary infection and shingles (zoster) after reactivation, is one of the most prevalent viral infections in humans [34]. While primary HSV infection may be asymptomatic, primary VZV infection is usually symptomatic in form of varicella, which in the Western world is a common childhood infection. Unlike HSV, the skin lesions are generally distributed over the upper part of the body and may, in addition to epidermal location, go deeper and engage dermis. Like other alphaherpesviruses, VZV can establish latency in sensory ganglia (along the entire neuroaxis) from where the virus reactivates to cause herpes zoster. In zoster, ulcers and pain usually appear along the dermatome of a sensory nerve of the specific ganglion from which the virus has reactivated, while virus remains latent in other ganglia along the neuroaxis. VZV spread is truly airborne, and both varicella and zoster patients can transmit infection to a seronegative person, although zoster patients are regarded as less contagious than varicella patients. However, it is believed that varicella outbreaks can be triggered by transmission from zoster patients, for example a grandparent meeting with a seronegative grandchild. Unlike HSV, VZV primary infection usually results in viraemia, and while HSV is thought to access neuronal
ganglia only from the axons in the skin, VZV may also access neurons via immune cells during viraemia (reviewed in [53]).

Like HSV, VZV can infect the CNS following both primary infection and reactivation, and such infections can occur without any signs of skin manifestations. In Sweden, VZV is reported to be the second or third most common cause of viral CNS infection [54] and this virus is related to many different clinical CNS manifestations, including encephalitis, myelitis and cerebellitis, which are described later in the thesis.

2.2 Structure of a herpes virion

All herpesviruses share a common structure (Figure 2). In the centre, a DNA core with linear double-stranded DNA is located, which is surrounded by an icosahedral capsid. Outside of this, a space called the tegument is located, which contains proteins and enzymes that facilitate initiation of replication. The tegument in turn is enclosed by an envelope where several different glycoproteins are inserted.

*Figure 2. Structure of a herpes virion. Double-stranded, linear DNA is located inside the icosahedral nucleocapsid, which is surrounded by tegument proteins. This capsid is in turn surrounded by the viral envelope on which surface viral glycoproteins are exposed. Note that in de-enveloped virions, the genome is circularized [39].*
Viral genome

Alphaherpesvirus genomes commonly share four structural components: the unique long (UL) and the unique short (US) regions, the terminal repeat (TR) regions, flanking the ends of the genomes, and the inverted repeat (IR) regions, linking the unique regions together (Figure 3). While the UL regions encode single-copy genes, the IR regions may code for diploid genes, as well as sequences required for viral DNA cleavage and package.

Interestingly, alphaherpesviruses display isoforms of their genomes. HSV have four isomeric forms, present in equal proportions, while VZV show two predominant isoforms. The isomeric forms of the genome have emerged from inversion of the unique sequences relative to each other. For HSV, both the UL and the US can be inverted. Technically, VZV do have four isoforms, but the inversion of UL only occurs in around 5% of the total genomes, due to the existence of much shorter TR and IR adjacent to the UL region [55]. Thus, the two dominant VZV isoforms are due to inversions in US.

As can be seen in Figure 3, the genomes of HSV-1 and HSV-2 are considerably larger than the VZV genome. Furthermore, the TR and IR adjacent to the UL region are much longer for HSV than for VZV. In addition, the G+C content (Figure 3) is high for HS but low for VZV, which also underscores the large differences between the viruses and supports the genetic diversity demonstrated in the phylogenetic tree (Figure 1) [55].

The genome of HSV-2 is more homologous to HSV-1 than to VZV, but there are nevertheless large differences between the two HSV genomes as well. The overall nucleotide identity between HSV-1 and HSV-2 is approximately 50% [56], and the differences in the genomes, and the resulting differences in structure and functions of glycoproteins, could explain the type-specific preferences for sites of latency and lytic infection.

**Figure 3.** Human alphaherpesvirus genomes. UL = unique long region, US = unique short region, IRL = internal repeat long region, TRL = terminal repeat long region, IRS = internal repeat short region, TRS = terminal repeat short region. G+C represents the content of guanine and cytosine in the genome, indicative of the number of three-hydrogen bonds in the DNA chain. High G+C percentage indicates that the HSV chromosome is more stable than VZV DNA with its low G+C percentage.
HSV glycoproteins and their role in cell entry

Envelope glycoproteins are decisive in early virus-cell interactions, in attachment to and fusion with the host cell and in the immune escape of the virus. Alphaherpesviruses encode a multitude of their own glycoproteins, which are glycosylated by the host cell machinery. Glycans attached to the viral glycoproteins can either be N-linked (attached to asparagine residues) or O-linked (attached to serine, threonine or tyrosine residues). While HSV-1 and HSV-2 encode for at least 12 glycoproteins [57-59], the VZV genome is smaller, in particular the Us region, and encodes fewer glycoproteins [28]. The VZV cell entry is less well studied than that of HSV, and the entry receptors are partly different, but as the major focus of this thesis is HSV-1, only the HSV glycoproteins and their cell entry procedures will be described here.

Five glycoproteins participate in HSV cell entry: glycoprotein B (gB), gC, glycoprotein D (gD) and the complex of glycoproteins H and L (gH/gL). For HSV-1 and HSV-2, gB, gD and gH/gL are essential glycoproteins in cell culture while gC is dispensable. The functions of all known glycoproteins of HSV are described in Table 1. Glycoproteins involved in cell entry are also described further below.

During HSV entry, the virus can bind at least four different receptors with its glycoproteins, as is illustrated in Figure 4. Replication of herpesviruses is initiated when the viral glycoproteins interact with the surface receptors of the host cells.

Figure 4. Viral entry into cells assisted by glycoproteins.
(1) An HSV-1 virion approaches the cell surface.
(2) Viral glycoproteins interact with host cell surface receptors. Glycoprotein C (gC) and partly gB binds to HS, which brings the virion closer to the cell membrane, where gD can bind HVEM, Nectin-1 or -2, or a modified form of HS and gB can bind PILRα as a co-receptor.
(3) Virion attaches to host cell surface, where binding to receptors induce a conformational change in gD, which activates gB and gH/gL.
(4) Virion envelope fuses with the cell plasma membrane, assisted by gB and the complex of gH/gL. This leads to release of the capsid into the cytoplasm along with the tegument content.
The primary interaction is between the viral attachment protein gC and heparan sulphate (HS) [60, 61] or chondroitin sulphate (CS) when HS is absent [62, 63]. Although gC is known to be non-essential for cell-entry, HSV-1 virions deficient in gC (gC-1 negative virions) are rarely found in nature and have been shown to display reduced infectivity [60]. HSV-1 gB (gB-1) can also interact with HS although, in gC-1 wild-type virus, this interaction is less important than gC-1 binding [64]. For HSV-2 virions, gC may not be as important during the initial attachment phase as for HSV-1, and gB-2 has instead been suggested to be of greater importance for HSV-2 binding to HS [65, 66]. Special focus on gC will be given in a separate paragraph.

After initial attachment, gD can bind to any of the natural receptors: nectins, herpesvirus entry mediator (HVEM) or 3-O-sulfated HS (Figure 4). Furthermore, the paired immunoglobulin-like receptor α (PILRα) can be used as a co-receptor for cell entry through binding via gB [67]. The next paragraph will discuss the different entry receptors in detail.

After binding to the host cell, a conformational change in gD is induced, which activates gB and gH/gL-mediated fusion between the virion envelope and the plasma membrane (Figure 4). This in turn leads to a release of the capsid into the cytoplasm, while the content of the tegument (enzymes and transcription factors for initiation of viral transcription) also finds its way into the cell. Docking of the capsid with the nuclear membrane leads to release of the genome into the nucleus where it circularizes, and can be transcribed for replication. The transcription process is performed by a cellular RNA-polymerase, but the procedure is controlled both by virus-encoded and cellular nuclear factors. Here, the biological decision of HSV lytic replication or latency is determined and the expression of responsible genes is triggered. Since latency will be described in detail in the chapter on viral infections in the CNS, only the fate of lytic infection will be described in this section.

In a lytic infection, the infected cell produces infectious virions. HSV and VZV encode for their own DNA polymerases and other relevant enzymes, such as the viral thymidine kinase, which promote viral DNA replication. Newly synthesized viral DNA enters the empty procapsids within the nucleus, and the virus exits through the nuclear membrane. After this, the virus obtains its envelope with newly produced glycoproteins during its passage through the Golgi and ER networks. Finally, the virus exits the cells by exocytosis or lysis of the cell membrane, to spread to new cells in the same host, or to a different host. In lytic infection, the virus regulates the metabolism of the host cell as well as the protein synthesis, the cell cycle and intrinsic and innate cell responses [22].

Histopathologically, lytic infection is associated with swelling of cells and degeneration of the cell nuclei and loss of intact plasma membranes, leading to
formation of multinuclear, giant cells and cell lysis. Lytic infection leads to recruitment of an intensive inflammatory response, though this activity is significantly lower during recurrent disease.

<table>
<thead>
<tr>
<th>Glycoprotein</th>
<th>HSV-gene</th>
<th>Gene function</th>
</tr>
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<tbody>
<tr>
<td>gB</td>
<td>U1:27</td>
<td>Fusion protein that, together with gH and gL [68, 69] is essential for infectivity of virions and fusion between cells. gB is required for cell entry but also participates in the initial interactions with the cell surface HS [60] together with gC. gB can bind to PILRα [67], and is essential for infection in cell culture.</td>
</tr>
<tr>
<td>gC</td>
<td>U1:44</td>
<td>Mediates the attachment of virions to cells through binding to HS [60] or CS [63]. gC-1 can also bind complement component 3b (C3b) and block binding of complement components C5 and properdin to C3b, a function which gC-2 lacks on whole virions [70].</td>
</tr>
<tr>
<td>gD</td>
<td>U5:6</td>
<td>Defines viral tropisms through interactions with the entry receptors HVEM [71], 3-O sulphated HS [72] nectin-1 [73] and nectin-2 (particularly relevant for wild-type HSV-2, although specific mutations in HSV-1 gD (gD-1) can also induce binding via nectin-2 [10]). gD initiates a conformational change leading to exposure of domains involved in fusion, thereby allowing gB, gH and gL to complete the fusion between the virion envelope and the plasma membrane [68]. gD is essential for infection in cell culture.</td>
</tr>
<tr>
<td>gE</td>
<td>U5:8</td>
<td>Forms a heterodimer with gl, but gE is the major constituent in the viral Fc receptor and is involved in antiretroviral defence [74, 75]. gE is essential for HSV anterograde spread along with gl [74, 76, 77], and important for axonal targeting and retrograde transport [74, 78].</td>
</tr>
<tr>
<td>gG</td>
<td>U5:4</td>
<td>Precise function unknown, but HSV-2 gG (gG-2), which is considerably larger than gG-1, has been show to bind chemokines and may be of importance for immune evasion [79]. gG-1 may enhance apical infection of polarized cells [80].</td>
</tr>
<tr>
<td>gH</td>
<td>U1:22</td>
<td>A fusion protein that is essential for the infectivity of virions and fusion between cells. gH interacts in a complex with gL and is essential for infection in cell culture [81]. gH can induce neutralizing antibodies [82].</td>
</tr>
<tr>
<td>gI</td>
<td>U5:7</td>
<td>Forms a heterodimer with gE, where the complex forms a viral Fc receptor for immunoglobulin G (IgG) [75]. In polarized cells, such as epithelial cells, the complex can assist in basolateral spread of progeny virus. gI is essential for HSV anterograde spread along with gE and probably also with Us9 [77].</td>
</tr>
<tr>
<td>gJ</td>
<td>U5:5</td>
<td>Blocks apoptosis [83].</td>
</tr>
<tr>
<td>gK</td>
<td>U1:53</td>
<td>Inhibits fusion between infected cells and adjacent cells. Appears to be of importance in the interaction between gB and PILRα [84]. Reported to promote viral egress [85].</td>
</tr>
<tr>
<td>gL</td>
<td>U1:1</td>
<td>Appears to regulate the fusogenic activity of gH and is thereby essential for cell fusion [86]. gL is not anchored to the plasma membrane [87] but is found in complex with gH and is essential for infection in cell culture.</td>
</tr>
<tr>
<td>gM</td>
<td>U1:10</td>
<td>Might be required for package of gN into virions, with which it interacts [88, 89]. gM is suggested to be required for efficient membrane fusion during viral entry and spread [90].</td>
</tr>
<tr>
<td>gN</td>
<td>U1:49.5</td>
<td>Reportedly blocks endogenous antigen presentation. gN interacts with gM [89].</td>
</tr>
</tbody>
</table>
**HSV entry receptors**

As mentioned above, there are three known classes of entry receptors for gD, one receptor for gB and two attachment receptors for gC (and partially gB).

HS, highly sulphated carbohydrate polymers covalently linked to proteins (so called proteoglycans, see below) or to lipids inserted into the cell membrane, usually function as attachment molecules for HSV via gC and gB, but gD can also utilize the modified form of 3-O-sulfated HS as its specific receptor. Members of the 3-O-sulfotransferase enzyme family modify HS, and can be found in the brain but also elsewhere in the body [91, 92]. The presence of this enzyme in the brain suggests the importance of this specific HS receptor for HSV-1 infection in the CNS [72], though the exact entry mechanisms are still unclear. HS and CS, as mentioned above, are constituents of proteoglycans with a cell-associated core protein (carbohydrate backbone) and GAG chains bound to serine residues on the backbone [93]. The GAG chains consist of numerous linear repeats of disaccharide motifs, synthesized via a dynamic process in three phases, which can include different enzymes depending on cell type and stage of cell differentiation [94]. Depending on which sugar residues that are combined in the initial disaccharide motif of the GAG chain, different families of enzymes are activated, resulting in synthesis of either HS or CS chains [93]. Virus can bind to sulphated oligosaccharide motifs on GAGs, which are negatively charged due to the sulphate groups. Therefore, electrostatic forces are of importance in the attachment interactions between the viral glycoproteins and the proteoglycans [93].

HVEM is a cell-surface receptor belonging to the tumour necrosis factor receptor (TNFR) superfamily; it is expressed by T lymphocytes as well as by epithelial and neuronal cells [71, 95-99]. The natural function of HVEM appears to be regulation of the mucosal microbiota and the epithelial barrier [100]. Upregulation of HVEM expression via the latency-associated transcript (LAT) has recently been suggested to enhance reactivation of HSV from latency [101].

The intracellular adhesion molecules in form of nectins are found on epithelial and neuronal cells [96-99, 102-105]. Nectins are members of the immunoglobulin (lg) superfamily and complexes can be formed between nectins on adjacent cells [73, 106]. HSV-1 binds mainly to nectin-1 while wild-type HSV-2 and HSV-1 strains with specific gD-1 mutations also can bind to nectin-2 [10, 101, 107].

Finally, PILRα can trigger viral fusion in certain cell types upon binding to gB [67]. PILRα is found on monocytes, macrophages and dendritic cells and normally delivers inhibitory signals to the host cell. Binding of gB to PILRα may therefore also provide the virus with an immune escape route (reviewed in [108]) but the significance of this receptor needs further investigation.
**Glycoprotein C of HSV-1**

All three alphaherpesviruses carry gC (the UL44 gene) on their envelope, but while gC of HSV-1 and HSV-2 (gC-1 and gC-2, respectively) are highly conserved (65% similarity, where most divergence is seen in the N-terminal region), VZV gC only display 30% genetic similarity with gC-1. Nevertheless, VZV gC might also utilize HS as an initial receptor [109]. gC is a type-1 membrane glycoprotein, which is highly glycosylated by both N-linked (attachment via nitrogen atoms on amino acid (aa) residues) and O-linked (attachment via oxygen atoms on aa residues) glycans.

As mentioned in the previous section, HSV gC can mediate the binding of HSV to cell surface GAGs such as HS and CS. gC-negative virus can still bind to GAGs on the cell surface via gB [60], though it has been reported that gC-1 deficient virus has a reduced infectivity compared with wild-type virus. Furthermore, although gC-1 may be non-essential in cell culture experiments, the gC-1 glycoprotein appears to have an important function for HSV-1 infection in humans, where purified gC-1 alone has been demonstrated to mediate binding to HS in the absence of other viral glycoproteins [112]. The interaction between HS and purified gC-2 has been demonstrated to be superior in binding strength to that of HS and gC-1 [65, 66]. This feature is most likely related to the divergence between gC-1 and gC-2 in the N-terminal region. Here, a mucin-like region, rich in O-linked glycans can be found on gC-1 (Figure 5, 6); this domain is absent in the gC-2 genome. Instead for HSV-2, a mucin-like region similar to that of gC-1 is found on gG-2. The mucin-like region of gC-1 has been found to be essential for the interaction with GAGs, where it functions as a negative binding modulator for gC-1 as compared with gC-2; and this property could regulate viral tropism [113]. In the presence of other glycoproteins such as gG-2, the interactions

![Figure 5](image-url). Map of gC-1. Mucin-like region glycosylation in enlargement, adapted from [110]. Hexagonal stick corresponds to location of N-linked glycans. Rhomb-shaped, green stick corresponds to O-linked glycan pattern as identified in [111] Monoclonal antibody B1C1 binds to the loop structure in antigenic site II. Red line and aa marked (129-155 + 247) represent the GAG-binding site. TMR = transmembrane region, GalNAc = N-acetylgalactosamine, Gal = galactose, SA = sialic acid, Ser = serine, Thr = threonine.
between HS and HSV-2 are reduced, most likely due to shielding of the gC-2 binding site through the mucin-like region of gG-2.

Two antigenic sites, representative of the epitopes of gC-1, have been mapped [114]. Antigenic site II was localised to aa 129-247, adjacent to the mucin-like region, while antigenic site I is found at aa 307-373 (Figure 5). The actual GAG-binding site of gC-1 is located carboxyterminally of the mucin-like region within the protein (Figure 5), where the basic and hydrophobic residues at the loop structure in the antigenic site II participate in the GAG-binding domain [115]. The monoclonal antibody (mab) B1C1, which was used to define antigenic site II [116], has been demonstrated to block the interaction between gC-1 and cell surface HS efficiently in vitro as well as in vivo, thereby neutralizing viral infectivity [117].

The mucin-like region (Figure 5) is a region rich in threonine (Thr) and serine (Ser) residues, which are densely decorated with O-glycans [118]. The O-glycosylation sites of gC have recently been identified [111], where the localisation of nine sites were determined within one-half of the mucin-like region. Norden et al. [110] described the stepwise addition of the glycans to the mucin-like region, where N-Acetylgalactosamine (GalNAc) transferases initiate the dynamic O-linked glycosylation with addition of GalNAcs to only a few specific Ser and Thr residues. This initiation is then followed by GalNAc modification in an ordered “seed-and-spread” pattern, before other monosaccharides can extend the residues as seen in Figure 5.

The function of the mucin-like region, also containing basic aa:s, has been attributed to electrostatic and modifying interactions with the GAGs on cell surfaces, and to cell-to-cell spread of the virus [113, 119] (Figure 6). Furthermore, the region appears to participate in induction of selectin ligands via carbohydrate

![Figure 6](image-url). The electrostatic interactions between negatively charged GAGs on the cell membrane and the positively charged binding site of glycoprotein C on HSV-1 (gC-1) is modulated by the mucin-like region. HS = heparan sulphate. CS = chondroitin sulphate.
bindings, which could possibly influence viremic spread of HSV-1 [120, 121]. In addition, O-linked glycosylation of a mucin-like domain on a gammaherpesvirus has been shown to shield vulnerable epitopes on viral glycoproteins from neutralizing antibodies [122]. This could also be true for the mucin domain of gC-1. In addition, a recent study on HSV-2 has demonstrated that viral O-linked glycans, for example present on gG-2, were recognized by chemokines at epithelial surfaces early in infection, before the actions of interferons [123].

In addition to its GAG-binding function, gC-1 has in cell culture experiments been shown to function as a receptor for complement component 3b (C3b), a part of the innate immune response. By introduction of four non-relevant aa residues in a walking manner, the interaction with C3b was localised to four gC-1 regions: aa 124-137, aa 279-292, aa 339-366 and aa 223-246 [124]. Interestingly, the first C3b binding region is located within the antigenic site II, and, like GAG binding, this interaction can be blocked efficiently by the mab B1C1 (Figure 7) [125]. Deletion of the mucin-like region (aa 33-123) does not reduce binding of C3b, but appears to prevent binding of other complement components to the C3b complex [126] and also to reduce the affinity for HS [127]. Interestingly, the mucin-like region has not been shown to not participate in the binding of C3b, but instead it interferes with another complement factor, properdin [126].

The binding of C3b has also been located on three sites of purified gC-2 [70], but not on the surface of HSV-2 virions [124, 128, 129]; this suggests that the manner in which the glycoprotein is presented on the viral envelope might influence the binding of C3b. Nevertheless, gC-2 can also block the complement-mediated neutralization [130]. Further implications of the C3b binding will be discussed below in the section on the complement system.

Interestingly, gC-1-negative strains are very rarely isolated from patients, further implicating the importance of gC-1 for viral replication and infectivity in vivo. In addition, it has been suggested that the structural variations of HS could contribute to the wide cell and tissue tropism presented by HSV-1 [131], which would mean that gC-1, and the mucin-like region, is highly involved in viral tropism.

![Figure 7](image-url)

*Figure 7.* The four regions of HSV-1 gC-1 binding to C3b as demonstrated in [70]. Monoclonal antibody B1C1 bind to C3b-binding region I. C5/P blocking domain corresponds to the mucin-like region. P = properdin. TMR = transmembrane region.
2.3. Viral infections in the CNS

The CNS is a part of the body normally relatively well protected from external microbial invasion by several defence mechanisms including the blood brain barrier (BBB). Therefore, CNS infections are rare in comparison with, for example, infections involving the respiratory tract and the gastrointestinal system, but when opportunities to enter the CNS arise for a pathogen, the spread can occur through three different pathways: through neuronal or haematogenous spread, or locally via eyes, nose or sinuses.

The CNS has its own defence against infections, including microglia and astrocytes that release, among other substances, chemokines and cytokines to recruit immune cells from the systemic circulation. Despite this defence, pathogens in the form of bacteria, protozoa, viruses and fungi, which succeed in passing across the BBB, can establish severe infections with risk for lethal outcome. Furthermore, many CNS infections can result in residual symptoms or sequelae that can permanently affect the everyday life of the patient. Detection of microbial agents causing the infection can be done through analysis of cerebrospinal fluid (CSF), serum/plasma and vesicular fluid, or, in some instances, urine, faeces and/or nasopharyngeal secretions. Earlier, brain biopsies subjected to virus culture were utilized for diagnosis, but with the introduction of sensitive and specific polymerase chain reaction (PCR) methods, far more cases than before are linked to specific viral pathogens [132].

Viral CNS infections

CNS infections can be caused by a variety of viral agents and are most often acute but can, on some occasions, be chronic. The aetiology of these infections, which can be sporadic or endemic, can vary in different geographical regions. Zoonotic viruses common in warmer regions such as Zika virus [133], Japanese B encephalitis virus [134], Dengue virus [135], Yellow fever virus [135], West Nile virus [136] and Rabies virus [137] are regularly detected in a global setting. Viruses more common in Northern European settings, such as enteroviruses [138], herpesviruses [132], tick-borne encephalitis (TBE) virus [139], adenoviruses and, on rare occasions, influenza viruses [140], have all been associated with viral CNS infections. Several childhood infections such as rubella [141], morbilli [142], parotitis [141] and polio [137] may also cause CNS disease, but these infections have been successfully defeated due to general vaccination programs. The clinical picture itself is rarely enough to determine which virus has caused the CNS infection in question [132]. Other symptoms of infection, such as fever,
respiratory difficulties and gastrointestinal manifestations may occur concomitantly with viral CNS infections, but can be absent and therefore laboratory diagnosis of such viral infections is important. Included among routine diagnostic methods is detection of viral nucleic acids by PCR, followed by gene sequencing for identification of viral strains and, to a lesser degree, isolation of virus in cell culture and antigen detection. As an indirect method, demonstration of IgM and IgG antibodies in the CSF and serum is useful [132].

Determination of the viral agent causing the infection is successful in around 50-60% of all patients [143-145], and in many of these cases, the diagnosis leads to initiation of antiviral treatment to target the responsible virus.

Viral CNS infections are manifested in several clinical entities, including encephalitis, meningitis and myelitis. Encephalitis is often severe, while viral meningitis normally is a milder condition (especially as compared with bacterial meningitis) and can in most cases resolve after 7-10 days. Although the CSF laboratory findings are different in viral meningitis as compared with those in bacterial meningitis, clinical symptoms including headache and nuchal rigidity are often similar. Viral encephalitis on the other hand is often associated with focal symptoms and neurological sequelae, which may also occur in bacterial meningitis [146-149]. Apart from the distinct conditions of encephalitis and meningitis, meningoencephalitis, as a condition involving both the brain and the meninges, is commonly reported for many neurotropic viruses [139, 150-155].

The national surveillance of polio and other CNS viruses requires that all viral meningoencephalitis cases are to be reported to the Public Health Agency of Sweden (Folkhälsomyndigheten, FoHM). Each year statistical reports are presented on their website, available also for the general public. However, not all cases of viral CNS infections are reported to the FoHM, apart from those caused by polio, other enteroviruses and TBE. Moreover, the definition of meningoencephalitis held by the FoHM [156] does not appear to correspond to the previously described definition, possibly resulting in inclusion of the less severe cases of meningitis as well. Between 2010 and 2015, the approximate average number of reported cases of meningoencephalitis per year was 880, a number that includes both domestic and imported cases (Figure 8).

Enteroviruses, which constitute 30-40% of all reported cases of viral meningoencephalitis (Figure 8), are the most common cause of viral meningitis in Sweden. Enteroviruses display a seasonal appearance, being more common during the end of summer and the beginning of autumn, as compared with the incidence during the rest of the year [54]. The virulence of the circulating enterovirus strains determines the number of CNS infections caused by these viruses and explains the variable incidence between years. Second to enteroviruses, the most common cause of viral meningoencephalitis in Sweden is TBE, being the causative agent
in 20-30% of the total number of cases (Figure 8). In fact, in 2015, CNS infections due to TBE virus even marginally exceeded those caused by enteroviruses.

In the Western world, the most common cause of sporadic, focal viral encephalitis is HSV-1, while HSV-2 induces meningitis that may be recurrent. In contrast, VZV shows diverse clinical manifestations within the CNS including meningoencephalitis, encephalitis, cerebellitis, meningitis, myelitis and focal neuropathies including post-herpetic neuralgia (PHN). VZV is reportedly the most commonly detected alphaherpesvirus in CSF samples from patients with CNS symptoms in western parts of Sweden [157]. Furthermore, other herpesviruses, including HHV-6, EBV and CMV, are also detected in clinical studies of viral

*Figure 8.* Reported cases of viral CNS infections in Sweden 2010-2015, adapted from data collected by the Public Health Agency of Sweden (FoHM) [54]. Note that only about 50% of all meningoencephalitis cases in Sweden are reported to the FoHM. * = HSV type undefined. The group “Other herpesviruses” includes HHV-6, EBV and CMV. The group “Other viruses” includes adenovirus, West Nile virus, Japanese encephalitis virus, JC virus, Toscana virus, mumps virus and parechovirus.
meningoencephalitis; in together they represent a substantial proportion of viral CNS infections (Figure 8).

Up until now, the majority of meningoencephalitis cases in Sweden have been contracted domestically. However, increased travel and immigration from countries where vaccination coverage is insufficient and also affected by the global warming leading to an introduction of arthropod-borne virus (arbovirus) infections to geographic locations previously not stricken (reviewed in [21]), might change the aetiology of viral meningoencephalitis in the future. Indeed, during the last six years, a few meningoencephalitis cases caused by “exotic” viruses, including West Nile virus and Japanese encephalitis virus, have been reported in Sweden [54]. However, the Swedish cases are substantially fewer than what is seen in other Western countries, such as the US and the southern parts of Europe, where these infections have become domestic. This has been described, among others, for West Nile virus [158], Dengue virus [159] and most recently for Zika virus (in Florida) [133].

Cell culture and animal models for studying alphaherpesvirus infections in the CNS

Although human alphaherpesviruses show a wide tropism and therefore can infect many cell types, CNS infections in cell culture models can only be studied in cells derived from that lineage. To date, few such cell culture systems adequate for studies of the pathogenesis of these viruses have been described. Cultured oligodendrocytes, astrocytes and whole trigeminal ganglia have all been infected 

\textit{in vitro} \textit{with alphaherpesviruses, with demonstrable cytopathogenic effect} [160-163]. One promising method recently introduced is the reprogramming of a cell culture towards human induced pluripotent stem cells (iPSCs) [164]. Subsequent differentiation into neuronal cells results in a cell culture driven towards the cell type of interest [165-168]. A few studies with alphaherpesvirus infection of iPSCs have been presented to date [169-171]. iPSCs offer a great possibility for maturation into a large variety of cell types, but differentiation can only be driven towards cells within a specific embryonic layer (Figure 9). In line with this, astrocytes, neurons and oligodendrocytes are all of interest and can be produced from the same iPSCs as they all originate from the ectoderm [166, 169]. However, microglial cells, which originate from mesoderm, will not appear in the same stem cell-derived culture as other neuronal cells.

Several animal models have been used to study alphaherpesvirus CNS infections \textit{in vivo}, including those of rats, mice, guinea pigs and rabbits [28, 39]. Both the olfactory bulb and the trigeminal ganglion can be involved in
establishment of persistent infections [172], but the routes these viruses exploit for further spread within the CNS have been less extensively examined [173, 174].

One of the most challenging questions of infection with alphaherpesvirus in animal CNS models is the understanding of how the virus can infect the CNS without inflicting damage to the surrounding structures. Many animal studies have used intracranial (i.e.) inoculation to ensure that the virus is distributed to the right location, though this is most likely an unnatural route of infection [175-177]. For VZV, animal models outside primates have been difficult to find. Interestingly, studies have demonstrated that HSV-1 and HSV-2 behave differently in CNS after infection of rats, as regards neurovirulence and neuropathology; this demonstrates that rats are a useful model for discrimination between the CNS manifestations of these two viruses [175].

Latency and reactivation – neuronal transport and the fate of the infected neuron

After lytic infection with alphaherpesvirus in cell culture, the infected cell is usually destroyed. However, in sensory neurons, the alphaherpes virion can also
enter a latent state after infection, in which the genome is found in its circularized state in the nucleus of the neuron [178]. During latency, the expression of viral lytical genes is restricted, while a subset of genes, including LAT, is expressed [179, 180]. Here, the viral genome is maintained and protected from the destructive mechanisms of cellular and host defences.

The virus can reactivate from the latent state in the sensory ganglia to replicate, and to be transported peripherally to induce a lytic state in the innervated skin region during which new infectious virions are produced. The precise mechanisms responsible for the virus reactivation from a latent state are unknown. Interestingly, the phosphorylating activity of viral thymidine kinase (TK; targeted by antiviral treatment) is thought to be of importance in mature neurons, as these are post-mitotic and lack expression of the cellular TK [39]. Triphosphorylated nucleoside precursors are required for DNA synthesis, and without the activity of the viral TK, viral replication would probably not occur in neurons.

The fate of the neuron after reactivation of an alphaherpesvirus has been debated, but most probably, the neurons survive, at least in HSV infection. This has been based on two observations; patients with frequently recurrent lesions at the same site do not suffer from local anaesthesia; and virus is shed from microscopic lesions between clinical manifestations in women with recurrent genital herpes [39].

The immune response has been suggested to play a role in preserving the latent infection, where CD8+ T-cells and interferon γ (IFN-γ) can block viral gene expression and replication [181, 182]. Although there have been many studies presented on this subject, the role of especially the innate immune mechanisms of the host needs to be investigated further.

Already during the 1920s, Goodpasture and Teague observed that HSV could spread via axonal transport, and demonstrated that retrograde neuronal transport from the periphery may introduce the infection into the CNS [183, 184]. This observation was later confirmed by other studies in animal models and two-chamber cell culture systems of sensory neurons [185-191], where infection of virus in the periphery mostly leads to retrograde transport along the axons to the nerve cell body (Figure 10). In reactivation of virus from latency, axonal transport occurs in an anterograde fashion (Figure 10), to the skin and this might explain how reactivated virus can enter the brain. Interestingly though, while latent virus usually is found in the cell body of sensory neurons, HSV DNA findings have been reported from the brain during autopsy of patients without any symptoms of HSV CNS infections [192, 193].

Retrograde and anterograde HSV transport can be used for neuronal tracing to determine the distribution of axons and dendrites, where retrograde spread of infection is defined as spread of virus from a postsynaptic neuron to an uninfected
Presynaptic neuron and anterograde spread originates from a presynaptic neuron and proceeds to an uninfected postsynaptic neuron (reviewed in [194]).

Furthermore, in an experiment where mice were pre-treated with capsaicin (the substance causing burning sensation in chili pepper) before infection with HSV-1, it was demonstrated that the mortality rate was reduced in both acute and latent infections [195]. This outcome might be explained by the capsaicin treatment. Such treatment could cause degeneration of unmyelinated sensory neurons in the trigeminal ganglia, resulting in decreased availability of surrounding glial cells for infection, in turn a confirmation of neuronal transport for HSV-1. Interestingly, a more recent study has also revealed that in vitro, capsaicin can reactivate a latent HSV-1 infection in a neuronal cell culture [196].

Figure 10. Top: Establishment of alphaherpesvirus latency in sensory neurons of ganglia. The alphaherpesviruses enters the neuron from the site of a lytic infection in another cell/tissue (represented here by epithelial cells) and the virion is transported along the axon to the cell body (soma) via retrograde transport. Bottom: Reactivation of alphaherpesvirus from latency. The virion travels via anterograde transport to a synapse where the virus infects post-synaptic cells (represented here by epithelial cells) lytically.

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Neurotropism

Unlike VZV, which can infect several regions of the CNS and result in a variety of CNS manifestations [197], HSV-1 and HSV-2 most often are associated with infection in distinct and different areas, i.e. they show a hitherto unexplained type-specific neurotropism. In neonatal children, HSV-1 and HSV-2 can cause meningoencephalitis. In both manifestations, the brain and the meninges are involved, but the infection is less severe when HSV-1 is diagnosed [198].

When causing encephalitis in adults, HSV-1 preferably infects the limbic system and the temporal lobes. This infection results in severe damage including necrosis in the affected area [132]. Memories, language, olfaction, behaviour and emotions are functions associated with these regions of the brain, and the affected areas are often linked to neurological sequelae that can be life-long.

In contrast, the meninges are the main target during HSV-2 infection of the CNS in adults [132]. Consisting of three membranes, the meninges surround and protect the brain and spinal cord, and infection leads to acute symptoms such as severe headache, neck pain and nuchal rigidity. Importantly, many of these patients develop recurrent meningitis [199]. In addition, focal symptoms such as urinary retention in patients with HSV-2 meningitis may be linked to myelitis in the lumbosacral region [200]. Despite such attacks, adults with primary and recurrent HSV-2 CNS infections of the CNS rarely display long lasting symptoms or permanent sequelae [201].

Alphaherpesvirus infections in the CNS

Herpes simplex encephalitis

HSE, with at least 95% of cases caused by HSV-1, is the most severe CNS infection caused by alphaherpesviruses in adults [202, 203]. HSE is seldom caused by HSV-2, but there are a few case reports connected to invasive surgery and brain trauma such as ischemic stroke [204, 205]. In addition, some few cases of HSV-2 encephalitis have been presented without any concurrently inflicted brain trauma or underlying immunodeficient conditions [204, 206]. In some studies HSV-2 is reported to be involved in 10% of all HSE cases, but it is unclear from where this data originates, or if neonatal HSV CNS infection is included in this percentage. Closer to the truth is probably that HSV-1 causes most HSE cases after the neonatal period, based on the different neurotropism of the two viruses as described above.
With an incidence of 2-4 cases per million inhabitants per year (Figure 11), HSE is the most common sporadic viral encephalitis in the Western world [140, 207, 208]. Before the introduction of antivirals HSE was associated with a 70% mortality rate, but despite reduced mortality to around 10-20%, morbidity after antiviral treatment is still high, where the majority of patients are left with remaining neurological sequelae [207, 209-212].

HSE can be caused either by primary or recurrent infection [203] and can occur in all age groups, although it is more common in elderly (Figure 11). Recurrent infection is likely if antibodies against HSV-1 are present at onset of neurological symptoms. This is the case for at least 2 of 3 HSE patients, both adults [203] and children [213]. However, it is unclear if the infection is due to reactivation of virus from the site of latency, activation of virus already in the brain or infection with a new, neurovirulent strain of the virus.

On a cellular level, the infection in HSE patients is mainly found in the cerebral cortex [214, 215], which is the outer layer of neural tissue in the brain. The

![Figure 11](image-url). Top: HSE cases in Sweden between 2006-2014 [54]. Bottom: Age distribution of HSE in Sweden 1990-2001 (adapted from [207]).
composition of the cerebral cortex, the outer layer of neural tissue in the brain, is grey matter, which is selectively damaged in the infected regions [216]. Particularly, neurons in lamina V are affected (Figure 12).

The suggested routes of entry for HSV-1 into the CNS are via either the olfactory tract or the trigeminal nerve (Figure 13), and both these routes have been described in the literature [214, 217, 218]. Interestingly, in an autopsy material from HSE patients that died early after onset, olfactory bulb infection was prominent [214].

HSE usually engages the limbic system and the temporal lobes (Figure 13), where symptoms and sequelae are related to the extent and severity of damaged regions. Symptoms and sequelae include (although are not restricted to) focal neurological symptoms, memory loss, epilepsy and seizures, aggression, dysphasia and hemiparesis. In almost all HSE patients, lesions are detected in hippocampus, the region in the brain that is responsible for converting short-term memory to long-term memory. This has been reported by Damasio et al. [216], who describe an HSE patient with major amnesia syndrome due to extensive destruction of the temporal lobe and the limbic system. The patient experienced HSE ten years prior to the report, and had not since been able to acquire any new memories. Neither did the patient have any extensive recollection of memories prior to the infection.

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2. INTRODUCTION
To identify the areas affected by HSE, methods such as magnetic resonance imaging (MRI), computed tomography (CT) and electroencephalogram (EEG) are
used. While MRI can detect pathological changes caused by HSE during the entire course of the infection, CT cannot always detect abnormal changes in initial infection [209, 220]. EEG is of particular importance to identify epilepsy in patients with decreased consciousness. The early finding by MRI and CT in HSE is oedema (Figure 14), and haemorrhages in the encephalitic area are also quite common. The presentation on neuroimaging may therefore mimic bleeding or acute cerebral infarction, although not systematically described hitherto [221] (Figure 15). Initially, the infection is unilateral, with herpetic lesions only being present in one brain hemisphere, but may spread to both hemispheres in the later stages of infection. As expected, herpetic lesions are found in the temporal lobe and the hippocampus, related to the neurological symptoms and sequelae seen in HSE patients (Figure 14). However, lesions can also involve the frontal, parietal and occipital lobes.

In one study, increased intracranial pressure (ICP) which peaked at day 12 after onset of symptoms was reported for one third of all HSE patients [222].

Figure 14. Left: MR scan in the acute stage from a 53-year-old woman with HSE and onset of disorientation on the day of admission. Six days after admission, coronal T2-weighted images show a widespread oedema in the ventromedial part of the right temporal lobe, dominating in the hippocampus region and in the right insula region. A minor oedema is seen in the left hippocampus region. Right: CT scan on the day of admission from a 58-year old woman with HSE with disorientation at arrival to hospital. An expansive low attenuated lesion in the right temporal is seen with an oedema in the temporal and parietal lobes. The appearance is concordant with HSE, but could be mistaken for low-grade astrocytoma. Published with permission [219].

Figure 15. CT scan without contrast in the acute stage from a 27-year-old man with HSE with symptoms of fever, headache, and progressive disorientation during 10 days before admission to hospital. An expansive intracerebral haemorrhage in the insular region of the left temporal lobe is seen with a large oedema causing compressing of the left ventricle.
HSE is mainly affecting immunocompetent patients, but may also cause opportunistic infection in CNS in immunocompromised hosts [223]. In the latter group, the clinical findings often show a different picture (reviewed in [224]). These patients often present with an atypical infection pattern, where extensive involvement of other parts of the brain, including the cerebellum and the brain stem, are described. One study attributed this to the ineffective immune response in these patients, allowing the virus to spread more uncontrolled [225]. Furthermore, immunocompromised patients tend to present with fewer prodromal symptoms and less focal deficits than immunocompetent patients [225]. Non-inflammatory CSF profiles and negative CT scans can also be seen; findings that have led to speculation that HSE might be underdiagnosed in immunocompromised patients due to difficulties in recognizing symptoms [223, 226]. Despite reports of poor outcome [225], there have also been reports of patients who have recovered from HSE, despite their immune deficient state, without neurological sequelae [227].

Occasionally, HSE patients can have clinical relapses, which can be either a “true” relapse (where HSV DNA can be detected in the CSF) or an immune-mediated relapse (where no HSV DNA can be found in the CSF) [228-231]. HSE relapses often occur within a few weeks to months of discontinuation of antiviral therapy and immune-suppressant drugs are administered since immune-mediated mechanisms are suggested [228].

**Neonatal HSV infections in the CNS**

Unlike HSE, neonatal meningoencephalitis caused by HSV has less distinct distribution of virus and more extensive involvement of other parts of the brain.

HSV-infections in neonates most often result from a transmission from an infected mother to the baby and can be acquired at three stages: congenitally (in the uterus), perinatally (during delivery) or postnatally (after delivery) [39]. Perinatal transmission is the most common and can occur during delivery when the baby may come in direct contact with infected vaginal secretions. In mothers with primary infection close to delivery date, the risk for transmission of virus to the baby is increased compared with mothers with established HSV infection (reviewed in [232]).

The three forms of neonatal HSV infection, CNS infection, multi-organ disseminated disease and disease located in skin, eye or mouth (SEM), differ slightly in incidence and prognosis [233]. In Sweden, the incidence of neonatal HSV infection is approximately 1:12 000 to 1:13 000 births [234], while in the US the incidence is much higher [232].
Initially, the symptoms of neonatal CNS infection are non-specific, and can include fluctuations in body temperature, apathy, respiratory distress and feeding difficulties, which can later develop into apnoea, disseminated intravascular coagulation, hypotension and shock. In approximately two-thirds of all neonatal HSV CNS infections, skin lesions can be found. CT scans of neonatal CNS infection have revealed abnormalities in 55-65% of the cases [235], which could be localised to the temporal, frontal, parietal and subcortical regions.

While antiviral treatment has reduced the mortality in early HSV infection of the CNS, morbidity is still high, especially for HSV-2, which unfortunately is the most common cause in neonates (approximately 75-80% of all cases [198]). Many HSV-2 infected neonates show residual neurological sequelae or die after infection, while HSV-1 infected neonates in one study totally lacked sequelae at follow-up [198]. Despite the reportedly better outcome for neonates infected with HSV-1 as compared with those infected with HSV-2, there has been reports of neurological sequelae and mental retardation in long-time follow-up studies also after neonatal HSV-1 CNS infection [234, 236].

The morbidity can include delays in development, epilepsy, blindness, cerebral palsy and cognitive disabilities [198, 234]. Furthermore, microcephaly has been reported after neonatal CNS-infection, as well as after congenitally acquired infections [43, 198, 235, 236], a finding which also has been described for other viral infections such as rubella virus [237, 238] and more recently Zika virus [239, 240].

**Herpes simplex meningitis**

While HSE carries a high mortality rate, herpes simplex meningitis (HSM) has a milder course, where most patients recover within weeks to months. HSV-2 is the main cause of HSM, although a few cases are attributed to HSV-1 [145]. In primary HSV-2 meningitis, symptoms such as intense headache, neck stiffness, photophobia and nausea can be observed [241]. These symptoms are similar to what is found for bacterial meningitis, but the viral meningitis is less severe. Furthermore, around 30-40% of all HSM patients are reported to experience symptoms from the central, peripheral and/or autonomous nervous system [242]. Herpetic lesions may precede or occur in association with the HSM, but more than half of the patients report of no previous or current herpetic lesions.

Most patients suffering from HSM will not experience any long lasting complications, but 20-30% of all patients develop recurrent meningitis episodes. These episodes usually have a shorter duration than the primary episode and milder symptoms. HSV DNA can be detected and quantified by real-time PCR in the CSF in most cases. Immunocompromised patients are more prone to develop
neurological sequelae or even fatal meningoencephalitis if they do not receive treatment in early infection [201, 243].

HSM is responsible for almost 20% of all viral meningitis in Sweden, where most cases are found among younger adults [143]. Interestingly, the incidence of HSM is considerably higher in female patients than in male subjects [241, 244, 245]. This might be attributed to the larger area involved in the genital HSV lesions of women, and that a higher proportion of patients from this gender experience viraemia, and at a higher dose, during primary HSV-2 infection [246]. It has been observed that pre-existing antibodies against HSV-1 may protect against HSM caused by HSV-2 [242], similar to the reported asymptomatic genital infections of HSV-2 in patients with pre-existing HSV-1 antibodies [247].

Mollaret’s meningitis

Mollaret’s meningitis is a rare type of recurrent meningitis in which recurring aseptic meningitis occurs over several years and even decades. Patients experience recurrent episodes of headache, stiff neck, fever and CSF pleocytosis [248]. These episodes normally resolve after 2-5 days and the duration between episodes can vary from a few weeks to several years (reviewed in [249]). Although diagnostic evidence has not been found in all described cases, HSV-2 DNA [250] is often detected in CSF and this virus has been suggested to be the most common causative agent.

Varicella zoster CNS infections

As mentioned previously, VZV can cause a wide spectrum of CNS infections of diverse severity, and with a more scarcely documented occurrence of neurological sequelae as compared with HSV infections [132]. Adults with reactivated VZV (zoster infection) are more common as a trigger for neurological disease, but acute VZV infection (varicella) can also be complicated by CNS manifestations [132]. In children, the infections are often diagnosed in association with vesicular rash, however reactivation of the virus is not necessarily accompanied by skin lesions. Several of the CNS manifestations caused by VZV infections, as described below, can also be observed in patients with active HSV-infection, but are less common. Furthermore, some of the described CNS manifestations may occur simultaneously.

Due to its appearance in association with either primary infection or reactivation as zoster, VZV encephalitis is most commonly diagnosed in elderly immunocompetent persons [157]. In the AIDS-era, the prevalence of encephalitis
due to VZV has been increased, but other causes of immunosuppression can contribute to this complication, where it is found regardless of age [251]. In a small retrospective study of VZV encephalitis cases in France, it was found that despite antiviral treatment, mortality and morbidity rates were similar to those seen in antiviral-treated HSE [252]. Parallel findings have also been seen in Swedish patients [157] and have been presented by others in follow-up studies [144], although these reports also included encephalitis cases caused by other viral agents. The neurological sequelae after VZV encephalitis tend to be subcortical, where cognitive processes, memory, emotions and behaviour have reportedly been affected (reviewed in [197]).

*VZV cerebellitis*, in the form of *acute cerebellar ataxia* (ACA) is a type of encephalitis that affects the cerebellum, and is mainly seen in young children within three weeks of primary infection [253]. ACA affects the coordination of muscle movement and in particular balance, coordination, and eye movements. Besides its known motor functions, cerebellum has also influence on non-motor regions of the cerebral cortex (reviewed in [254]) suggesting a role in cognition, which may also be affected by ACA [253]. Furthermore, nausea and headaches can also occur [197]. Some studies report of patients recovering fully within a few months [255, 256], while other studies describe remaining sequelae in some patients [253].

*VZV brainstem encephalitis* is a special form of encephalitis where the brainstem is affected. The brainstem, adjacent to the spinal cord, normally controls vital life functions, including respiration, cardiovascular control and consciousness, and infection here may lead to serious consequences.

*VZV meningitis* can occur in all age groups [132]. Meningitis caused by VZV is mainly due to zoster reactivation. Furthermore, reactivation of the VZV vaccine strain in immunocompetent children has been reported to cause meningitis [257]. Most patients, irrespective of age, experience a favourable outcome after VZV meningitis, with no or mild neurological sequelae.

VZV can also cause *meningoencephalitis*, which, like VZV encephalitis, is more common in older patients [132]. Dysfunctions in the brain (without diagnosed encephalitis) that can lead to paresis, motor deficits or altered consciousness, can be due to *VZV encephalopathy* [157].

*Ramsay-Hunt syndrome* is a complication of VZV reactivation in the geniculate ganglion (a bundle of nerve fibres and sensory neurons of the facial
Ramsay-Hunt syndrome is normally unilateral, and is mainly seen in older age groups. Patients commonly present with vesicular (zoster) rash in the ear, along with other ear-associated symptoms like ear pain, vertigo, tinnitus and hearing loss. Furthermore, acute paralysis of the facial nerve develops and is an important part of the syndrome. Despite reactivation of virus, lesions are not always found, which can hamper diagnosis. Without antiviral treatment, only 10-20% of all patients fully recover and even with treatment, 40% show rest symptoms. In the CSF, a pattern of increased biomarkers for neuronal damage and astrogliosis was recently found, and the amounts of these factors correlated to the detected VZV DNA quantities [259]. Hence, it was suggested that early diagnostics and start of treatment during Ramsay-Hunt syndrome is of importance for the outcome.

Myelitis is an infection of the white or grey matter in the spinal cord that can result in paresis of extremities, reduced functions in bladder or bowel or sensory deficit, depending on which nerve roots that are affected by VZV. Immunocompromised patients are overrepresented in the VZV myelitis cases, and reportedly the outcome is worse, with higher mortality or degree of disability, compared with immunocompetent patients (reviewed in [260]). Another infection close to the spinal cord is radiculitis, which is an inflammation of the spinal nerve roots combined with severe pain along the associated nerve [261].

CNS vasculopathies can be caused by VZV in both children and adults. Large or small cerebral arteries can be infected with VZV, which can predispose for brain haemorrhage, aneurysms or ischemic stroke. It is thought that VZV vasculopathy might be underdiagnosed (reviewed in [197]) especially in the elderly population where the CSF of stroke patients seldom is analysed for signs of recent VZV infection. In contrast to what is seen for HSE, some researchers have suggested that VZV encephalitis is caused primarily by vasculopathy rather than by direct infection of the brain [262, 263], but this question remains to be resolved.

2.4 Immune responses in the CNS

The CNS is normally protected from direct entry of pathogens by the BBB. Furthermore, microglia are a type of glial cells that function as the initial form of active immune defence in the CNS and they are also known as the residential macrophage cells. Microglia are very sensitive even to minimal pathological changes in the CNS and are active both in the search for damaged neurons and
synapses, plaques and pathogens (reviewed in [264]). Due to the sensitivity of the nervous tissue in the CNS, microglia are required to act quickly both to decrease inflammation and neuronal damage and to destroy the invading pathogen. To be activated and to communicate with other CNS cells and immune cells, microglia can be assisted by different parts of the innate immune response, where cytokines are very important [264]. Microglia have also been reported to be involved in neuroinflammation and neurodegeneration, where different pro-inflammatory factors may put microglia in a continuous active state [265, 266].

**Antibody response**

Antibodies are proteins secreted by B-cells that can recognize and identify pathogens. To bind antigens, the antibody has a Y-shaped form, where the tail region (known as the fragment crystallisable (Fc) region) can interact with Fc-receptors on cell surfaces or with proteins of the complement system to alert the immune system of pathogens, thereby activating it [3].

There are five different classes of antibodies, IgA, IgD, IgE, IgG and IgM, of which IgG and IgM are of particular interest in viral CNS infections. Depending on cell developmental stage and activation, different classes are produced by different types of B-cells. IgM is an early participant in the B-cell mediated immune response to pathogens, before IgG is produced (Figure 16). Class switching and maturation of the antibody response is required for generation of IgG [3] and this antibody is thus a part of the secondary immune response. In early primary infection, levels of IgM are high, and levels of IgG do not increase until later in infection (Figure 16). IgG can usually be detected also after primary

![Figure 16. Top: IgM and IgG structure. Bottom: Antibody response after primary and secondary antigen exposure (such as after primary and recurrent alphaherpesviral infection in the CNS).](image-url)
infection, which is used in serology to confirm a diagnosis retrospectively. At a second exposure to the antigen, the IgG response normally is higher, as B-cells producing antibodies against the antigen already circulate in the body (Figure 16).

Interestingly, intrathecal antibodies in the form of IgG against HSV can be found many years after HSE, a finding not reported for any of the other alphaherpesvirus CNS infections [267, 268].

The complement system

The complement system is an important part of the innate immune response, which has pro-inflammatory, immune enhancing and cell membrane-attacking features. The major production of complement components, which include fluid-phase proteins and cell-surface receptors, occurs in the liver, but there have also been reports of a separate production in the CNS, both by glial cells and neurons (reviewed in [269, 270]). It is not so surprising that the CNS has separate synthesis of complement components, given the separation of the CNS from the general circulation via the BBB, which hinders the passage of larger proteins.

Activation of the complement system both leads to release of anaphylatoxins (peptides with pro-inflammatory features) and to the formation of the membrane attack complex (MAC) which results in membrane disruption. Complement activation mainly occurs via three different pathways: the classical pathway, the lectin pathway and the alternative pathway. In addition to these, other activation pathways have been identified in the more recent years, including activation of complement component 5 (C5) via thrombin [271] and via properdin (reviewed in [272]).

The classical pathway is activated by antigen-antibody complexes, where antigen bound to both IgM and certain IgG isotypes are recognized by and interact with C1q. Binding to C1q (in a complex with C1r and C1s) triggers activation of two associated serine proteases that in turn cleave C4. Binding of C2 to C4b and a second cleavage then result in the formation of C4b2a, the C3 convertase of the classical and lectin pathway (Figure 17).

The lectin pathway is initiated by pattern recognition receptors such as mannose-binding lectin (MBL). Invading microorganisms and foreign cells are recognized by MBL via carbohydrate patterns on their surface. Binding to this surface activates the MBL-associated serine proteases, which then proceed to cleave C4 and eventually leads to the formation of C4b2a (Figure 17).
The alternative pathway is separated from the two other pathways in that it produces another C3 convertase: C3bBb. Furthermore, the alternative pathway does not require binding to an antibody or pathogen for activation. Rather, activation occurs via spontaneous breakdown of C3 to C3a and C3b (or C3-H2O). C3b is bound to complement factor B (CFB), which is then cleaved by the serine protease factor D, followed by binding of properdin and the alternative C3 convertase is formed (Figure 17).

*Figure 17.* The three major activation pathways of the complement system and the terminal pathway.
Both of the C3 convertases can cleave C3 to C3a and C3b, where C3b then can attach covalently to target surfaces. C3b can also bind to the C3 convertases, leading to the terminal pathway. Here, the convertase specificity changes from C3 to C5, where C5 cleavage results in production of C5a and C5b. The C6-C9 components then bind to C5b, resulting in the formation of the MAC and membrane disruption.

The anaphylatoxins C3a and C5a are released during complement activation and can interact with specific receptors to stimulate both the innate and the adaptive immune response. C4a is also accounted as an anaphylatoxin, but is much weaker than C3a and C5a, and its actions and significance have not yet been elucidated [273].

While the complement system is important for targeting and eliminating pathogens, it has also been reported to have a negative role in demyelinating and neurodegenerative diseases, like multiple sclerosis (MS) [274], Parkinson’s disease [275, 276] and Alzheimer’s disease [277]. The negative role of the complement system on these conditions may be mediated through a long-term immune response that attacks the cells of the brain, leading to cell damage. The negative effects of the complement system have mainly been attributed to over-activity of the anaphylatoxins, which have pro-inflammatory features and recruit inflammatory cells to the area (reviewed in [278, 279]).

The importance of the complement system in CNS infections has only recently come into focus, where bacterial meningitis has been more extensively examined than viral CNS infections [280-283]. In bacterial meningitis, increased activity of different complement components is associated with positive or negative outcome depending on the specific pathogen that is involved [284]. Furthermore, in vitro studies indicate that neuronal cells are less susceptible to complement attacks than are skin cells, suggesting a contribution to the latency of HSV-1 in neurons [285].

**Complement component 3b can bind to glycoprotein C of HSV-1**

As described earlier gC-1, but not gC-2, functions as a receptor for C3b on whole virions [124, 286]. The C3b-binding activity of gC-1 has been suggested to have a protective effect for the virus on antibody-independent immune activation and neutralization. It has been shown that while gC-deficient HSV-1 and HSV-2 strains are neutralized by the complement system, regardless of presence of anti-HSV-antibodies, the infectivity of wild-type strains with fully functioning gC-regions is unaffected in the presence of complement components.

Binding of C3b to wild-type gC-1 induces blocking of other members of the complement cascade, which includes properdin and C5 [287]. As shown in Figure 18, properdin functions as a stabilizer of the C3 convertase of the alternative
pathway. Therefore, inhibition of properdin binding to C3b would protect HSV-1 from complement-mediated lysis via the alternative pathway. Interestingly, the properdin-binding property of gC-1 is not found for gC-2, not even in its purified form [129, 287]. As interactions between properdin and the mucin-like region of gC-1 have been demonstrated [126], a region missing in gC-2, these results are not surprising. Furthermore, the mucin-like region also appears to interfere with the binding of C5 to C3b [287]. No reduced effect on lysis via the classical pathway has been reported in studies [288]. As activation of the alternative pathway does not require antibody binding, the complement blocking effect of gC-1 might be most important early in HSV-1 infection, as has also been suggested by Friedman et al. [289].

Interestingly, the mab B1C1, recognizing the amino-terminal loop structure of the gC-1 protein, has been shown to reduce the complex of C3b, properdin and C5, indicating that the GAG-binding structures of gC-1 also are important for C3b binding [125]. All in all, the interactions of gC-1 with C3b, properdin and C5 indicate the importance of complement blocking for HSV-1 immune evasion.

Toll-like receptors

Like the complement system, toll-like receptors (TLRs) are an important part of the innate immune response. Expressed on the membranes of leukocytes, fibroblasts, epithelial cells, glial cells and endothelial cells and within endosomes, TLRs are single membrane-spanning receptors that recognizes structurally conserved molecules from microorganisms [291-293]. Through the recognition, TLRs activate immune cells by recruiting adapter proteins, in order to increase the antigen-induced downstream pathways and subsequent activation of other
downstream proteins including protein kinases and cytokines, leading to cell proliferation and increase in adaptive immunity [292].

TLRs can be found in the CNS, where they participate in the innate immune response against pathogens, as they do in the rest of the body. However, studies have also indicated negative effects of TLRs in the CNS, where they participate in autoimmunity, neurodegeneration and tissue damage [291].

Several TLRs have been reported to be involved in HSV-infection. Cell expression of TLR3 and TLR9, among others, have been of interest in HSV-induced CNS infections, where increased responses have been noted [294]. Although few host mutations have been found to increase the risk for alphaherpesvirus CNS infections, deficiencies in TLR3 have been shown to increase the risk for HSE in otherwise healthy children [19, 295, 296]. However, only about 5-10% of all cases of HSE in children can be related to TLR3 mutations. One case report describes a TLR3-related mutation in a young adult HSE patient [18] and a recent study identified TLR3-related mutations in adult HSE patients [297]. These reports indicate that deficiencies in the TLR3 pathway might also increase susceptibility to HSE in adults. Hence, TLR-3 mediated immune response may be of importance in the control of HSV infection in the CNS.

Cytokines

Cytokines are a category of small cell-signalling proteins which play an important role in immunomodulation through their effects on surrounding cells [298]. Their actions are mediated via receptors where they modulate the balance between the humoral and the cell-based immune response, and also regulate the maturation, growth and responsiveness of certain cell populations. Furthermore, some cytokines can influence the actions of other cytokines via a signalling cascade [299]. Cytokines are produced by numerous cell types; macrophages (including microglia), B-lymphocytes, T lymphocytes, mast cells, endothelial cells, fibroblasts and other connective tissue cells [300]. Chemokines, interferons (IFNs), interleukins (ILs), lymphokines and tumour necrosis factor (TNF) all belong to the cytokine group, where a specific cytokine can be produced by more than one cell type. Pro-inflammatory cytokines, such as IFNs, ILs and chemokines can be induced by oxidative stress and can be induced by and contribute to oxidative stress [301].

Cytokines all have matching cell-surface receptors, where binding leads to intracellular signalling cascades that can alter different cell functions, including upregulation and downregulation of several genes and their corresponding
transcription factors. This in turn can induce production of other cytokines, increase the production of surface receptors for other molecules, or suppress their own effect through a feedback inhibition loop [298].

Interestingly, apart from their immune-modulating properties, cytokines have been shown to be involved in several developmental processes during embryogenesis (reviewed in [302, 303]).

In the CNS, cytokines assist in activating microglia and in stimulating the adaptive immune response [300]. Although cytokines are important in the immune response to infections, they can also promote pathological changes in our bodies. Such adverse events have been linked to different disease entities including schizophrenia, major depression and Alzheimer’s disease [300, 304]. Pro-inflammatory cytokines are also reported to contribute to the continuously active state of microglia in CNS during infections and inflammation, which can result in neurodegeneration [305]. Furthermore, over-secretion of cytokines can trigger a cytokine storm (or hypercytokinemia) which is a potentially fatal immune reaction where the usual regulatory feedback loop between cytokines and leukocytes is disturbed [299]. A cytokine storm leads to over-activity of immune cells in a specific area in the body, and such activity can cause extensive damage to tissues and organs in this area. The cause of a cytokine storm is still not elucidated, but it has been suggested that an exaggerated immune response occurs when a new and highly virulent pathogen invades the host. Interestingly, cytokine storms have been connected to numerous viral infections, including Ebola [306-308], avian influenza [309] and smallpox [310]. The extensive effects of the 1918 pandemic on the younger population are believed to be due to the stronger immune response in a healthy immune system, thereby leading to increased cytokine levels [311].

**Interferon gamma**

As the only member of the type II class of interferons, IFN-\(\gamma\) is critical for both the innate and the adaptive immune activity against viral, bacterial and protozoal infections [312]. IFN-\(\gamma\) is secreted by T helper cells type 1 (T\(_h1\) cells), cytotoxic T cells and NK cells, and has both antiviral and immune-regulatory properties [312]. Furthermore, secretion of IFN-\(\gamma\) from T\(_h1\) cells results in a positive feedback loop that promotes CD4+ differentiation to T\(_h1\) cells. In the CNS, IFN-\(\gamma\) can also activate microglial cells, which then results in a cytokine-induced cascade that activates surrounding microglia. In response to IFN-\(\gamma\) activity, NK-cell and macrophage activity is promoted, along with expression of major histocompatibility complexes (MHCs), and induction of intrinsic defence factors leading to direct antiviral effects [312].
IFN-γ normally binds to the heterodimer interferon-γ receptor, leading to activation of cellular responses [312]. IFN-γ can also bind to HS, one of the receptors HSV may use to enter a cell. The biological significance for interaction between HS and IFN-γ is not extensively described, although it has been suggested that this interaction with HS may protect IFN-γ from proteolytic cleavage [313].

Interestingly, increased IFN-γ production has been found in the acute phase of HSE [314, 315]. In animal models, deficient IFN-γ production has been connected to a poor outcome after HSV infection, associated with increased apoptosis [316]. When IFN-γ instead is present, neuronal death is reduced, indicating the protective role for this cytokine in HSV infection. However, in patient studies it has been shown that higher initial values of IFN-γ in HSE are related to a poor outcome [315].

In connection with deficiencies in IFN-α and IFN-β, impaired production of IFN-γ has been shown to increase vulnerability for viral infections, including the risk for HSE (reviewed in [317]). Therefore, it might be inadvisable to completely block the production of IFN-γ during the initial phase of HSE.

**Tumour necrosis factor alpha**

TNF-α is mainly produced by activated macrophages, but other immune cells, as well as glial cells and neurons, can also contribute to TNF-α production [318, 319]. As a part of the so-called acute phase reaction, TNF-α regulates immune cells, but this cytokine can also induce apoptosis, inflammation and inhibit viral replication [318]. For cell signalling, TNF-α can bind two receptors: TNF receptor type 1 (TNFR1) and TNF receptor type 2 (TNFR2). While TNFR1 is expressed in most cells, TNFR2 is mainly express in cells of the immune system [318]. Furthermore, the receptor types respond differently to different trimeric forms of TNF-α, where the role of TNFR2 has been less extensively examined [318].

In HSE, levels of TNF-α have been shown to peak not in acute infection but rather during early convalescence [314]. TNF-α has been shown to be toxic to myelin, which could contribute to the damage seen in CNS in HSE patients. Furthermore, several other diseases, including Alzheimer’s disease, cancer and depression have been connected to dysregulation in TNF-α production, either through mutations or through anti-TNF-α treatment via monoclonal antibodies.

**Aquaporins**

Aquaporins constitute a family of membrane-bound water channels, whose function is to form pores in the cell membrane to enable rapid transport of water
through cells while preventing passage of ions. The transport of water over membranes is needed in order to maintain the osmotic balance in the cell. Some aquaporins only transport water across the cell membrane, while other aquaporins with larger-sized pores also can transport small, uncharged solutes like urea, glycerol, ammonia and CO₂ and are known as aquaglyceroporins. The human aquaporins AQP3, AQP7, AQP9 and AQP10 belong to this group.

In the cell membrane, aquaporins form tetramers, where the separate monomers all act as water channels. The aquaporin proteins consist of six transmembrane α-helices, which have both the amino end and the carboxyl end of the sequence located in the cytoplasm (Figure 19). To date, thirteen types of aquaporins are known and defined in mammals, but more are suspected to exist. The aquaporins differ from each other in their peptide sequences, and these sequence variations result in different pore sizes.

In the brain, three main aquaporins are reported to function in the brain: AQP1, AQP4 and AQP9. AQP1 is mainly found in the choroid plexus cells [320, 321], suggesting a role for AQP1 in CSF formation, and has also been described in neurons in the trigeminal sensory ganglia [322]. Presence of AQP9 has been reported in astrocytes [322-325], vessel-lining endothelial cells [325] and catecholaminergic neurons [325-327], but these studies are mostly from animal models. Nevertheless, the function of AQP9 to transport solutes like glycerol also suggests a contribution to the cell metabolism [323]. AQP4 is the most abundant aquaporin in the brain, and also the most well studied. It can be found in astrocytes throughout the brain, although the distribution varies in different brain structures, which could suggest that AQP4 may have further physiological functions apart from water homeostasis (reviewed in [328, 329]). Furthermore, AQP4 has been extensively examined in relation to neuroinflammation in neuromyelitis optica, where auto-AQP4- antibodies can be found in serum of these patients [330-332].

Due to their water transporting function, the CNS aquaporins not only regulate the brain fluid homeostasis but are also suggested to play a role in brain trauma.
and brain oedema, contributing both positively and negatively to the water homeostasis (reviewed in [328]). Furthermore, AQP4 and AQP1 has been suggested to be involved in the pathogenesis of HSE [333].

Autophagy

With the Nobel Prize in Medicine 2016 awarded to studies of autophagy, it is difficult to discuss immune activity in CNS infections without referring to this process that the cell can use to eliminate dysfunctional components or unwanted cargo [335, 336]. Autophagy occurs when a double-membrane vesicle (known as an autophagosome) absorbs either a part of the cytosol or ubiquitinylated cargo (including pathogens – a process known as xenophagy). The autophagosome then fuses with the lysosome where lysosomal enzymes can degrade the cargo (Figure 20).

Post-mitotic neurons, which cannot easily rely on cell-destructive or inflammatory responses due to the risk for neuronal death and neurodegeneration, have been shown to depend on autophagy instead to control intracellular

![Autophagy in viral infection of neurons](image)

**Figure 20.** Left: Impact of autophagy on viral infection in neurons. The virus is absorbed in an autophagosome (a double membrane vesicle) which then fuses with a lysosome, where the virus is degraded. This leads to decreased viral replication and production of interferon type 1, resulting in an overall increased neuronal survival. Right: Impact of viral infection on neuronal autophagy. When viral autophagy is inhibited, the viral replication is increased resulting in neuronal cell death via neurodegeneration and inflammation. Adapted from Orvedahl and Levine [334].
pathogens such as viruses. Furthermore, autophagy is necessary for neuronal differentiation and homeostasis as well as prevention of neuronal cell death.

Autophagy has been suggested to play a vital role in neuronal control of HSV infections, although the exact mechanisms are unclear. A recent study has suggested that autophagy, rather than IFN signalling, could dominate in the neuronal anti-HSV response [337]. In addition, it was demonstrated that autophagy was required to control HSV replication in neurons, unlike what was seen in mitotic cells [338, 339]. Furthermore, another study has demonstrated upregulation of autophagic clusters during HSV infection, but mainly in neurons that did not exhibit viral antigen expression [340].

HSV can respond to autophagy via HSV-encoded autophagy-modulating proteins, such as ICP34.5. This evasion is important for establishment of encephalitis, where the prevention of neurodegeneration via autophagy is inhibited (Figure 20) (reviewed in [334]). This also suggests that the balance between latency and lytic replication in neurons is related to autophagy.

Anti-N-methyl D-aspartate receptor encephalitis

One type of encephalitis that has come into focus in recent years is the autoimmune anti-N-methyl D-aspartate receptor (NMDAR) encephalitis [341]. NMDARs are ligand-gated cation channels found on the cell membrane of neurons, and they are important in synaptic transmission and plasticity [342]. During anti-NMDAR encephalitis, NMDAR antibodies reduce the expression of glutamate receptors on neurons in post-synaptic dendrites [341].

Anti-NMDAR encephalitis, which can present with seizures, autonomic dysfunction, abnormal or involuntary movement and changes in psychiatric behaviour, have mortality and morbidity rates (25% in total [343]) that are lower than those for HSE. The aetiology of anti-NMDAR encephalitis is not yet entirely known, although in approximately half of the cases malignancies are present [341, 344]. However, a link between relapsing HSE and anti-NMDAR antibodies has been presented in recent years [345, 346], and there have also been reports of possible connections between anti-NMDAR antibodies and CNS infections by VZV [347] or morbilli virus [348]. In some anti-NMDAR encephalitis cases the autoimmune activity is suggested to be triggered by viral infection. Furthermore, a pathogenic effect on neurons has been reported for NMDAR antibodies [349] and while this could explain the sequelae seen in HSE patients, another hypothesis suggests that the neuronal damage developed during HSE provides a setting for development of NMDAR antibodies, without actually being an anti-NMDAR encephalitis.
Relationship between neurological sequelae and immune system activity in CNS infections

Although alphaherpesvirus infections in CNS can directly attack and kill various neuronal cells, findings described in this subchapter imply that the immune system activity itself can inflict damage on brain tissue. Studahl et al. [350] described increased levels of neurofilament in the CSF of HSE patients also on follow-up. As neurofilament is a sign of neuronal lysis, the immune system must be active also after the elimination of lytic HSV infection in the brain, thereby damaging the infected area further through neuroinflammation and neurodegeneration.

That alphaherpesviruses can persist in the neurons in a latent state without damaging the cells is in fact quite logical. These viruses does not benefit from attracting the immune defence, which would lead to apoptosis and immune activity, and instead the post-mitotic neurons are necessary for viral replication during reactivation.

2.5 Diagnostic methods in alphaherpesvirus CNS infections

For diagnostics of alphaherpesvirus CNS infections, quantitative polymerase chain reaction (qPCR) on CSF to detect viral DNA is the gold standard. In qPCR, a set of primers and a probe target a conserved sequence of the genome that will be amplified and quantified.

qPCR as a diagnostic procedure can be used in the acute phase of the CNS infection, where virus usually can be detected in CSF in the initial phase of CNS disease and up to one-two weeks after onset of disease. However, PCR cannot be used in later stages of the infection, and in recurrent episodes of for example HSM, viral DNA is not always detectable. Furthermore, at least in HSE, PCR results may be negative for HSV in the beginning of the infection (days 1-3), but a second CSF sample taken a few days later might confirm the diagnosis [351, 352].

In later stages of infection, serology to identify antibodies in CSF and serum samples can be used to demonstrate intrathecal synthesis of IgG specific for the different alphaherpesvirus antibodies. The sensitivity of the serological tests increases with time. Acute infection can be diagnosed with elevated IgM levels in serum or seroconversion from negative to positive (where an antibody levels in an early sample are compared with those in a later sample). Intrathecal antibody production can also be analysed, by calculating the ratio between IgG levels in serum and in CSF, where increased levels indicate ongoing infection.
For neonatal HSV infection, PCR on material from herpetic lesions, serum, CSF and conjunctival swabs are used. Positive PCR findings are valid for diagnostics in new-borns during their first month. It has been demonstrated that uninfected new-borns can present with positive IgG levels from a mother who has experienced HSV infection during the perinatal period, due to maternal antibodies delivered across the placenta [353].

2.6 Vaccines and treatment in alphaherpesvirus CNS infections

Vaccines

Out of the three human alphaherpesviruses, vaccination is currently only available for VZV. A live varicella vaccine (Varilrix® or Varivax®) has been available for around 20 years and is mainly given to children to prevent varicella infection. Several countries around the world, including USA and Germany, have childhood vaccination programs including varicella [354, 355]. In Sweden however, although varicella vaccine is available, the government has not yet decided to include it in the general childhood vaccination program [356]. The existing varicella vaccine is a live attenuated vaccine based on a clinical VZV isolate, the Oka strain [357]. Vaccination induces VZV-specific cellular immunity and primes the host response, which modify the severity of the disease. Although one dose of the vaccine to children reportedly induces cellular immunity in more than 90% of all recipients, a second dose is even more efficient. In addition, due to fear of severe primary VZV infection during pregnancy and immunosuppression, this vaccination is also available for adults. In these patients, two doses of the vaccine need to be administered to achieve high rates of seroconversion. However, despite the two-times administration of the vaccine, adults have lower cellular immunity to VZV and lower IFN-γ response compared with children that only have received one dose of vaccination.

In at least 94% of vaccine recipients, the VZV IgG and T-cell responses is persistent for 7 to 10 years after immunisation. However, long-term VZV immune evaluation has not been performed following vaccination, and it is unclear if booster doses are needed. The incidence of CNS complications associated with varicella has been reduced in countries where varicella vaccination is recommended [256]. This is related to the successful protection against primary infection, but whether this vaccine will protect also against reactivated VZV infections in adults is still an open question. Vaccine-related reactivation of VZV
has been described in a few cases of immunocompetent recipients, but reactivation of vaccine virus in recipients with suppressed immunity is reported to occur less often than wild-type VZV reactivation in unimmunised patients.

Moreover, a high-dose version of the live vaccine (Zostavax®) is available for prevention of herpes zoster, and is given to the older population to prevent reactivation during waning immunity. Here, a higher dose than that administered to children is needed, but the protection against zoster and PHN is limited [358]. The vaccine decreased the incidence of zoster by 51.3% (p<0.001) compared with placebo recipients, and the incidence of PHN was decreased by 66.5% [358]. This data indicates the need for a new or improved zoster vaccine.

Recently, promising data from phase 3 clinical trials regarding protection against zoster was reported for a subcomponent vaccine based only on VZV glycoprotein E (gE) [359, 360]; this vaccine will probably reach the market in 2017. Selection of gE as the candidate antigen was based on the knowledge that gE is essential for replication and cell-to-cell spread. Furthermore, gE is the primary target for VZV-specific immune responses. The vaccine efficacy against zoster has been determined as 97.2% overall compared with placebo [360]; in participants over 70 years of age, the 4-year protection vaccine efficacy against zoster was approximately 90% and against PHN 88.8% [359]. However, one negative aspect of the vaccine candidate is the increase in reactogenicity seen in immunised patients in response to the vaccination, though most symptoms were mild-to-moderate injection-site reactions probably related to the adjuvant [360].

These findings demonstrate that protection through subcomponent vaccine against an alphaherpesvirus is possible, which may provide renewed optimism after the recent failure of an HSV-2 vaccine based on gD [361]. Promising results for gG-2 as a base for HSV-2 vaccine have been described recently by our laboratory [362-364]. So far, this subcomponent vaccine has only been tested in animal models. The primary goal for HSV vaccination has been a protection against genital herpes, but an efficient vaccine should also provide immunity against CNS infections, as has been described for VZV vaccine [256, 365], and also for morbilli vaccine [366].

Antiviral treatment

CNS infections caused by alphaherpesviruses have, unlike many other viral CNS infections, a highly recommended antiviral therapy available in the form of the nucleoside analogue acyclovir. Acyclovir is a structural analogue of 2-deoxyguanosine, apart from a modification in the cyclic ring where the 3’-
positioned carbon has been removed (Figure 21) [367]. The 3’ carbon is normally involved in creating a phosphodiester bridge to the following nucleotide.

The introduction of acyclovir in HSE therapy markedly reduced the mortality rate from 70% to the current 10-20% [209, 212, 368]. The effectiveness and non-toxicity of acyclovir is explained by the design of the drug. For acyclovir to be activated, an initial phosphorylation step is needed, which can effectively be performed by the viral TK (Figure 22), while host cell TK is one million times less capable to phosphorylate acyclovir [367]. Therefore, acyclovir is selectively activated in infected cells and is almost harmless to non-infected cells, providing a non-toxic profile. After the initial activation, the acyclovir molecule is further phosphorylated by cellular kinases to its active state. The activated form of acyclovir can then be incorporated into the viral DNA chain, where the activity of viral DNA polymerase is selectively inhibited and further elongation of the chain is blocked due to the blocking in the 3’ carbon position of the molecule.

Acyclovir is very effective when given intravenously, but has a low bioavailability when given orally [367]. However, the bioavailability is greatly improved when the aa valine is connected to the molecule, resulting in an L-valyl-ester prodrug of acyclovir known as valacyclovir (Figure 21). Once inside the systemic circulation, valacyclovir is transformed to acyclovir via esterase, and therefore valacyclovir is preferred in oral therapy [367].

For HSE and HSV myelitis, the recommended duration of intravenous (i.v.) acyclovir is 14 days to 21 days while for HSM and VZV meningitis the duration of therapy is usually 7 days, where only more severe symptoms or extensive

![Figure 21](image1.png)  
*Figure 21. Left: Structural formulas of 2’deoxyguanosine. Middle: acyclovir. Right: valacyclovir.*

![Figure 22](image2.png)  
*Figure 22. Activation mechanism of acyclovir.*
vomiting results in a recommendation of i.v. acyclovir, otherwise oral medication is used [132]. Treatment of primary HSM can be performed with antiviral therapy, although the CNS infection can often heal by itself (one exception is in immunocompromised patients [243]). In recurrent episodes, antiviral treatment can be beneficial, but the severity of the symptoms may determine if antiviral therapy is needed.

In neonatal HSV infections, acyclovir is given i.v. for 21 days, and followed by 6 months oral treatment. Antiviral therapy improves the outcome of neonatal HSV infection, especially if administered early. Better antiviral response has been reported in HSV-1 infected neonates than for HSV-2 infected neonates [198], although studies have demonstrated that neonatal patients can experience neurological sequelae after antiviral treatment regardless of virus [234, 236].

For VZV, few treatment studies on CNS infections are available, and the recommendations are often based on case studies or case reports. In myelitis, meningoencephalitis, encephalitis, brain infarction and ACA (especially in severe cases), i.v. acyclovir is recommended [132], otherwise oral valacyclovir can be used. Importantly, VZV is less sensitive to acyclovir than is HSV, due to inferior ability for VZV to phosphorylation in the first activation step of the nucleoside analogue, and therefore a higher dose might be needed [367].

Drug resistance against acyclovir is unusual in immunocompetent patient (<1%), but is more common for immunocompromised (3.5-10%) [369-371]. Resistance is most commonly due to mutations in the viral TK, although clinical isolates with mutations in the viral DNA polymerase also have been identified [372, 373].

The effects of long-term follow-up use of valacyclovir to reduce morbidity in HSE patients was tested in a recent study [374]. The hypothesis was that reduction of persistent, low-level HSV replication in CNS after the initial i.v. acyclovir treatment with oral administration of valacyclovir would reduce the neuropsychiatric sequelae and improve the outcome. However, no significant differences were found between the intervention group and the control group given placebo. Interestingly, the authors mention that as many as 44% of the enrolled HSE patients (regardless of treatment group) had no or only mild sequelae 90 days after the completion of the i.v. acyclovir treatment, which in sequel studies are around 50%. The authors referred to limitations of the study design that may have contributed to these results; only patients able to self-medicate with oral medication were enrolled; this would exclude patients with the least favourable outcome according to the Mini-Mental Status Examination (MMSE).

Furthermore, long-term treatment with valacyclovir to prevent recurrent HSM has also been tested, without success [242]. The reason behind this failure remains to be clarified.
Corticosteroids

Despite the significant reduction in mortality, acyclovir treatment for HSE patients has not reduced morbidity to a comparable degree. Recently, the sequelae seen in these patients have been attributed to an inflammatory process rather than the infection itself [375]. Cytokines and chemokines excreted from glial cells in the infected area in the HSE brain trigger an immune response that apart from attacking the virus also can damage the surrounding brain structures. Due to this inflammatory process, corticosteroid treatment has been suggested as a suitable complement to antiviral therapy in HSE patients to reduce the high morbidity still associated with the infection. This is motivated by the reduction of the inflammatory response and brain oedema by the steroid treatment [375]. However, mostly case studies and case reports have been published on the subject [376-378], apart from animal studies [379].

Therapeutic corticosteroids are synthetic analogues of steroid hormones produced in the adrenal cortex of the body, where synthetic glucocorticoids can be used to suppress inflammatory disorders as well as autoimmune disorders. They have potent anti-inflammatory effects but can also cause immunosuppression, where the function and numbers of lymphocytes are decreased, as well as suppressing cell-mediated immunity in the form of cytokines and the humoral immunity. The anti-inflammatory effects of glucocorticoids are mediated through suppression of the pro-inflammatory products prostaglandins and leukotrienes, the two main products of inflammation. Due to the many properties and mechanisms of glucocorticoids, long-term therapy is associated with many adverse events, where for example the long-term immunosuppression can cause an immunodeficiency. Furthermore, as glucocorticoids resemble human-produced hormones, therapy for more than one week leads to suppression of the patient’s adrenal glands due to exogenous suppression of corticosteroid hormones. To avoid withdrawal symptoms, it is therefore recommended to slowly decrease the dose for a few days in longer therapy, rather than to interrupt immediately.

An early retrospective study on the matter provided statistically evidence of slightly improved outcome in HSE morbidity with combination therapy of acyclovir and corticosteroids [380]. However, the study group was rather small and non-randomized and to be able to include gender and age as other influencing variables, the patient material needs to be extended to make accurate predictions. Furthermore, dosage and length of corticosteroid treatment differed between patients. The same research group later described predictors for a prolonged clinical course in HSE patients [381], where they indicated that corticosteroid treatment might have beneficial protective effects against prolonged HSE course.
In a recently presented retrospective follow-up study [377], the difference in clinical outcome of children with HSE receiving antiviral treatment only, or a combination with acyclovir and corticosteroids, was analysed. Although few patients were included in the study, the authors reported a reduced morbidity in steroid-treated patients, but the group treated with corticosteroids still had mild sequelae. However, the follow-up period in this study was longer for the patients only receiving antiviral treatment compared with the steroid-treated patients, and although the authors state that steroid treatment was given “early during the disease” for approximately 2 weeks, the exact period of time was not mentioned.

In 2008, the German trial of acyclovir and corticosteroids in herpes simplex virus encephalitis (GACHE) [382], a randomized, double-blind multicentre study, was initialized, where acyclovir treatment was to be combined with either corticosteroid therapy or placebo, to evaluate the effect on morbidity. The purpose of the GACHE trial was to provide important information on the safety and effectivity of corticosteroid treatment in HSE therapy, under more controlled conditions than prevalent in previously reported studies. Unfortunately, however, the study has been halted due to low enrolment numbers [383].

Despite the positive results presented in case studies, caution has been raised when considering corticosteroid treatment as there have been reports of reactivation of latent HSV after suppression of immune response (reviewed in [375]). This may not be surprising as the immune response is an important first line of defence in the combat against the virus infection. Therefore, corticosteroid treatment during the early phase of HSE of immunocompromised patients seems motivated, but the discussion has nevertheless created a resistance among some medical groups to administer immunosuppressive therapy even to immunocompetent HSE patients.

Treatment regimens of viral CNS infections in general do not include any recommendations of corticosteroid treatment, due to this lack of clinical studies. However, in clinical practice, high ICP generally justifies the use of corticosteroid treatment to reduce the oedema, as does a clinical picture of viral myelitis (reviewed in [132]). The general recommendation here is early treatment for a short period, 3-5 days, to avoid the adverse effects associated with corticosteroids.

Future and experimental therapy in CNS infections

Experimental treatment with the anti-secretory factor peptide 16 (AF-16), which corresponds to the 16 aa amino-terminal part of the endogenous protein anti-secretory factor (i.e. the S5A component of the proteasome), has in two animal studies of HSV-1 CNS infection reduced the ICP and the lateral ventricle
enlargement [384, 385]. Through decrement of the ICP with AF-16, all infected animals survived when therapy was started 4 days after infection, in contrast to vehicle-treated animals that showed a mortality of 80-90% after the experimental infection. Despite that AF-16 did not reduce viral replication or affect antigen distribution, the AF-treated animals did not develop neurological dysfunctions. The endogenous AF protein has been shown to reduce inflammatory reactions; this may explain why neurological dysfunctions are not seen in AF-16 treated animals with experimentally induced HSE. As a small peptide, AF-16 can easily pass the BBB to enter the brain, and it has been demonstrated that intranasal delivery, as used in the studies described above, further facilitates the entry of drugs into the CNS [386]. Therefore, AF-16 is a highly interesting option in future therapy of viral CNS infections, although clinical studies are needed to confirm the findings from the animal model.

Two other compounds, related to each other, PG545 and muparfostat (also known as PI-88), that mimic the structure of GAG chains, have been shown to have anti-HSV activities by our lab [387-389]. While the effect of muparfostat on HSV cell-to-cell spread only has been demonstrated in cell culture [389], PG545 has also been shown to have a virucidal effect on the lipid envelope of HSV particles and to be protective against genital HSV-2 infection in mice [388]. However, these compounds have not been tested in CNS models, so the efficacy and outcome has not been evaluated.

One problem associated with new medical therapy directed to the CNS is the passage over the BBB of the molecule of interest. Such passage requires the substance to display certain properties, where molecular weight, lipophilicity, polar surface area, charge, hydrogen bonding and molecular flexibility need to be optimised. So developing new therapy against CNS infections is often cumbersome. Instead, finding new use of drugs already known to pass the BBB is often of interest. Furthermore, the route of administration, i.e. subcutaneous, intranasal, oral, i.v. or i.c. needs to be considered.

What has been clearly demonstrated over the years is that antiviral therapy alone is not sufficient to significantly reduce the morbidity of the more severe alphaherpesvirus CNS infections, despite a prominent reduction in mortality. In this thesis, corticosteroid therapy has been discussed, which usually is the first suggestion for immunomodulation. Although only few studies have been published for alphaherpesvirus [390], limited trials have also been performed on other viral CNS pathogens, for example on influenza virus and West Nile virus, where immune-modulating therapy has been tested experimentally or in a small clinical scale [391, 392], with positive outcome. Instead of corticosteroids, Srivastava et al. [391] has presented an experimental model where immunoglobulins have been used against West Nile virus encephalitis with
positive results in controlling the CNS inflammation. However, as with all immunomodulatory therapy, cautions must be raised about possible activation of other pathogens.

To conclude, the future therapies for CNS infection might be evolving toward brief immunosuppressive therapy combined with antivirals (when available), and the restrictions for drugs to passage over the BBB need to be overcome when administering a drug to the CNS.
3. Material and methods

Detailed descriptions of material and methods used for each paper are found in the enclosed articles of this thesis. Stefan Lange adapted the rat HSE model from a model described previously by Johnson [393], using intranasal instillation of virus in the olfactory region. The in vitro model for infection of differentiating neuronal cells was developed in collaboration with Petra Bergström and Lotta Agholme at the Department of Clinical Chemistry. To study the binding properties of gC to GAGs, a model based on surface plasmon resonance (SPR) was developed by Marta Bally and Noomi Altgärde at Chalmers University of Technology.

3.1 Viruses and cell cultures

In paper I, the clinical strain HSV-1 2762 was used. This strain, obtained from a brain biopsy of a male patient with fatal focal encephalitis [368], was previously shown to be highly neurovirulent in in vitro experiments [187] as well as in animal experiments [394].

In paper III, HSV-1 strain 2762 and another clinical strain, HSV-2 VF-1181 [187], were used. HSV-2 VF-1181 is also a neurovirulent strain, obtained from a patient with HSM caused by HSV-2. These neurovirulent strains from clinical cases were used for infection of the iPSC and differentiating neuronal cultures. The rationale was that these viruses would induce a virulent infection in stem cells similar to that seen in the CNS after HSV infection, as compared to the more non-neurovirulent cell culture-adapted laboratory strains. Green fluorescent protein (GFP)-labelled virus HSV-1 K26-GFP [395] was used in immunocytochemistry studies (paper III). K26-GFP is a strain based on HSV-1 KOS, where a GFP-tag has been inserted into the capsid protein VP26 to facilitate visualisation of the infection.

In paper IV, the laboratory strain HSV-1 KOS [396] was used to determine the importance of the mucin-like region of gC. For investigation of the function of gC, two mutants of KOS were also used. The first mutant had a deletion of the gC-region of the virus (HSV-1 KOS-gCdef) due to a frame-shift mutation of cytosine at position 366 in the gC-genome (Ekblad, unpublished data). The second mutant (HSV-1 KOS-gCΔmuc) was derived from a marker transfer procedure where the mucin-like region of gC (aa 33-116) had been deleted; this strain is described in more detail in section 3.5.
Green monkey kidney (GMK) cells [397] were used in cell-based assays to assess the yield of infectious extracellular (EX) and cell-associated (CA) virus (paper III and IV), and to test the effect of different compounds with antiviral effect on HSV-1 variants (paper IV).

In paper III, GMK-cells and human fibroblasts were used for control assays of viral infection. Results were compared with results from infected cell cultures at four different stages during differentiation to cortical neurons from human induced pluripotent stem cells (iPSCs). These cells were included to demonstrate the differences in how the viruses act in different cell cultures.

3.2 DNA and RNA extraction and quantification

For extraction of viral DNA (papers I-IV), cell culture, patient and animal material was run on a MagNA Pure LC robot using the MagNA Pure LC DNA Isolation Kit I (cell and patient material) or MagNA Pure LC DNA Isolation Kit II Tissue (Animal tissue). Prior to DNA extraction, animal tissues were homogenized in a MagNA Lyser instrument by using MagNA Lyser Green Beads and a tissue lysis buffer [398].

Quantification of viral DNA was performed with qPCR for HSV-1 (papers I-IV) or HSV-2 (paper III). Highly conserved regions of HSV-1 gB and HSV-2 gB, respectively, were targeted with a pair of primers and a virus-specific probe [398]. Detection and amplification of HSV-1 and HSV-2 DNA resulted in specific cycle threshold (Ct) values; a standard curve with known concentrations of HSV-1 or HSV-2 in order to determine the number of respective virus DNA copies in each sample.

For detection of gene expression in rat brain tissue, homogenization was performed in a MagNA Lyser instrument using MagNA Lyser Green Beads (Roche) and QIAzol lysis reagent (Qiagen AB, Sollentuna, Sweden) and RNA was then extracted using the RNeasy Lipid Tissue kit from Qiagen. This combination of extraction methods was chosen due to the lipid nature of brain tissue.

RNA quantification of AQP9 and 18S (a house-keeping gene) was performed in a reverse transcription reaction by using the SuperScript III One-step RT-PCR system from Invitrogen and commercially pre-developed TaqMan Gene Expression Assays (Applied Biosystems) on a 7300 real-time PCR system. 18S was used as the housekeeping gene based on information from previous studies indicating that the gene is expressed constitutively in herpesvirus-infected cells [399]. The ΔΔCt-method (as described by Livak and Schmittgen [400]) was used to determine relative concentrations of AQP9 in total RNA samples.
3.3 The animal herpes simplex encephalitis model

For the animal HSE model presented in paper I, male Sprague-Dawley (SD) rats were used. The infective dose of HSV-1 virus was instilled in the right nostril of the rat under deep anaesthesia. The model provided the virus with a rapid entry route into the brain via the olfactory bulb, and resulted in a CNS infection resembling the pattern seen in human HSE. Rats have previously been suggested to be a relevant animal model for studies of HSV CNS infection because there are type-specific differences of HSV-1 and HSV-2 invasion of different locations within the rat brain; these type-specific differences parallel what is observed in humans [175]. Rats were sacrificed at days 1 to 6 post-infection, when tissue samples were taken for DNA and RNA extraction and quantification, and selected brain sections were investigated by immunohistochemistry staining. Infection studies did not extend beyond 6 days as the rats at this time point demonstrated severe symptoms. Tissues and sections from the olfactory bulb and the entry zone of the trigeminal nerve were of particular interest to observe which route the virus used to enter the CNS. For AQP9 RNA quantification, sections from the olfactory bulb, the AC and the hippocampus were of special interest in order to detect any upregulation of gene expression in HSV-1 infected animals.

The animal model was chosen as the viral inoculation, when HSV-1 was instilled to the olfactory mucosa, did not inflict any damage to the skin or the mucosal barrier that more invasive models can do (i.e. when virus is injected i.v. or i.c.). Such more invasive models can recruit inflammatory cells to the area of damage, which in our model could have affected the extent of the viral infection in the skin and mucosa and also the viral spread. In addition, i.c. injections are not useful for study of neuronal routes used by the virus as these would provide the virus with an unnatural route for entry and spread.

Immunohistochemistry was utilized to document the viral spread within the rat brain. Furthermore, it also functioned as a complement to viral DNA quantification since this method could indicate where in a specific brain section the virus would be found. When viral DNA is extracted from a specific tissue, the viral DNA quantification can indicate a DNA concentration in one area of the tissue section that does not correspond to the concentration in other regions of brain. Therefore, staining by immunohistochemistry could indicate where the virus was located and actually expressed antigens within the investigated section.

3.4 Patient material

In papers I and II, CSF and serum samples from HSE patients were analysed with enzyme-linked immunosorbent assay (ELISA) kits for AQP9 concentrations.
and presence of complement components, with ethical approval from the Regional Research Ethics committee in Gothenburg.

Due to the rarity of HSE cases, a retrospective study design was adopted to obtain a study group large enough to enable evaluations and predictions. The patients included in the group had been admitted to the Sahlgrenska University Hospital Östra Sjukhuset between 1995 and 2014, with CSF and serum samples that either had been sent to the Virology laboratory for immediate analysis or had been stored at the clinic for later analysis. Patients (n = 23 for paper I; n = 35 for paper 2) were included based on the medical records of clinical symptoms and diagnoses, along with detection of HSV-1 DNA in at least one CSF sample. If available, serial CSF samples from several occasions were included in analysis to obtain information about activity over time for the analysed components of interest. Patient information collected was age, gender, HSV-1 DNA concentration in each sample (both CSF and serum), time after onset of neurological symptoms for each sample and score on the Glasgow outcome scale (GOS).

In addition to the HSE patient group, a control group of healthy subjects was used for the analysis (n = 19 for paper I; n = 11 for paper II). However, due to the dissimilarities in age and gender between the two groups, another control group of so-called “patient controls” with demographic distribution closer to the HSE group was also included in paper II (n = 28). This group consisted of subjects that had sought medical care with initial suspicion of CNS infection due to their symptomatic profiles, though such infections were later deemed less likely. The healthy controls were used to set a baseline of values in the healthy population while the patient controls were included to distinguish differences between patients with proven HSV-1 CNS infection and undiagnosed patients with other symptoms.

Serum samples were included in the ELISA analyses both as positive controls and for determination whether increases or decreases of components of interest (AQP9 or complement components) only were present in the CNS or were a result of passive transfer from the general circulation to the CSF.

The ELISA method used for the analysis was of a sandwich ELISA model (commercially available). For the kits used in paper I and II, the capturing antibody is a monoclonal and the detecting antibody is a polyclonal (personal communication with the manufacturing companies). The commercial ELISA kits were chosen as they were sensitive, i.e. could identify even low concentrations of antigen, and specific due to the use of two separate antibodies for capture and detection of the antigen of interest.
3.5 A model for infection of cortical neurons differentiating from induced pluripotent stem cells

![Diagram of differentiation process and viral infection with HSV-1 or HSV-2 or mock-infection at four time points during differentiation: day 0, day 30, day 60 and day 90. At time points 0h, 24h and 48h post-infection four different procedures were performed: supernatant was removed to obtain extracellular (EX) virus in supernatant, cell and viruses were harvested for cell-associated (CA) virus, lysis buffer was added to cells to obtain a lysate, and cells were incubated with a tetrazolium compound to measure cell viability in a colourimetric assay. CA and EX virus was then subject to viral titration and viral DNA quantification to obtain viral titre (measured in plaque forming units/ml) and viral DNA (measured as DNA copies per ml). Supernatant was also assayed for lactate concentration, a measurement of the metabolic activity in the cells. Finally, the lysate was subject for extraction of genomic DNA (gDNA) and total RNA. The total RNA was transcribed into cDNA and gene expression was analysed.](image)

**Figure 23.** Differentiation of human induced pluripotent stem cells (iPSCs) into cortical neurons. Infection of cells with HSV-1 or HSV-2 or mock-infection at four time points during differentiation: day 0, day 30, day 60 and day 90. At time points 0h, 24h and 48h post-infection four different procedures were performed: supernatant was removed to obtain extracellular (EX) virus in supernatant, cell and viruses were harvested for cell-associated (CA) virus, lysis buffer was added to cells to obtain a lysate, and cells were incubated with a tetrazolium compound to measure cell viability in a colourimetric assay. CA and EX virus was then subject to viral titration and viral DNA quantification to obtain viral titre (measured in plaque forming units/ml) and viral DNA (measured as DNA copies per ml). Supernatant was also assayed for lactate concentration, a measurement of the metabolic activity in the cells. Finally, the lysate was subject for extraction of genomic DNA (gDNA) and total RNA. The total RNA was transcribed into cDNA and gene expression was analysed.
In paper III, a previously described cell culture model for differentiation of human iPSCs into cortical neurons [401, 402] was used. At four time points during differentiation, representative of different stages during neuronal development, cells were infected with HSV-1 or HSV-2. The morphological appearance of the cells at different stages of differentiation is shown in Figure 23.

Infected or mock-infected cells and conditioned media from these cultures were analysed for viral titres and viral DNA, cellular gDNA and total RNA, gene expression, cell viability, cell metabolism and complement activity (Figure 23). This cell culture model was chosen based on its resemblance with neuronal development in utero, and is a relevant model for development of human neuronal cells. We were interested in distinguishing if the viruses would infect the cell culture more profoundly at any particular stage during differentiation and in analysing the effects of infection on cellular gene expression of differentiation markers. Detection of any discriminating difference between HSV-1 and HSV-2 infectivity in these cells would be of considerable interest, particularly with our knowledge of the different characteristics of the infection in neonates, older children and adults.

As we were unable to determine the number of cells in each well before infection, we decided to infect all wells with the same concentration of virus. The CA viral titres and viral DNA concentrations were normalized to the cellular genomic DNA (gDNA) extracted from the same well in order to obtain an equal estimate of the viral infection for each time point of interest. The concentration of gDNA was chosen for normalization as it is estimated to be consistent in all cells for most organisms and would therefore be similar for all four time points of interest, and also for the control cell cultures GMK cells and fibroblasts. This provided information to calculate viral titre and viral DNA in relation to the gDNA concentration, a ratio of plaque forming units (pfu)/gDNA (for viral titre) and viral DNA/gDNA (for viral DNA).

3.6 Construction of an HSV-1 strain lacking the mucin-like region of glycoprotein C

Using a marker transfer assay that has been described previously [113], an HSV-1 strain mutant deficient in the mucin-like region of gC (Δ33-116) was produced in paper IV (Figure 24). Here, a mutant virus that had been selected as deficient in the mucin-like region through multiple passaging of HSV-1 KOS in GMK cells in the presence of the compound muparfostat (PI-88) [113] was recombined with a gC-negative virus (HSV-1 KOS-gCdef, described in section
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3.1) (Figure 25). This was done to ensure that the mucin-like region was the only altered part of the genome, as the multiple passaging could also have introduced other mutations in the viral genome. To ensure proper marker transfer, cells infected with the resulting mucin-like region-deficient strain (KOS-gCΔmuc) were immunostained for gC-positivity using the monoclonal antibodies B1C1, C2H12 and C4H11 directed towards different epitopes of the gC protein. The viral DNA was sequenced to confirm that the defined mutation was present in the genome.

The gCs isolated from wild-type HSV-1 KOS and from the mucin-like region deficient HSV-1 KOS-gCΔmuc (Figure 24) were extracted from lysates of EX virus and virus-infected cells by using immunoaffinity chromatography [66].

![Image](image-url)

**Figure 24.** Top: Wild-type gC-1 with the mucin-like region (aa 33-116). Bottom: gC-1 Δmuc with a deficient mucin-like region, where the aa 33-116 residues have been deleted, thereby removing numerous O-glycosylation sites.

**Figure 25.** Marker transfer assay of HSV-1 lacking the mucin-like region of gC into HSV-1 deficient in gC through co-infection in GMK- cells.
To assess the contribution of sialic acid (SA) to GAG-independent interactions, HSV-1 KOS and HSV-1 KOS-gCΔmuc were treated with neuraminidase for desialylation. This was performed to determine whether SA could interfere with the binding between GAG and virus.

The two viral strains were examined for yield of infectious virus following infection of GMK cells. Here, as was done for differentiating neuronal cells, separate analysis was performed for the amount of virus produced by infected cells (CA virus) and virus released into cell medium (EX virus). Cells infected with the two viral strains were studied with electron microscopy.

3.7 Surface plasmon resonance experiments

To assess the binding properties of gC to GAGs in regards to kinetics and equilibrium in Paper IV, surface plasmon resonance (SPR)-based interaction experiments were performed. For this method, a sensor chip functionalized with biotinylated GAGs (hyaluronic acid (HA), CS or sulphated HA) was used. GAGs were biotinylated at the reducing end to enable an immobilized end-on configuration to a streptavidin surface, which would mimic the attachment of GAGs to proteoglycans on the cell surface. A constant stream of running buffer containing isolated gC either from KOS or KOS-gCΔmuc was then added, where

\[ \text{Running buffer} \]

\[ \text{Sensor chip} \]

\[ \text{Polarized light} \]

\[ \text{Reflected light} \]

\[ \text{Output} \]

Figure 26. Surface plasmon resonance (SPR). A running buffer containing isolated gC-1 from either wild-type or mutated virus was allowed to run in a constant stream over a sensor chip. The sensor chip had previously been functionalized with biotinylated GAGs to which the isolated gC could bind. The binding was recognized by a detector that recorded the reflection of polarized light directed towards the base of the chip.
the isolated gC could bind to the biotinylated GAGs and the binding was detected (Figure 26).

For inhibition experiments, the gC1-specific monoclonal antibody B1C1, in which the binding site overlaps that of the HS-binding region of gC, as shown in Figure 5, was mixed with isolated gC and then injected over the functionalized sensor chip.

To estimate the contribution of electrostatic interactions in binding of gC-1 to the GAG, different ionic strengths were used for the gC-1 containing running buffers. In addition, to obtain binding affinity of gC, a low flow of running buffer was used until equilibrium; this was repeated with increasing concentrations of gC.

### 3.8 Effect of antiviral compounds on HSV-1 infection

Cells were infected with HSV to determine the effect of antiviral compounds in a viral plaque number reduction assay (paper IV). This was performed using either heparin or muparfostat, two compounds with antiviral effect. Muparfostat is an oversulfated oligosaccharide that mimics GAGs; heparin is a GAG closely related to HS. Working dilutions of KOS or KOS-gCΔmuc virus were added serial five-fold dilutions of the compounds; these mixes were held at room temperature for 10 min. GMK cells were infected with the virus-compound mixes, and cell culture plates were incubated for 3 days. The number of plaques formed in the plates inoculated with each compound premixed with virus was counted. From this, the 50% inhibitory concentration (IC$_{50}$) was calculated.

Neuraminidase-treated viruses were also tested for their sensitivity to heparin to establish the effect of GAG interactions after this modification, following the procedures described above.
4. Results and discussion

Appropriate cell and animal models for studying alphaherpesvirus infections in the CNS are scarce and can seldom mimic the entire course of the disease. The role of the viral glycoproteins during cell entry and egress has been studied previously, but the role of the so-called mucin-like regions of some glycoproteins is not well characterized. Furthermore, little is known of the role the immune responses have in the damage seen in the brain after infection, but more and more evidence seems to indicate that the virus alone is not the cause of the symptoms and sequelae related to alphaherpesvirus CNS infections (reviewed in [403]). In this thesis, viral spread within the CNS, viral adhesion and release, the sensitivity to HSV-1 and HSV-2 infections of developing neuronal cells and the activity of the innate immune response were investigated.

4.1 A pathway for contralateral spread of HSV-1

We used a rat model to provide information on routes of transport for HSV-1 spread in HSE (Paper I). Here, we found that after intranasal instillation in the right nostril only, virus distribution, as detected in the form of HSV-1 DNA, was observed early both in the right trigeminal ganglion and lamina cribrosa (Paper I: Fig. 1). The latter region is a sieve-like structure of the ethmoid bone, between the nasal cavity and the brain, through which the olfactory nerves pass. Viral spread to the parallel structures on the left side of the brain was delayed by 2 to 3 days. Antigen detection by immunohistochemistry supports the findings of a delayed spread to the contralateral hemisphere (Paper I: Fig. 2-3). Contralateral viral spread may be limited by an anticipated transport of virus across synapses. This was further illustrated by findings in the olfactory bulb, which was initially reached from the olfactory mucosa through passage of synapses (as illustrated in Paper I Fig. 10a). In the right olfactory bulb, HSV-1 DNA was not found until rather late after infection but surprisingly, HSV-1 DNA in the left olfactory bulb was found near the time of its detection in the right bulb. One explanation to this finding was provided by immunohistochemistry where HSV-1 antigen was detected in the anterior commissure (AC). This is a bundle of nerve fibres for contralateral communication found in an evolutionary conserved part of the brain. The AC provided the virus with a shortcut for rapid spread to the left olfactory bulb and to the limbic system (Paper I: Fig. 4). The suggested spread of virus in rat brain after infection with HSV-1 in the olfactory mucosa is illustrated in Figure 27.
Immunohistochemistry demonstrated that infected cells in the AC morphologically resembled oligodendrocytes. In addition, these cells were not positive for markers specific for neurons, astrocytes or microglia (Paper I: Fig. 5). This finding may be important for explanation of the rapid contralateral spread of the virus. Given the difference in time for the virus to reach the right olfactory bulb compared with the right trigeminal ganglion, synapse passage may be a time-limiting step in the spread of HSV-1.

Initially, we did not have a suitable immunohistochemistry marker for oligodendrocytes. To identify oligodendrocytes as the cells targeted by HSV-1 in the AC, morphological resemblance combined with no apparent co-staining of other glial cells was used. However, later we discovered that a polyclonal antibody for AQP9 co-stained with the presumed oligodendrocytes in the AC (Figure 27).

Figure 27. Left: routes for HSV-1 entry and spread in rat brain. Dashed arrows indicates suggested routes for spread. Right: Staining for AQP9 in the anterior commissure (AC). Top right: Combined staining for AQP9, HSV-1 and nuclei. Second top right: Staining for nuclei with DAPI (blue). Second bottom right: Staining for AQP9 with a rabbit polyclonal antibody (red). Bottom right: Staining for HSV-1 with a mouse monoclonal antibody (green).
In addition, immunohistochemical observations showed enhanced staining for AQP9 in HSV-1 infected animals as compared with uninfected controls, indicating that AQP9 expression may be increased in these animals (data not shown).

Unlike corpus callosum, the main route for communication between the two brain hemispheres in placental animals but not in other vertebrates, the AC commissure is present throughout the animal kingdom. However, due to the existence of corpus callosum, the AC in placental animals is less extensively developed compared with for example in marsupials [404, 405]. This knowledge encouraged us to suggest that the use of the AC for spread could indicate that HSV, and maybe all alphaherpesviruses, targeted this route in the brain since long in evolution.

Although it has been previously demonstrated that HSV-1 can enter the brain via both the trigeminal nerve and the olfactory bulb [214, 217, 218, 406], the further spread to the limbic system, where much of the cell damage of the infection is localised [407], is less well known. When evaluating whether the results from our rat model reflect the actual viral spread in HSE patients, the autopsy findings by Esiri [214], where HSV-1 antigen was found both in the trigeminal ganglion and the olfactory bulb in HSE-patients deceased early in infection, should be considered. This indicates that the route of infection seen in the rat model is similar to that found in humans. Esiri also found evidence of HSV-1 antigen being expressed in the AC of some subjects, again of relevance for our findings in the rat model.

Cultured oligodendrocytes have previously been tested for their susceptibility to HSV-1 and were judged to be highly sensitive as compared to other cells of different origins [96, 161, 408]. However, HSV-1 infection of oligodendrocytes has rarely been described in clinical reports. This is in contrast to observations made in astrocytes and neurons [214, 409]. Our findings suggest that the myelin sheath produced by oligodendrocytes may provide the virus with the opportunity to avoid a possible delay associated with viral transport over synapses.

Next, due to the noted increased presence of AQP9 in infected animals, we were interested in investigating the gene expression of AQP9 in rat brain tissue. Here however, no increase in AQP9 gene expression was seen in infected rats compared with control rats, but rather, the gene seemed to be constitutively expressed during infection (Paper I: Fig. 9).

Our next step was to investigate AQP9 concentrations in samples of CSF and serum from HSE patients, to relate the increased presence of AQP9 antigen detected in the animal model to a clinical setting. CNS aquaporins, including AQP9, have been implicated in the development of brain oedema during trauma as well as during infection with other pathogens [410, 411]. As brain oedema is a
common finding in HSE patients, which often can contribute to the neurological pathology if left untreated [211], we were interested in investigating if AQP9 antigen concentrations differed between HSE patients and healthy controls. We also analysed concentrations of the other two CNS aquaporins, AQP1 and AQP4, in the CSF and serum samples by ELISA assays. In contrast to our findings on gene expression, AQP9 concentrations were significantly increased in CSF from HSE patients as compared with healthy controls (Figure 28). Notably, AQP9 concentrations were below the minimum for detectable concentration in CSF from all healthy controls. The increase in AQP9 concentrations was seen in early HSE, but not in later stages when HSV-1 DNA was undetectable. No significant differences in protein concentrations in CSF were revealed either for AQP1 or for AQP4 between HSE patients and healthy controls (Figure 28).

Although AQP9 is one of the three aquaporins expressed in the CNS, this ion channel protein has not been extensively investigated in patients or in animal models. This is in contrast to AQP1 and, in particular, to AQP4. In a previous animal study, the gene expression of these two CNS aquaporins was studied in HSV-1 infected mice. Interestingly, the results demonstrated that AQP4 expression was downregulated during acute HSV-1 infection [333] and increased in late infection, while the opposite pattern was demonstrated for AQP1. This may suggest that if we had examined the gene expression in the brain rather than the protein concentrations in CSF for these two aquaporins, our results could have been different.

The increased AQP9 levels we observed in HSE patients have previously been noted in animal models utilized to investigate other brain trauma (reviewed in [412]). One explanation proposed for the increased aquaporin concentrations

![Figure 28](image)

Figure 28. Aquaporin concentrations in samples of CSF from control subjects (n= 15) and herpes simplex encephalitis (HSE) patients (n = 19). Left: AQP1 concentration in CSF. Middle: AQP4 concentration in CSF. Right: AQP9 concentration in CSF. ns = non-significant, ****= p<0.0001. The Mann-Whitney U test was used to obtain p-values.
presented here, and also for findings recorded in patients with other brain trauma, is that this elevation constitutes a functional response to brain oedema in order to reduce the increased ICP. On the other hand, AQP9 has also been reported to be expressed in leukocytes [413] and, as HSE normally leads to an increase in CSF and brain leukocytes, the observed increase in AQP9 noted here could be explained by a cellular inflammatory response in this disease.

4.2 The complement system is activated both in acute and late herpes simplex encephalitis

The persistent neurological sequelae seen in HSE patients have previously been suggested to be caused by the immune response rather than by the infection itself. In Paper II, we examined the activity of the complement system, a part of the innate immune response, both in acute and long-term HSE to investigate if the activity can be related to neurological complications. One reason for this investigation was the reports from cell culture and animal studies of a direct binding interaction between C3b and gC on HSV-1, where gC-1 functions as a receptor for C3b [414]. Since these discoveries were made in the end of the 1980s and beginning of the 1990s, several cell culture experiments have been performed with the aim of elucidating the gC-C3b bindings at the molecular level, but studies of the clinical importance of this interaction during HSE are lacking.

For our study, we tested seven different complement components (C1q, CFB, C3a, C3b, C4b2a, C5 and C5a), representative of the three different activation pathways of the complement system as well as of the terminal pathway (Figure 17). Results for HSE patients were compared with those for patient controls and healthy controls, where the healthy controls were utilized for baseline concentrations and patient controls were used to identify any differences or similarities between HSE patients and undiagnosed but symptomatic subjects (Paper II: Fig. 1). Here, we observed that concentrations for C1q and CFB were similar in CSF for all three studied groups. Although C4b2a concentrations were significantly lower in HSE patients compared with patient controls, only a few patients contributed to this difference. Therefore, these results received less attention, especially as there was no difference seen between healthy controls and HSE patients. C3a, C3b, C5 and C5a concentrations on the other hand were significantly higher for HSE patients as compared with healthy controls. A significant increase in C3a and C5a was also detected in HSE patients as compared with patient controls. In addition, we found that the concentrations of C3a, C5, and C5a were not significantly increased in serum from HSE patients as compared with healthy controls (no data was available for C3b levels in serum from healthy
controls). These findings indicated that the complement components were produced intrathecally rather than being recruited from the general circulation. Intrathecal complement production during CNS infection has also been reported by others [283, 415].

The anaphylatoxins C3a and C5a can, among other functions, recruit the adaptive immune response to the site of damage and thus have a pro-inflammatory effect. We were therefore interested in analysing the differences in complement activity during acute and prolonged HSE. Here, we found that C3a concentrations tend to increase over time, while C5a levels slightly decreased during follow-up (Paper II: Fig. 4). Nevertheless, the two anaphylatoxins were significantly increased as compared with both control groups in early as well as in late HSE (Paper II: Fig. 2).

Then, we analysed our results in regards to GOS-reported outcome scores for HSE patients, where no differences in complement concentration were found in the initial sample in relation to outcome (Paper II: Fig. 5). This was unexpected, as our hypothesis was that either increased, or reduced, complement activity could affect the extent of neurological damage in the HSE patients, as was previously reported in bacterial meningitis of different origin [284]. However, a separation of the HSE patients into five groups of GOS score gave low numbers of cases in each group, making interpretation of the results difficult. Furthermore, complement measurement in late HSE could be biased as patients with GOS score 1 had a shorter follow up since they only could be sampled until they deceased, which we could not correct for in our analysis.

In paper I, AQP9 was found to be increased in CSF of HSE patients and was suggested to contribute to ICP. The increased activity of the complement components in Paper II could therefore be a response to leaking cells, and brain oedema, as well as to other damage caused by the virus.

During the work with Paper II, we also studied CSF and serum material from patients with other viral CNS infections for comparison, although these data have not yet been published. Amongst the CNS infections, this material included patients with TBE. While almost all HSE patients had follow-up samples, only a few TBE patients had samples collected over time. Here, we found that CSF concentrations of C3a were significantly increased in HSE patients as compared with TBE patients (Figure 29), while C5a and C3b concentrations were similar between HSE patients and TBE patients. Interestingly, C1q levels were significantly higher in TBE patients as compared with HSE patients (Figure 29). This finding was particularly intriguing as C1q did not differ significantly for HSE patients as compared with either of the two control groups (Paper II: Fig. 1), indicating that in TBE virus infection, the classical pathway of the complement system might be preferentially activated in the CNS.
Neurological sequelae, which are commonly diagnosed in HSE patients, have also been reported in 30-50% of TBE patients in long-term follow-up [416-419]. Unlike the HSE patient material, where serial samples were obtained for up until two years after onset of symptoms, the TBE material we had access to was mainly sampled within the first 20 days after onset of symptoms. Therefore, any predictions of long-term complement activity, and eventual relation to outcome for TBE patients, are difficult to make. However, the increased concentrations of C3a for HSE patients as compared with TBE patients could be related to the increased levels of neuronal and astroglial cell damage markers reported in a previous study [350].

Interestingly, C1q, which was found to be increased in TBE patients, has been reported to interact with the non-structural glycoprotein 1 (NS1) on the surface of Dengue virus, another flavivirus related to TBE virus [420]. This could indicate that the increased concentrations of C1q in TBE patients actually reflect the
differences in complement activation pathways as suggested above. If so, flavivirus glycoproteins may bind C1q, in a manner similar to the function of HSV-1 gC as a receptor for C3b. NS1 of different flaviviruses (including West Nile virus and Zika virus) have recently been demonstrated to be directly involved in the interactions of the MAC [421]. These findings indicate that as many viruses function as receptors for complement components, the importance of the complement system in response to a viral infection may previously have been underestimated.

Our findings of complement activity both in acute and prolonged phases of HSE could indicate that immunosuppressive therapy may be beneficial in HSE patients in combination with antiviral treatment, as has been suggested in different case reports. Despite of the negative effects of corticosteroid therapy, where the CNS infection by alphaherpesviruses can be triggered by the immunosuppression (reviewed in [375]), or of reports where corticosteroid treatment has not had the desired effect [381, 422], the benefits on the outcome of corticosteroid treatment, initiated after PCR confirmation of HSE, appears to be promising. If the immune activity in CNS could be reduced for as long as HSV-1 DNA can be detected in CSF, the cortical damage could possibly be diminished, even if not completely abrogated. However, to find the appropriate dose for and duration of immunomodulating therapy, clinical trials like the GACHE study initiative [382] will be needed.

### 4.3 Differentiating neuronal cells vary in their vulnerability to HSV-1 and HSV-2 infection

To reduce the need for animal models of HSE, we tried to identify a new cell culture model to study HSV-infection. In Paper III, HSV infection of cortical neurons differentiating from iPSCs was described. This model may imitate neuronal differentiation under the gestation period, where proliferation of cells is high in early differentiation, while the cells have reached a post-mitotic phase in later stages of differentiation (Paper III: Figure 1).

We infected cells at four representative stages of differentiation, with CNS-derived isolates of HSV-1 or HSV-2. Between the four differentiation stages, we found differences in cell viability and virus production after infection with these viruses (Paper III: Figure 2-4). The main finding held for both viruses was that the cytopathogenic effect was most pronounced in cells infected on day 30 after onset of differentiation, although virus yields reached a maximum in cells infected on day 90 of differentiation. Many different processes occur during cell division that may contribute to these findings. Possibly, proliferating cells, commonly observed
during early differentiation (and present in cultures of human fibroblasts and GMK-cells) are more vulnerable to HSV-infection than post-mitotic cells seen in late differentiation and may contribute to the cytopathogenic effect on day 30. One factor that argues for this hypothesis is the latent infection that HSV can establish in mature neurons through expression of genes that suppress autophagy in these cells (reviewed in [334]), where the virus is protected and maintained in a persistent state. On the other hand, undifferentiated iPSCs did not show high production of infectious virus, nor was their cell viability affected, despite their high frequency of mitosis. Therefore, other factors apart from proliferation, for example a changing pattern of expression of viral surface receptors during differentiation, could also contribute to the varying susceptibility to viral infection. Another possibility is a beneficial effect of synapse formation on viral replication and cell viability. In late infection, the neurons are connected via synapses, which facilitate the transmission of virus to other cells, while these connections are lacking in early infection. One suggestion here is that without synapses, the egress of progeny virus is hampered, which would increase the pressure of the neuron/neuronal progenitor cell in the direction of cytopathogenicity.

The aim of **Paper III** was to characterize eventual differences between HSV-1 and HSV-2 in a novel cell culture model of CNS infection, to elucidate the neuropathogenicity of the two viruses during specific stages of neuronal development. We observed reduction in cell viability in early differentiation for both HSV-1 and HSV-2, as compared with uninfected control cells (Paper III: Figure 4). This reduction was greater for HSV-2 infected cells despite similar titre after 48h (Paper III: Figure 2, 3). Our results do not explain why neonates are more severely harmed by HSV-2 infection than by HSV-1 infection, but do indicate that our cell model is useful for studying HSV infection in the developing brain.

As was seen in **Paper I**, the importance of the sensitivity of additional brain cells, not just neurons, to infection with HSV must be considered [423-425]. Our cell cultures contained mainly neurons (at least 95%), with appearance of only a few astrocytes in late stages of differentiation. In addition, by electron microscopy we have observed a cellular structure that we interpret as being an oligodendrocyte (Figure 30). These three cell types, neurons, astrocytes, and oligodendrocytes, develop from the neuroectoderm. Microglial cells, on the other hand, are not expected to be found in the differentiated iPSCs cultures as they originate from another embryonic layer. Therefore, to get a better understanding of the behaviour of HSV-infection in differentiating neuronal cells, and thereby in developing brains, cell cultures with both neurons and all glial cells are needed.
Figure 30. Electron microscopy of cortical neurons (day 90 of differentiation) infected with GFP-tagged HSV-1. Top right: Overview. Boxes labelled A to D indicate enlarged sections. (A) HSV-1 virion in exocytic vesicle at the peripheral part of cytoplasm. Note presence of a single virion in this vesicle and of several virion-like enveloped structures lacking typical viral capsid in another exocytic vesicle. (B) Presence of virion-like enveloped structures in exocytic vesicles. Note absence of typical viral capsids inside these structures and presence of tubulo-vesicular (elongated vesicles) forms of these exocytic vesicles. (C) Oligodendrocyte-like cell attaching to cortical neuron. Note presence of rough endoplasmic reticulum structure in cortical neuron just below the cell plasma membrane fragment contacting the oligodendrocyte outer membrane. (D) Inside the nucleus there are >10 capsids of which two are empty (no DNA inside). In the perinuclear space, on the right side of the nucleus, there are a substantial number of empty capsids and some complete viral particles.
As we found in Paper II, the immune system is activated during HSV CNS infection. Besides the complement system, increments of other signalling proteins that can stimulate the adaptive immune response have also been described. To assess the potential complement reactivity of the HSV infected iPSC system, the cells were examined in the ELISA complement assays as outlined in Paper II. However, no significant differences were detected between infected cells and their controls (Paper III: Figure 5). Despite reports of production of different complement components in different cells of the CNS, a full response of this system requires the presence of all types of CNS cells. Furthermore, to inflict the damage to cortical neurons that is found in the brains of HSE patients, presence of an immune response might be needed, which could explain why the cell viability was almost unaffected by infection in late differentiation.

Our study shows that cortical neurons can be infected with a low dose of HSV, and can replicate the virus efficiently. Therefore, this model does resemble the infection in HSE patients where initial virus input to the brain is probably very low, but increases through replication. The absence of effect on cell viability after infection in cell culture of mature cortical neurons may again indicate that other cells than neurons need to be present to provide for cytopathogenic virus replication, as the neurons were devoid of complement activation and were less vulnerable to infection at this stage of differentiation.

Given the fact that HSV-2 infection in adults is associated with meningitis, while HSV-1 is associated with the more severe HSE, several factors must influence the virus preferences for site of infection, including glycosylation patterns on the host cell surface, which will be discussed in the next section. Why HSV-2 affects neonates more severely than adults is unclear and proper explanations are difficult to give. Furthermore, why HSV-2 after the neonatal period infects the meninges rather than the actual cerebrum, as HSV-1 does, is unclear, but could depend on the intrinsic, type-specific, properties of the two viruses during their replicative cycle.

4.4 The mucin-like region of glycoprotein C contributes to virus binding/entry and release of progeny virions from cell surface

HSV-1 and HSV-2 differ in their interactions with the cell surface attachment receptors known as GAGs [66]. These differences in virus-cell interactions are of potential importance for viral tropism and pathogenesis. To define the role of the mucin-like region of gC in attachment to and release from GAGs on the cell
membranes during viral entry and egress, the influence of the highly O-glycosylated mucin-like region on GAG-binding properties was studied in Paper IV. For this purpose, we utilized a previously defined mutant virus of HSV-1 KOS that lacked the mucin-like region (i.e. aa 33-116) after selection during repeated passages with the heparin analogue muparfostat (PI-88) and compared its GAG-binding properties with that of the parental strain [113].

Firstly, the reactivity with three gC-targeting antibodies (B1C1, C2H12 and C4H11) was tested for both the mutant and the wild-type virus, to establish that deletion of the mucin-like region had not extensively modified the conformation of the gC protein. The ELISA results showed no major alterations in reactivity and hence in conformation between the mutant and the wild-type virus. The specific viral infectivity was then calculated by dividing the number of viral DNA copies/ml (which reflects the total number of viral particles) by the infectious titre (pfu/ml; reflects the total number of infectious viral particles) in preparations of purified virus. Here it was found that the deletion of the mucin-like region affected the virus moderately, requiring around twice the amount of viral particles per infectious unit compared with the parental virus, i.e. one out of seventeen mutant virions and one out of ten wild-type virions were infectious. Altogether, these results indicated that while the conformation of gC is not greatly altered by a mutation in the mucin-like region, as compared with its wild-type strain, the mutation somehow affected the infectivity of the virus, indicating the importance of the mucin-like region in HSV-1 infectivity.

Using a cell culture assay, we then tested the effect of two compounds with antiviral properties (GAG-mimetic muparfostat and heparin), that function as inhibitors of virus-GAG interactions (Paper IV: Fig. 2). Here we found that the mutant virus required almost three times higher concentrations of the compounds, as compared with the wild-type virus, to reach the IC$_{50}$. In addition, even at the highest tested concentrations of the compounds, the infectivity of the mutant virus was less inhibited than that of wild-type HSV-1. The interference on the GAG-binding interactions of the virus by SA, which is a terminal sugar residue found on different viral glycoproteins including gC-1 and its mucin domain, was tested by treating the virus with neuraminidase. This enzymatic cleavage resulted in slightly reduced sensitivity to heparin (IC$_{50}$ of approximately 1 µg/mL) for the wild-type virus devoid of sialic acid as compared to untreated virus (IC$_{50}$ of approximately 0.5 µg/mL). In contrast, an increased sensitivity to heparin (but only at low concentrations) was observed after neuraminidase treatment of the mutant virus. Together, these results confirmed the importance of the mucin-like region of gC for modulation of its GAG-binding activities during viral attachment. Furthermore, mutations in the mucin-like region can alter the binding of gC to GAG-based inhibitors.
Next, the yield of newly produced infectious mutant and wild-type viruses was tested in cell cultures to determine the amount of infectious virus retained by the cell (cell-associated (CA) virus), and the amount of virus released from cells into culture medium (extracellular (EX) virus) (Paper IV: Fig. 3). Here, we found that the amount of CA virus produced by the mutant virus was approximately 2-5 times less than that of the wild-type virus, while the amount of EX virus was reduced by approximately 20-600 times for the mutant, depending on the time after infection when the comparison was made. These significant differences, especially in viral egress, could suggest that newly produced virions of the mutant virus, devoid of the mucin-like region, were trapped by GAG chains present on the surface of infected cells. To verify this assumption, cells infected with mutant virus or wild-type virus were examined by electron microscopy. Viral particles were found to be present on the surface of infected cells for both viruses, but for the mutant virus approximately two times more viral particles were retained on the cell surface (Paper IV: Fig. 4). Altogether, these data strongly supported the importance of the mucin-like region of gC both in attachment of HSV-1 to cells and in release of newly produced virions from the surface of infected cells.

To study the interactions between gC and GAGs on a glycoprotein level, particularly in relation to the mucin-like region, binding studies were performed using a surface-based assay. Here, using an SPR-based platform with GAGs biotinylated at the reducing end attached to the sensor surface (Paper IV: Fig. 1), the binding of gC molecules purified from the mutant and wild-type viruses to the GAGs was investigated. We found that both the mucin-like region-deleted mutant gC and the wild-type gC bound to sulphated GAGs (CS and sulphated HA), which indicated that the presence of the mucin-like region was not required for binding to GAGs per se (Paper IV: Fig. 5). This is not surprising, as the GAG-binding region of gC is situated downstream of the mucin-like region. In addition, neither the mutant nor the wild-type gC bound well to non-sulphated GAGs, confirming that the interaction between gC and GAG requires the presence of sulphated groups on the GAG molecule.

Using B1C1, a monoclonal antibody recognizing a region partly overlapping the HS and CS binding site on gC [117], a competitive assay was performed to study the nature of the gC-GAG interaction. When the glycoprotein was mixed with the antibody prior to the SPR-based experiments, B1C1 effectively reduced binding to sulphated GAGs for both the mutant and the wild-type gC. This confirmed that also in the present model, the interactions between gC and GAGs involved the previously mapped GAG-binding site (Paper IV: Fig. 6). Furthermore, we tested the contribution of electrostatic interactions for binding of gC to GAGs. For this, a hypertonic running buffer was used, where binding levels of the glycoprotein at saturation were compared with that of runs performed with
isotonic buffer. It was found that binding to CS was reduced almost equally for the mutant and the wild-type gC, while for sulphated HA, the reduction in binding was greater for the wild-type virus than for the mutant. These results indicated an important role for electrostatic interactions in binding of gC to GAGs, at least under the conditions of this experiment, while the deletion of the mucin-like region may decrease the sensitivity for gC to a hypertonic solution in binding to some GAGs. Also, treatment of virus with neuraminidase lead to decreased binding of the mutant gC to CS, while the binding of the wild-type gC increased (Paper IV: Fig. 6). In the cell-based experiments with viral particles, the desialylated virions were less dependent on the GAG-specific interactions for attachment and rather used other virus-cell interactions. This could not be observed in the SPR-based experiments with gC due to the lack of other receptor molecules than GAGs in that system.

Next, the binding affinities between gC and GAGs were determined for both the mutant and the wild-type gC (Paper IV: Fig. 7). Here, it was found that the mutant gC had a weak cooperative behaviour, but no such property was found for wild-type gC. This indicated that binding of one mutant gC molecule to a GAG chain will facilitate the binding of the next gC. More importantly, the affinity of mutant gC to sulphated GAGs was found to be lower than for wild-type gC. However, testing the dissociation of gC from sulphated GAGs, the complex between mutant gC and GAG, in contrast to the wild-type gC-GAG, was not found to dissociate under the conditions of the experiment (Paper IV: Fig. 8). Therefore, these results again confirmed what was observed in cell-culture experiments, that the mutant gC was less likely to bind to GAGs with the specificity observed for the wild-type gC. However, once the complex between gC and GAG is formed, the mutant gC-GAG complex is more stable, which agreed with the observations by electron microscopy showing enhanced trapping of mutant virus particles on the surface of infected cells (Paper IV, Fig. 4).

As SPR-based experiments only could be used to study the interactions on a glycoprotein lever, total internal reflection fluorescence (TIRF) microscopy was used to study the binding of the whole virus particle to GAGs (Paper IV: Fig. 9). In line with the data obtained using purified gC molecules, the association of the mutant virus particles to sulphated GAGs was found to be decreased as compared with the wild-type virus particles.

Overall, the findings in Paper IV demonstrated that the mucin-like region is important for the gC-GAG interactions both during attachment of virus to cell and during release of newly produced virions from the surface of infected cells. While virus with a deletion of the mucin-like region of gC can still bind to cells, the infectivity and the spread of virus is not as efficient. This could suggest that the modulatory interactions between cell surface GAGs and the mucin-like region of
gC may induce a conformational change in the GAG-binding site of gC to promote a well-balanced gC-GAG interaction of importance for specific HSV-1 attachment to cells and release of progeny virions from cell surface. However, other explanations such as steric hindrance and/or slight repulsion offered by the mucin region might contribute to the efficiency of full gC in GAG binding.

The experimental model in Paper IV did not include HS, the GAG that probably is the most important receptor in HSV-1 attachment to cells. However, it has been shown in previous studies that HS and CS bind to approximately the same region of gC [63] and that in the absence of HS, HSV-1 can utilize CS for attachment instead [62, 63]. Furthermore, the kinetic properties of gC have been assessed in a previous study [127], where a mucin-deleted virus with a larger deletion than our virus mutant was used. In this study, it was found that the mutant gC had lower affinity for HS as compared with the wild-type gC, while the complex between HS and mutant gC was less likely to dissociate than that with the wild-type gC. Both observations were in line with our findings. However, while we used full-length gC, where the transmembrane region was intact to preserve the native conformation of the protein, the other study used gC without the transmembrane region. This, in addition to the different GAGs used in the experiments, could explain some of the differences in affinity noted between the two studies. Recently, the mobility of HSV-1 particles on immobilized GAGs has been described [426], where it was demonstrated that both the degree of and pattern of sulphation on GAGs were associated with the movement of virus along the surface. This was hypothesised to resemble the in vivo movement during cellular infections, allowing the attached viral particles to search for the next receptor of the entry cascade. It was found that both HS and CS efficiently promoted lateral movement of HSV-1 particles in comparison to an artificially sulphated GAG in form of over-sulphated HA that rendered viral immobility after binding. Hence, we suggest that the burst-like pattern of sulphated stretches on natural cell surface GAGs such as CS and HS is more important for mobility than the actual degree of sulphation.

The reason for using KOS, a laboratory strain, for preparation of the mutant virus was that the more neurovirulent virus 2762 did not produce a mutant deficient in the mucin-like region after repeated passages with muparfostat. However, the mutant virus used in this study will be tested in the animal model presented in Paper I. Despite the lower neurovirulence of the KOS strain, it would be of interest to investigate whether and how the differences in GAG interaction found both for mutant gC and mutant whole virions in vitro, would modulate the viral experimental infection in the more complex in vivo conditions in the presence of immune responses and several other binding molecules. The increased complement activity in the acute infection of HSE patients, as described in Paper
II, will probably also be present in the animal model, and therefore it might be difficult to distinguish the GAG-related activities from other functions of the mucin-like region of gC-1 in vivo. Complement component C3b can use gC-1 as a receptor and the mucin-like region can sterically hinder binding of properdin and C5 to C3b [287]. Therefore, in an in vivo condition, the mutant virus may appear to be more susceptible to complement degradation. The GAG-binding and C3b-binding regions of gC-1 overlap, as has been demonstrated by the mab B1C1 that blocks binding of both GAGs and C3b to gC [125].

In the differentiating neuronal cell model (Paper III), we found no extensive complement activity in the cell cultures. This suggests that the differentiating cells can be infected with HSV-1 where the mucin-like region of gC has been deleted, without any impact from this immune activity. In the absence of complement activity such as in the differentiating neuronal cell culture model, infection of cells with this mutant virus may help to elucidate the susceptibility to HSV-1 of neurons at different stages of differentiation and the role of GAGs in this process.

For HSV-1, the similarities in gC binding to complement components on one hand and GAGs on the other hand, as has been in focus of this thesis, may not be of utmost interest. Rather, the subtle differences between these interactions may be of interest in the future, where numerous questions are yet unanswered. Why does the virus use the same glycoprotein for blocking complement activity and for attachment to cells? Binding of C3b to gC-1 may impair the attachment to cells, as the GAG-binding region overlaps with the C3b-binding region. Can the complement components enter the cell upon infection along with the virus? For non-enveloped viruses, it was recently demonstrated that C3 can be carried into the cell through covalent attachment to the virus [427]. Is there an unknown effect of complement components intracellularly? For the intracellular C3-transfer with virus, cell autonomous immunity was activated [427]. This could possibly indicate that if complement components are transferred intracellularly with HSV-1 in neurons, both autophagy and other cell-autonomous immune functions, such as the proteasome, could be activated to protect the neuron.
5. Concluding remarks and future perspectives

The symptoms and sequelae of HSE and other alphaherpesvirus CNS infections have been well described in the literature. However, less is known of how the virus enters the CNS and finds its way to the specific locations where the infection is seen in MRI or CT scan. This thesis provides two models for studying alphaherpesvirus CNS infections – one rat model for spread of the virus in HSE, where a previously unreported route – through the AC – for transportation of HSV-1 has been suggested, and one stem cell-derived model for infection of cells at different stages of differentiation towards cortical neurons. These models may be of importance in a research area where accurate models are scarce.

CNS infections due to alphaherpesviruses have been demonstrated to be complex in their pathogenicity, and brain lesions are most likely caused by a combination of viral factors, cell properties and immune activities. No pathogenetic factor can be selected as the single most important, but these infections may rather arise from a series of unfortunate combinations of events.

The importance of the mucin-like region for viral attachment and release from cells provides a new target for antiviral treatment, where entry, egress and cell-to-cell spread of virus can be reduced. Moreover, without a functioning immune system, mitotic cells are more vulnerable to infection than are post-mitotic cells indicating the contribution of the immune system to the damage in CNS besides the actual effect of the viral infection.

The results presented in this thesis indicate that the innate immune response plays an important role in the acute and late stages of HSE. This implies that antiviral treatment alone is not sufficient to treat the CNS infection; a combined approach where immunomodulating treatment is used as complementary medication might be needed. Further research of prolonged innate immune responses in CNS is required, as this treatment approach might be valid, not only for HSE but also for other CNS infections with long-lasting sequelae. The findings of long-term complement activity in HSE patients can contribute to future evolvement in therapy and may influence new treatment recommendations.

Finally, although viral CNS infections are rare, their severity should not be neglected. With the increasing threat of CNS infections caused by arboviruses, such as TBE virus, Dengue virus, West Nile virus, and most recently Zika virus, the need for appropriate cell and animal models to study these infections, as well as methods for evaluating the immune response evoked, is constantly growing. Although findings presented in this thesis are related to the pathogenesis of
alphaherpesviruses, they can hopefully contribute to future research on other viral CNS infections.
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