

# Human Herpesvirus 6 (HHV-6) Induces Dysregulation of Glutamate Uptake and Transporter Expression in Astrocytes

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**Abstract** Human herpesvirus 6 (HHV-6) infects and establishes latency in the central nervous system (CNS). Reactivation of latent HHV-6 has been associated with neurologic diseases including epilepsy and multiple sclerosis (MS). In vivo, HHV-6 has been localized to astrocytes and can infect human astrocytes in vitro, suggesting that this virus may have a tropism for glial cells and may affect glial cell function. An essential role of astrocytes in the CNS is active maintenance of the excitatory neurotransmitter glutamate. Dysregulation of glutamate has been implicated as a potential mechanism of disease in both epilepsy and MS. Both disorders have demonstrated elevated glutamate in CSF and may be associated with dysregulation of glutamate signaling, uptake, and metabolism. This study demonstrates dysregulation of glutamate uptake in human astrocytes infected with both variants of HHV-6, A and B, with differential effects of HHV-6 in acute and persistently infected cells. Whereas astrocytes acutely infected with HHV-6 demonstrated increased glutamate uptake, cells persistently infected with HHV-6A and HHV-6B demonstrated impaired glutamate uptake. Functional dysregulation of glutamate uptake was associated with early increases in mRNA and protein expression of the glial glutamate transporter EAAT-2 followed by a sustained decrease in

mRNA expression in astrocytes infected with both HHV-6A and HHV-6B. Dysregulated glutamate uptake and transporter expression suggests a mechanism for dysregulation of glutamate levels in vivo and a potential mechanism for virus-associated neurologic disease.

**Keywords** human herpesvirus 6 · neurologic disease · human astrocytes

## Introduction

Human herpesvirus 6 (HHV-6) is a ubiquitous  $\beta$ -herpesvirus originally discovered in patients with lymphoproliferative disorders (Salahuddin et al. 1986). Exposure to HHV-6 normally occurs within the first 2 years of life, after which the virus establishes life-long latency. One site of HHV-6 latency is the central nervous system (CNS), and reactivation of the virus can occur after bone marrow transplant, particularly in association with immunosuppression (Fotheringham et al. 2007a). HHV-6 has been detected in brain material from patients with neurological disorders including multiple sclerosis (MS) and epilepsy (Cermelli et al. 2003; Challoner et al. 1995; Donati et al. 2003; Goodman et al. 2003). Although HHV-6 DNA can be found at low frequency in normal brains (Chan et al. 2001; Cuomo et al. 2001; Opsahl and Kennedy 2005) and may be a commensal pathogen in the CNS, reactivation associated with these disorders may contribute to disease pathology.

HHV-6 exists as two distinct variants, HHV-6A and HHV-6B, which differ in nucleotide sequence, cellular tropism, antigenicity, and etiology (De Bolle et al. 2005a). HHV-6B is the causative agent of the childhood febrile illness exanthem subitum (roseola) (Yamanishi et al. 1988). Although both variants of HHV-6 have been detected in

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human brain, MS has most often been associated with variant A (Akhyani et al. 2000; Alvarez-Lafuente et al. 2004, 2002; Fogdell-Hahn et al. 2005), whereas a subset of patients with mesial temporal lobe epilepsy (MTLE) have demonstrated exclusive detection of HHV-6B (Donati et al. 2003; Fotheringham et al. 2007b). A common mechanism that links these divergent diseases with HHV-6 is the observation that HHV-6 demonstrates tropism for astrocytes. Both HHV-6A and HHV-6B can infect primary astrocytes and glioma cells in vitro, although the two variants demonstrate different growth characteristics (Ahlqvist et al. 2005; Akhyani et al. 2006; De Bolle et al. 2005b; Donati et al. 2005; He et al. 1996; Yao et al. 2006). Expression of viral antigen in MS, MTLE, and post-bone marrow transplant brain can be detected in reactive astrocytes (Challoner et al. 1995; Donati et al. 2003; Fotheringham et al. 2007a; Fotheringham et al. 2007b; Goodman et al. 2003), and primary isolates of HHV-6 have recently been propagated in astrocytes cultured from freshly resected MTLE tissue (Fotheringham et al. 2007b). Infection of astrocytes with a large DNA virus like HHV-6 that codes for approximately 100 genes of known and unknown function (De Bolle et al. 2005a) raises the hypothesis that HHV-6 could encode multiple viral proteins that may affect the function of infected glial cells and thereby contribute to disease pathology.

Long thought to be strictly the scaffold or support cells of the brain, astrocytes are now known to be actively involved in intercellular communication and important regulators of brain function (Araque et al. 1999; Danbolt 2001; Matute et al. 2006). The active interaction between astrocytes and neurons regulates synaptic transmission, and alterations in this cell–cell relationship may contribute to CNS dysfunction. Astrocytes are crucial for maintenance of extracellular glutamate levels, the main excitatory neurotransmitter in the brain (Danbolt 2001). Active uptake of glutamate, intracellular metabolism, and release of glutamate and glutamine by astrocytes serve to preserve low extracellular glutamate levels, thereby protecting healthy cells from excitotoxicity (Danbolt 2001). Maintenance of extracellular glutamate is mediated primarily by the high-affinity, sodium-dependent glial glutamate transporters EAAT-1 and EAAT-2 (Danbolt 2001). EAAT-2 is the most abundant transporter expressed on astrocytes and is thought to mediate the majority of glutamate uptake in the CNS (Tanaka et al. 1997). A role for astrocytes and regulation of glutamate has been implicated in the generation of seizure (Cavus et al. 2005; de Lanerolle and Lee 2005; During and Spencer 1993; Tian et al. 2005). In addition, mutation or alternative splicing of glutamate transporters has been suggested to dysregulate glutamate uptake in epilepsy and promote the generation of seizures (Hoogland et al. 2004; Jen et al. 2005).

Regulation of glutamate, the major excitatory neurotransmitter in the brain, is associated with proper CNS function and is a primary function of astrocytes. Consequently, in this study, we examined the infection of primary human astrocytes with HHV-6 variants A and B and determined functional changes in glutamate uptake and changes in glutamate transporter expression. Infection of astrocytes with HHV-6A and HHV-6B induced dysregulation of glutamate uptake at acute and chronic time points and was associated with decreased expression of the glutamate transporter EAAT-2.

## Materials and methods

### Cell culture and viral infections

Fresh human brain tissue was obtained from NIH and Children's National Medical Center (Washington, DC) under protocols approved by the respective institutional review boards. Tissue was kept on ice in Hibernate A media (Brain Bits, Springfield, IL, USA) containing 2% B27 supplement, 0.5 mM glutamine, 1% penicillin/streptomycin, and 1:1,000 fungizone until processing. Tissue was dissociated manually with a razor blade and enzyme digested in Earl's balanced salt solution containing 0.1% DNase and 20 U/ml papain at 37°C for 1 h. Dissociated tissue was centrifuged at 1,500 rpm for 10 min and mechanically dissociated using a sterile glass pipette. After passing through a 40- $\mu$ m filter, the single cell suspension was separated on a percoll gradient by centrifugation at 15,000 rpm for 30 min. The glial cell layer was removed, washed, and plated on poly-L-lysine coated tissue culture flasks in Dulbecco's modified Eagle's medium/F12 supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 0.1% gentimycin. Fresh media was added daily until cell debris was completely removed. Purity of adult astrocyte cultures was confirmed by immunofluorescence staining for glial fibrillary acidic protein (GFAP) and cells were infected in six-well plates with HHV-6 at a density of  $2.5 \times 10^4$  cells/ml.

Primary human astrocytes and U251 astrogloma cells were infected with both variants of HHV-6, A and B. Briefly, cell cultures were incubated for 3 h with cell-free supernatants from T cells infected with HHV-6A (U1102 strain) or 6B (Z29 strain) (inoculum  $10^9$  viral copies/ $10^6$  cells), as described previously (Ahlqvist et al. 2005), or with cell-free supernatants first passed through a 0.1- $\mu$ m filter. Cells were washed three times with sterile phosphate-buffered saline (PBS) and fresh growth media was added. Infections were monitored for cytopathic effect and postinfection for viral load with quantitative polymerase chain reaction (PCR) and for viral RNA by reverse

transcriptase (RT) PCR. As demonstrated in previous studies, infection with HHV-6A induced cytopathic effect early during infection (Ahlqvist et al. 2005; Donati et al. 2005), and both HHV-6A- and HHV-6B-infected cells could be maintained and propagated in culture for 60 days (Ahlqvist et al. 2005).

#### Quantitative TaqMan PCR

DNA was extracted using the QIamp viral RNA kit for cell-free supernatants (Qiagen, Valencia, CA, USA) and the DNeasy kit (Qiagen) for cell pellets according to manufacturer's instructions. DNA from samples and controls was amplified using TaqMan technology on an ABI PRISM 7700 Sequence Detector System (Perkin Elmer, Waltham, MA, USA). Specific primers and probes for HHV6-A and HHV6-B and plasmids have been described (Nitsche et al. 2001). Results from cell lysates were normalized to  $\beta$ -actin.

#### Immunofluorescence

Astrocytes were grown on four-well chamber slides and fixed at  $-20^{\circ}\text{C}$  for 10 min in acetone/methanol (1:1). Slides were blocked for 10 min at room temperature with 3% BSA prepared in PBS. Primary antibody dilutions were prepared in PBS (1:100 mouse anti-gp116/54/64 (Advanced Biotechnologies, Columbia, MD, USA), 1:100 rabbit anti-GFAP (DAKO, Carpinteria, CA, USA), 1:40 mouse anti-HHV-6A immediate early 2 (IE2), a generous gift from Dr. Louis Flamand, and incubated on slides for 30 min at  $37^{\circ}\text{C}$  or for 1 h at room temperature. Slides were washed three times in PBS and incubated with secondary antibody [1:100 anti-rabbit IgG1 FITC, 1:100 anti-mouse IgG1 rhodamine, or 1:1,000 anti-mouse IgG2b rhodamine (Molecular Probes, Eugene, OR, USA)] prepared in PBS for 30 min at  $37^{\circ}\text{C}$  or for 1 h at room temperature. Slides were mounted with mounting media containing DAPI and were visualized using a fluorescence microscope (Carl Zeiss Microimaging, Thornwood, NY, USA) at  $20\times$  magnification.

#### Glutamate uptake

Astrocyte cultures were washed three times in warm Krebs's buffer (119 mM NaCl, 4.7 mM KCl, 1.2 mM  $\text{KH}_2\text{PO}_4$ , 25 mM  $\text{NaHCO}_3$ , 2.5 mM  $\text{CaCl}_2$ , 1.0 mM  $\text{MgCl}_2$ , 5.5 mM glucose, pH 7.2) and then incubated for 15 min with 2.5  $\mu\text{M}$  glutamate (0.5  $\mu\text{Ci}/\text{well}$ ) in Krebs's buffer at  $37^{\circ}\text{C}$ . Glutamate uptake was terminated by addition of ice-cold Krebs's buffer, and excess extracellular glutamate was removed by washing cells three times in ice-cold Krebs's. Cells were lysed overnight at room temperature in 0.25 mM NaOH containing 0.1% Triton  $\times 100$ . Cell lysates were harvested and radioactivity was determined using a Beta

counter. Protein concentration was determined using the detergent compatible protein assay from BioRad (Hercules, CA, USA) according to the manufacturer's instructions.

#### RT-PCR and TaqMan

RNA for RT-PCR and TaqMan was isolated from cell pellets using the RNeasy Plus kit as per manufacturer's instructions (Qiagen). The RNeasy Plus kit includes a spin step to remove genomic DNA, eliminating the need for DNase treatment of isolated RNA. RNA was reverse-transcribed using the First Strand Superscript III cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA), as per manufacturer's instructions, using random hexamer primers and 8  $\mu\text{l}$  of purified RNA. Two microliters of cDNA was PCR amplified for EAAT-1, EAAT-2, U12, U16/17, or actin (Table 1) using the Taq PCR master mix kit from Qiagen. RT-PCR products were run on 1.5% agarose gels and visualized with ethidium bromide staining.

Two microliters of cDNA from samples and controls was amplified for EAAT-2 expression using TaqMan technology on an ABI PRISM 7700 Sequence Detector System (Perkin Elmer). Specific primers and probes for EAAT-2 were custom synthesized by Synthegen, Houston, TX, USA (sense, CTCCTCATTCTGACAGCCGTG; antisense, CCCACAACATTGACTGAAGTTCTC; FAM/TAMRA probe, TGTCCAGCAGCCAGTCCACAGCCA). Results from cell lysates were normalized to expression of HPRT.

## Results

*HHV-6A actively infects primary human astrocytes* To characterize the infection of HHV-6A and HHV-6B in primary human astrocytes, viral loads in cell lysates and supernatant were quantitated by variant-specific real-time PCR at an early time point representative of acute infection

**Table 1** Primer sequences for glutamate transporter and HHV-6 viral gene expression

	Primer sequence	Product size (bp)
EAAT-1 F	CATGCACAGAGAAGGCCAAAA	498
EAAT-1 R	GTCACGGTGTACATGGCAAG	
EAAT-2 F	GGCAACTGGGGATGTACA	836
EAAT-2 R	ACGCTGGGGAGTTTATTCAAGAAT	702
U12 F	CACTGTCATTGAGCTGTCCAA	327
U12 R	ACCACATGAGCACAAAATCG	202
U16/17 F	CCTCTCCCCAGACAGAAAACA	240
U16/17 R	TTAATTGGCCGAAAAGTCCG	147
Actin F	TGGTGGGCATGGGTCAGAAG	410
Actin R	GTCCCGCCAGCCAGGTCCAG	

(e.g., day 5) and a later time point representative of a chronic infection (e.g., day 25). In primary human astrocytes, viral load in cell lysates from HHV-6A-infected cells increased from  $1.19 \times 10^8$  copies/cell at day 5 to  $4.87 \times 10^8$  copies/cell at day 25, suggesting active HHV-6A replication. This observation is consistent with a previous report demonstrating active infection and viral mRNA expression in primary human astrocytes infected with HHV-6A (Yao et al. 2006). Viral load in supernatant decreased from  $4.55 \times 10^5$  copies/ml at day 5 to undetectable levels at day 25 (Fig. 1a), indicating that, by day 25 postinfection, viral particles were not being released from HHV-6A-infected cells. In contrast to astrocytes infected with HHV-6A, primary astrocytes infected with HHV-6B demonstrated significantly lower viral loads in cell lysate and supernatant that decreased over time, suggesting HHV-6B-infection of primary astrocytes was nonproductive (Fig. 1a). This observation is supported by HHV-6B infection of an *in vitro* glioma U251 cell line, which demonstrated HHV-6B mRNA transcripts at days 1–4 that were no longer detectable by day 6 (Fig. 2a).

*Glutamate uptake is dysregulated in primary human astrocytes infected with HHV-6* To determine whether infection of human astrocytes with HHV-6 was associated with functional changes in glutamate uptake, we measured uptake of  $^3\text{H}$ -glutamate during early (day 5) and late (day 25) infection. In primary human astrocytes, infected with HHV-6A and HHV-6B, glutamate uptake was higher compared to mock during the early stages of infection (106.9 and 94.2% increase compared to mock, respectively; Fig. 1b). At later stages of infection, however, glutamate uptake in both HHV-6A- and HHV-6B-infected cells was decreased compared to uptake in mock-infected cells (62.2 and 45.3% lower than mock, respectively; Fig. 1b), suggesting a differential effect on uptake in early vs. late HHV-6 infection.

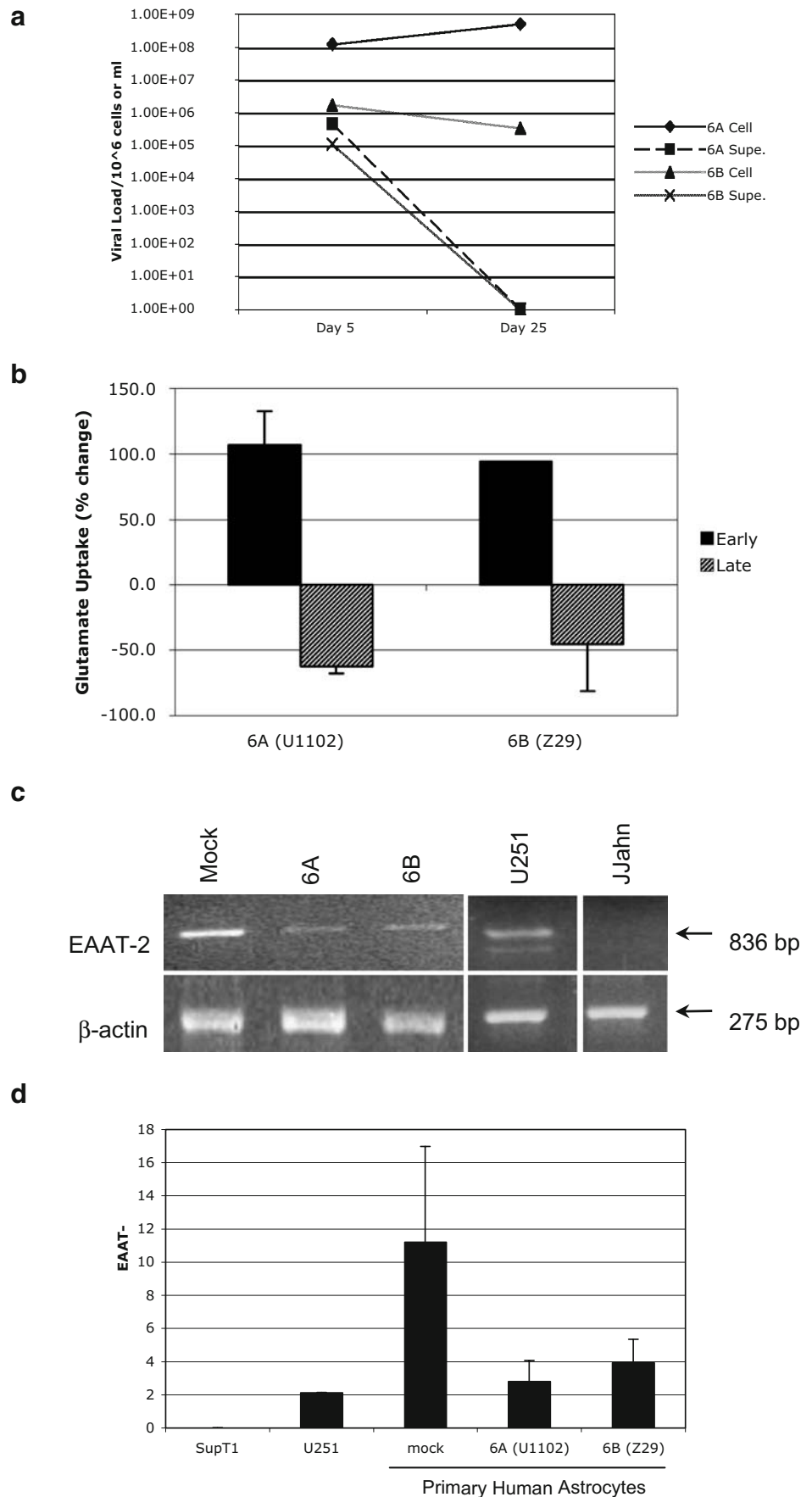
*Glutamate transporter expression is decreased in HHV-6 infected primary human astrocytes* To determine a mechanism by which HHV-6-induced dysregulation of glutamate uptake might occur, we measured mRNA expression of the glial glutamate transporters EAAT-1 and EAAT-2. As a positive control, U251 glioma cells expressed mRNA for both transporters, and also expressed the alternatively spliced variant of EAAT-2 (702-bp band), which was not expressed by uninfected (mock) primary astrocytes (Fig. 1c). JJahn T cells did not express mRNA for either transporter and served as a negative control (Fig. 1c,d). HHV-6A- and HHV-6B-infected U251 did not demonstrate any detectable change in EAAT-1 mRNA expression at day 5 postinfection (data not shown). Surprisingly, mRNA levels of EAAT-2 were decreased at day 5 postinfection in

both HHV-6A and HHV-6B infected astrocytes at the same time point that glutamate uptake was increased (Fig. 1b). The observation that glutamate uptake and EAAT-2 expression was discordant suggests that HHV-6-induced increases in glutamate uptake may occur through a different mechanism than decreased EAAT-2. Decreased expression of EAAT-2 mRNA in primary human astrocytes at day 5 postinfection with HHV-6A and 6B was confirmed by quantitative real-time PCR demonstrating a 4.0- and 2.9-fold decrease, respectively (Fig. 1d).

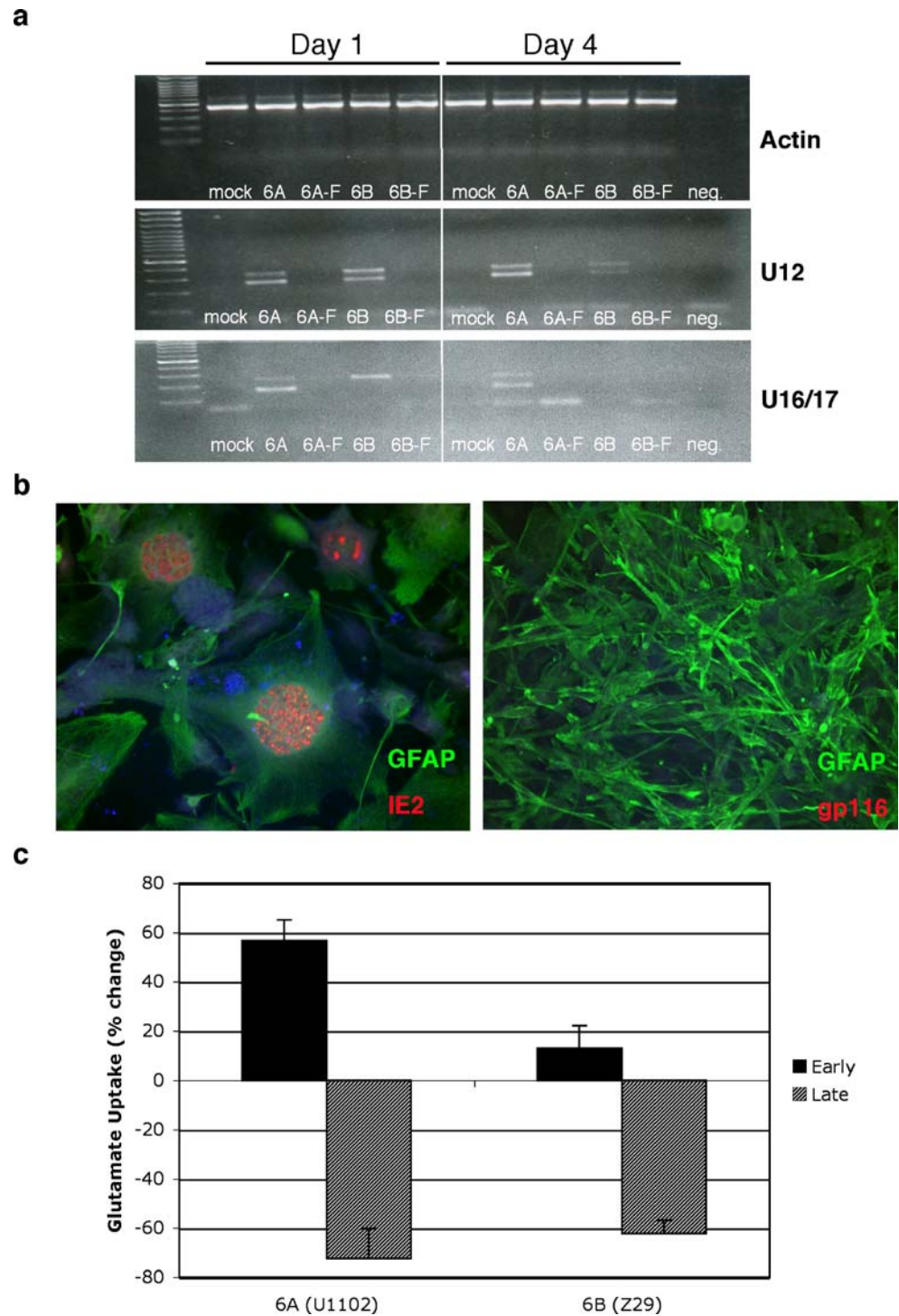
*HHV-6 actively infects U251 glioma cells* Because HHV-6A and HHV-6B infection of primary astrocytes had comparable effects on glutamate uptake even though HHV-6B infection appeared to be nonproductive, we investigated whether the effect on glutamate uptake was directly viral or caused by a virus-induced soluble factor (e.g., cytokines). Primary human astrocytes derived from a single brain are difficult to propagate in large quantities and grow slowly, and insufficient quantities are available to extensively investigate. As a result, we utilized the established model system of GFAP-positive U251 glioma cells that has been used to study HHV-6 (Akhyani et al. 2006; Yoshikawa et al. 2002) to confirm that the characteristics of HHV-6 infection and dysregulation of glutamate would mimic our observations in primary astrocytes.

To determine whether U251 astrocytes were actively infected with HHV-6, mRNA expression for the HHV-6 immediate/early genes U12 and U16/17 was measured by RT-PCR at days 1 and 4 postinfection. U251 astrocytes infected with HHV-6A and HHV-6B inocula expressed mRNA for U12 at days 1 and 4 (nonspliced transcript 327 bp; spliced transcript 202 bp), whereas only HHV-6A-infected cells expressed the spliced mRNA transcript for U16 (nonspliced transcript 240 bp; spliced transcript 147 bp) at days 1 and 4 (Fig. 2a). Expression of the spliced transcript suggests that U251 were actively infected with HHV-6A, consistent with our observation of active HHV-6A infection in primary human astrocytes. HHV-6B-infected cells expressed the nonspliced mRNA transcript for U16/17 at day 1 that was barely detectable by day 4, suggesting that U16/17 was expressed but not spliced. Expression of a nonspliced transcript is suggestive of a nonproductive infection. These results are consistent with the observation in primary human astrocytes that HHV-6A establishes an active infection, whereas infection with HHV-6B is nonproductive (Fig. 1a). Difference in viral gene expression in HHV-6A- and HHV-6B-infected glial cells suggests that infection with variant A may be more productive, as has been previously suggested (Ahlgqvist et al. 2005; Donati et al. 2005; Yao et al. 2006). Although HHV-6B can infect U251 astrocytes and induces expression of at least one viral gene, viral replication may be incomplete.

**Fig. 1** HHV-6 infects and dysregulates glutamate uptake in primary human astrocytes. Primary human astrocytes were mock-infected or were infected with HHV-6A (U1102) and HHV-6B (Z29) for 5 days. **a** Viral load in primary human astrocytes infected with HHV-6A or HHV-6B was measured by variant-specific TaqMan PCR 5 and 25 days postinfection. Viral load was quantitated as viral copies per  $10^6$  cells in cell lysates or as viral copies per milliliter in cell culture supernatant. **b** Primary human astrocytes were infected with mock, HHV-6A, or HHV-6B, and uptake of  $^3\text{H}$ -glutamate was determined during early (day 5) and late (days 25–44) infection. Glutamate uptake is expressed as percent change compared to mock ( $n=3$ ). **c** Semiquantitative expression of EAAT-2 was determined by RT-PCR in HHV-6A- and HHV-6B-infected cells at day 5. EAAT-2 and actin PCR product sizes are indicated by *black arrows*. **d** EAAT-2 expression was confirmed by quantitative real-time PCR. U251 glioma cells served as positive controls for both assays, the T lymphocyte cell lines JJahn and SupT1 served as negative controls.



**Fig. 2** HHV-6 infects and dysregulates glutamate uptake in U251 glioma cells. **a** mRNA expression for the HHV-6 immediate/early genes U12 and U16/17 was measured by RT-PCR in U251 glioma cells infected with filtered (6A-F, 6B-F) and nonfiltered HHV-6A and HHV-6B inocula at 4 days postinfection. Band sizes for actin, U12, and U16/17 PCR products are noted in Table 1. **b** Expression of HHV-6 viral protein in U251 glioma cells was determined 5 days postinfection with the HHV-6A variant specific antibody towards IE2 (red) and the nonvariant specific antibody towards gp116/54/64 (red). HHV-6 viral protein staining was colocalized with the astrocyte specific marker GFAP (green). Images are taken using a 20× objective. **c** Glutamate uptake was determined in U251 glioma cells infected with HHV-6A and HHV-6B during early (day 5) and late (day 32) infection. Glutamate uptake is represented as percent change compared to mock infection ( $n=4$ ).

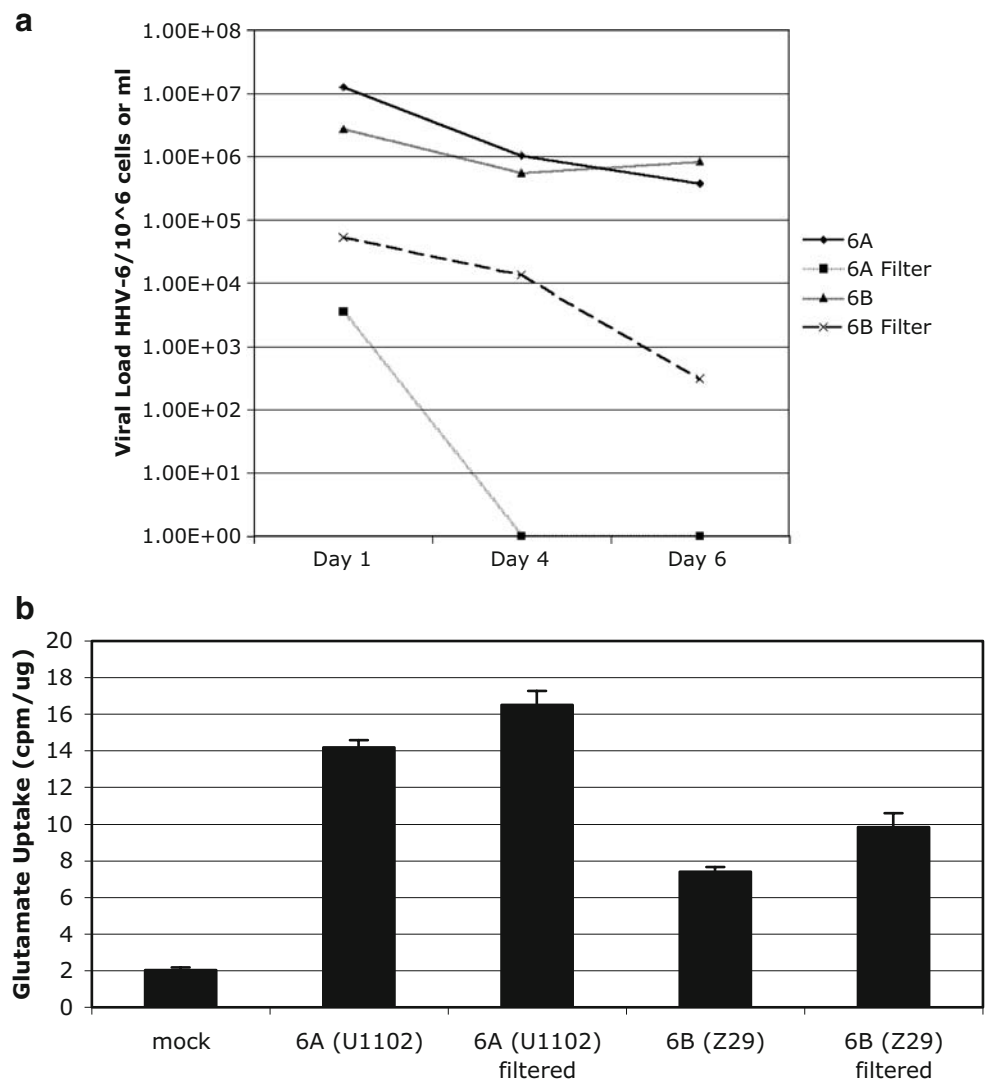


Finally, we determined examined viral protein expression of the immediate/early protein IE2 and the late HHV-6 glycoprotein gp116/54/64. As shown in Fig. 2b, U251 cells infected with HHV-6A for 5 days demonstrated punctate staining for IE2 localized to the nucleus of cells that were visibly enlarged (Fig. 2b). U251 cells infected with HHV-6B, however, did not demonstrate any staining for gp116/54/64 or any morphological changes indicative of active

infection, such as enlargement or syncytia formation (Fig. 2b). Expression of HHV-6 genes in infected U251 cells confirmed our finding in primary astrocytes that HHV-6A infection is more productive than HHV-6B infection.

*Glutamate uptake is dysregulated in HHV-6 infected U251 glioma cells* When glutamate uptake was compared in HHV-6A- and HHV-6B-infected U251 glioma cells during

**Fig. 3** HHV-6 Induced Dysregulation of Glutamate Uptake is Associated With a Virus-Induced Soluble Factor. U251 glioma cells were mock infected or were infected with filtered and nonfiltered HHV-6A (U1102) and HHV-6B (Z29) inocula. **a** Viral load was measured by variant-specific TaqMan PCR at 1, 4, and 6 days postinfection. Viral load was quantitated as viral copies per  $10^6$  cells. **b** Glutamate uptake determined at day 4 postinfection is represented as percent change compared to mock (representative experiment).



early (e.g., day 4) and late infection (e.g., day 32), the results mirrored those observed for HHV-6-infected primary human astrocytes (Fig. 1b). As shown in Fig. 2c, early infection with HHV-6A, and to a lesser extent, HHV-6B, increased glutamate uptake compared to mock-infected cells. Both HHV-6A and HHV-6B infected U251 showed decreased glutamate uptake compared to mock-infected cells during late infection (e.g., day 32). These data demonstrate that HHV-6 infection of primary human astrocytes and U251 glioma cells leads to increased glutamate uptake early in infection and decreased glutamate uptake later in infection. Collectively, these results demonstrate that HHV-6-infected U251 glioma cells are a faithful representation of virus-infected primary human astrocytes.

*HHV-6-induced dysregulation of glutamate uptake involves a viral-induced soluble factor* Because HHV-6A and HHV-6B infection of primary astrocytes and U251 cells dysregu-

lated glutamate uptake even though both cell types demonstrated nonproductive infection with HHV-6B and productive infection with HHV-6A, we investigated whether the changes in glutamate uptake could be associated with a virus-induced soluble factor rather than a direct viral effect. HHV-6 virions are approximately 160 nm in diameter, and filtration of HHV-6 inocula through a 0.1- $\mu$ m filter should eliminate infectious virus while allowing small, soluble factors to pass through. U251 glioma cells were infected with HHV-6A and HHV-6B that was filtered or nonfiltered, and viral load was determined over time. As expected, there was a significant decrease in viral load from days 1 to 6 in cells infected with both HHV-6A and HHV-6B filtered inocula, demonstrating that filtration eliminated a significant amount of infectious virus (Fig. 3a). To verify this observation, mRNA expression of the HHV-6 immediate/early genes U12 and U16/17 was determined in U251 cells infected with filtered inocula at days 1 and 4 postinfection.

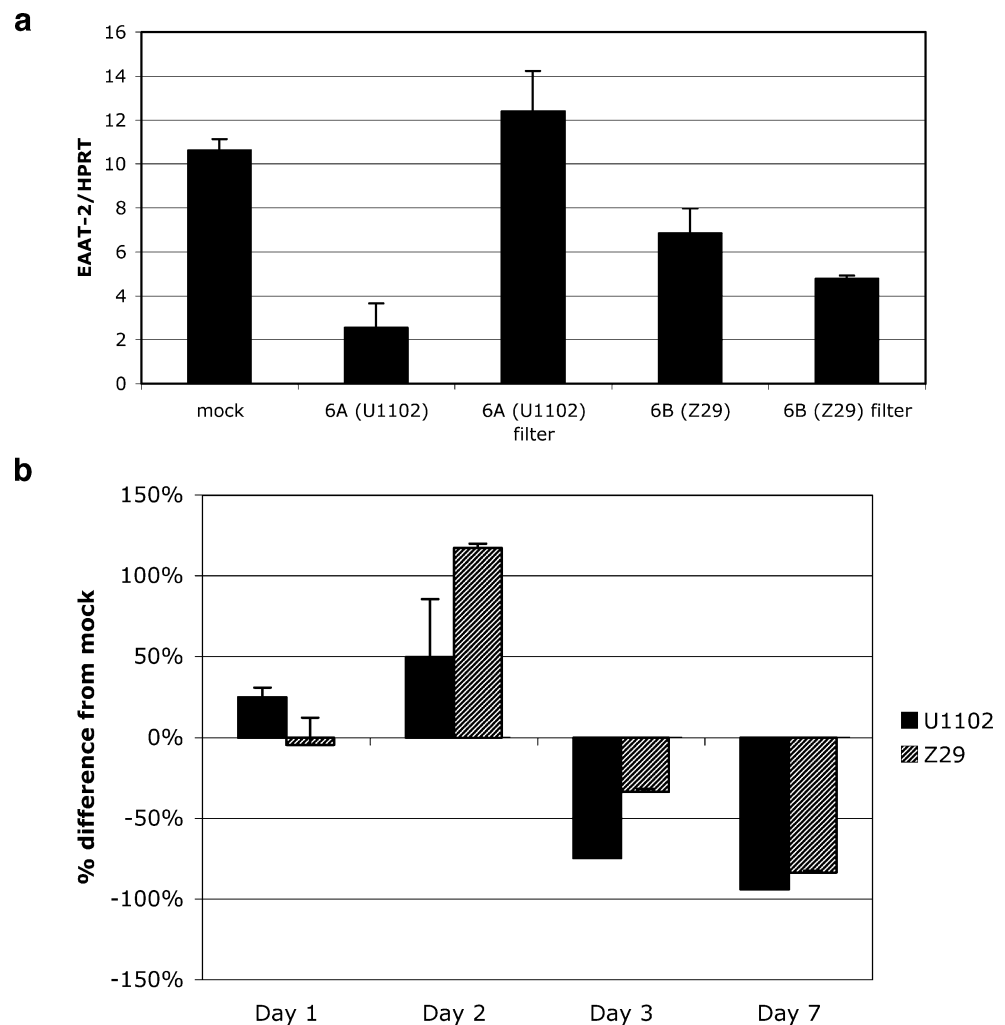
U251 cells infected with filtered HHV-6A or HHV-6B inocula did not express either viral gene, confirming that filtration of HHV-6 removed infectious virus (Fig. 2b).

As HHV-6 infection of primary human astrocytes and U251 glioma cells increased glutamate uptake, we investigated whether this effect was directly viral or associated with a virus-induced soluble factor. As shown in Fig. 3b, infection of U251 astrocytes with HHV-6A and HHV-6B for 4 days demonstrated increased glutamate uptake ( $p < 0.001$ ), again recapitulating the results observed with HHV-6-infected primary astrocytes (Fig. 1b). However, this increase in glutamate uptake was not affected by filtration of HHV-6 inocula (Fig. 3b;  $p < 0.001$ ), suggesting that, during early infection, increased glutamate uptake was associated with an HHV-6-induced soluble factor and may not be directly related to an active virus infection.

*HHV-6 infection of U251 glioma cells dysregulates glutamate transporter expression* To determine if HHV-6 infection of U251 glioma cells dysregulated EAAT-1 or EAAT-2

expression, glutamate transporter expression was measured in U251 infected with nonfiltered and filtered inocula at day 4 postinfection. RT-PCR determination of EAAT-1 mRNA demonstrated no detectable changes in HHV-6A- or HHV-6B-infected U251 glioma cells (data not shown). However, real-time PCR determination of EAAT-2 mRNA demonstrated decreased expression following infection with non-filtered HHV-6A or HHV-6B inocula (Fig. 4a;  $p < 0.05$ ), confirmed by RT-PCR (data not shown), consistent with the observation in primary human astrocytes (Fig. 1c,d). Filtration of HHV-6A inocula significantly reversed the decrease in EAAT-2 mRNA expression (Fig. 4a;  $p < 0.05$ ), suggesting that the effect of HHV-6A on EAAT-2 expression at day 4 was directly related to virus infection and not due to a virus-induced soluble factor. By contrast, filtration of HHV-6B did not reverse the decrease in EAAT-2 mRNA expression, suggesting that, unlike the observation with HHV-6A, the HHV-6B-induced decrease in EAAT-2 mRNA is associated with a soluble factor (Fig. 4a). The dysregulation of glutamate uptake and transporter expression

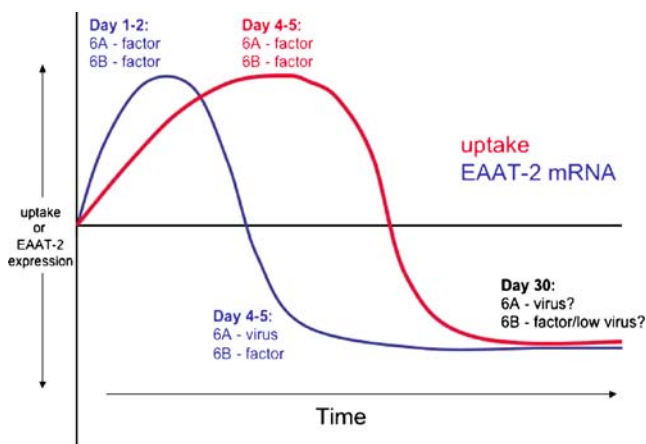
**Fig. 4** HHV-6 induces an early increase in EAAT-2 expression. U251 glioma cells were mock infected or infected for 4 days with filtered and nonfiltered HHV-6A (U1102) and HHV-6B (Z29) inocula. **a** EAAT-2 expression was determined by quantitative real-time PCR. **b** EAAT-2 expression was determined by quantitative real-time PCR at days 1, 2, 3, and 7 postinfection. Data are expressed as percent difference from levels of EAAT-2 expression in mock-infected cells.





(Figs. 3b and 4a) by an HHV-6B-induced soluble factor is consistent with the observation that HHV-6B is associated with a nonproductive infection in primary astrocytes and U251 cells (Figs. 1a and 2a,b).

Because infection of primary astrocytes and U251 glioma cells with HHV-6 increased glutamate uptake during early infection, at a time when mRNA levels for EAAT-2 were low, we investigated whether increased glutamate uptake was related to EAAT-2 expression. As shown in Fig. 4b, EAAT-2 mRNA expression was increased at day 2 in both HHV-6A- and HHV-6B-infected U251 cells compared to mock and would be consistent with the increase in glutamate uptake observed in HHV-6-infected primary astrocytes (Fig. 1b) and U251 cells (Fig. 2c) at days 4–5. The increase in mRNA levels at days 1 and 2 is likely associated with a virus-induced soluble factor because HHV-6A viral antigen expression, indicating full viral replication, is not detectable until day 5 (Fig. 2b) and infection with HHV-6B is nonproductive. By day 3, EAAT-2 mRNA levels begin to decrease following both HHV-6A and HHV-6B infection (Fig. 4b). This decrease in EAAT-2 transcript by day 3 is consistent with the decreases previously detected in primary astrocytes and U251 cells during early infection (Figs. 1c,d and 4a). The sustained decrease in EAAT-2 mRNA first detectable on day 3 may translate to the consistent decrease in glutamate uptake during late infection. The temporal relationship of mRNA for EAAT-2 and glutamate uptake in HHV-6-infected astrocytes is schematically represented in Fig. 5. In this model, the early (days 1–2) increase in EAAT-2 and uptake for both HHV-6A- and HHV-6B-infected astrocytes is



**Fig. 5** Schematic model of HHV-6-induced dysregulation of glutamate uptake and glutamate transporter expression. Changes in EAAT-2 mRNA expression are represented by the *blue line*, and changes in glutamate uptake are represented by the *red line*. Changes are displayed graphically over time. Mechanisms for HHV-6-induced changes in EAAT-2 or glutamate uptake are indicated as factor (soluble factor effect) or virus (direct viral effect). Only the mechanisms for day 30 are proposed.

associated with a soluble factor because the effect was not reversed by filtration of the inocula. Later in infection (e.g., day 30), the observed decrease in glutamate uptake and EAAT-2 expression appears to be directly related to virus for HHV-6A-infected cells because this effect was reversed by filtration. As we have demonstrated that HHV-6B is a nonproductive infection in glial cells, the decrease in glutamate uptake and EAAT-2 expression later in infection may be associated with an HHV-6B-induced soluble factor or with low levels of viral DNA that have been shown to persist throughout the culture (Ahlqvist et al. 2005).

## Discussion

HHV-6 infection in the CNS has been associated with neurologic disorders including epilepsy, MS, and encephalitis (Cermelli et al. 2003; Challoner et al. 1995; Donati et al. 2003; Fotheringham et al. 2007a; Goodman et al. 2003). Although active viral infection has been localized to astrocytes in resected tissue from patients with MTL and in MS lesions (Challoner et al. 1995; Donati et al. 2003; Goodman et al. 2003), an unanswered question is how HHV-6 infection of astrocytes may contribute to disease pathology. In this study, we show that HHV-6A and HHV-6B exhibit different infection characteristics in human astrocytes, and that both variants induce dysregulation of glutamate uptake and expression of glutamate transporters.

Active HHV-6 infection has been detected in astrocytes *in vivo* and *in vitro* (Akhyani et al. 2006; De Bolle et al. 2005b; Donati et al. 2003, 2005; Fotheringham et al. 2007a; Goodman et al. 2003; He et al. 1996; Yao et al. 2006). While HHV-6A and HHV-6B both infected human astrocytes, expression of only the nonspliced mRNA for immediate/early U16/17 and lack of detection of late viral protein suggests that HHV-6B replication was incomplete. Several published studies have demonstrated limited viral mRNA detection in HHV-6B-infected astrocytes compared to viral mRNA expressed following HHV-6A infection (De Bolle et al. 2005b; Donati et al. 2005). Similarly, our study found expression of the immediate/early gene U12 mRNA but expression only of the nonspliced U16/17 at day 1, and viral mRNA expression was lost by 4–6 days postinfection. These data, in combination with other published studies, suggest that HHV-6B can infect but does not establish a productive, active infection in astrocytes. Active infection of astrocytes with HHV-6A, however, is demonstrated by detection of spliced viral mRNA for U12 and U16/17, as well as protein expression of IE2. Both HHV-6A- and HHV-6B-infected cells had undetectable levels of viral mRNA by days 14 and 6, respectively, but stable levels of cellular viral DNA for up to 4–6 weeks postinfection,

suggesting that both variants establish persistent, but low-level, infection.

Although the characteristics of early infection were different for HHV-6A and HHV-6B, particularly with respect to mRNA expression and cytopathic effect, each infection demonstrated consistently high levels of glutamate uptake compared to mock-infected cells. After removing infectious virus from our inocula through a 0.1- $\mu$ m filter, the HHV-6A- and HHV-6B-induced increase in glutamate uptake was preserved, suggesting that a virus-induced soluble factor may be influencing uptake rather than being a direct effect of the virus in astrocytes. This suggests that an HHV-6-induced factor may trigger increased glutamate uptake. Glutamate uptake can be affected by proinflammatory cytokines including TNF- $\alpha$ , and HHV-6 can induce expression of proinflammatory genes (Mayne et al. 2001), suggesting a cytokine or a viral chemokine may cause the effect we report on glutamate.

During the chronic phase of infection, both HHV-6A and HHV-6B-infected astrocytes demonstrated an impaired ability to uptake glutamate, and this functional change was associated with decreased expression of the glial glutamate transporter EAAT-2. The early, acute phase of infection was associated with high glutamate uptake in astrocytes infected with either HHV-6A or HHV-6B. mRNA for EAAT-2 was increased at days 1 and 2 and decreased by day 3. The decrease in EAAT-2 mRNA expression at day 3 is sustained through the chronic phase of infection and may correlate with impaired glutamate uptake at later time points. The increase in EAAT-2 mRNA expression at days 1 and 2 likely translates to an increase in protein expression and in glutamate uptake that was detected at day 4. Whether the early increase in message and protein for EAAT-2 results from a soluble factor (may occur for 6B) or is directly viral (may occur for 6A), it correlates with the early expression of viral RNA and may be an early response of the astrocyte to HHV-6 replication. The long-term decrease in EAAT-2 expression correlates with a sustained decrease in glutamate uptake first detected at day 5 that is maintained throughout late infection and may be associated with low levels of viral DNA.

Glutamate dysregulation has been proposed as a disease mechanism in epilepsy and MS (Binder and Steinhauser 2006; Bolton and Paul 2006; Groom et al. 2003; Matute et al. 2006), and both disorders have been associated with HHV-6 infection of astrocytes (Cermelli et al. 2003; Challoner et al. 1995; Donati et al. 2003; Goodman et al. 2003). Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor blockers have demonstrated reduction of neurologic disability in rodent models of experimental autoimmune encephalomyelitis (EAE), and these studies have suggested AMPA-mediated excitotoxicity of oligodendrocytes as a mechanism of disease

(Pitt et al. 2000; Smith et al. 2000; Werner et al. 2000). In humans, high levels of CSF glutamate have been associated with exacerbation in relapsing–remitting MS, and decreased glutamate transporter expression has been shown on oligodendrocytes surrounding MS lesions (Pitt et al. 2003; Sarchielli et al. 2003). Based on animal and human studies of glutamate in EAE and MS, mechanisms have been proposed whereby glutamate levels in these disorders are not properly controlled. Dysregulation of glutamate can trigger glutamate-induced excitotoxic death of oligodendrocytes, which may lead to the development of MS lesions. Here, we suggest that HHV-6 infection of astrocytes in MS lesions (Cermelli et al. 2003) may likewise lead to impaired glutamate uptake through downregulated transporters, high glutamate levels, and excitotoxicity.

In studies of epilepsy, glutamate has long been considered a major contributor to disease pathology. High levels of glutamate are detected following seizure (Cavus et al. 2005; de Lanerolle and Lee 2005; During and Spencer 1993), expression of alternatively spliced glutamate transporters is increased (Hoogland et al. 2004), expression of glutamate-metabolizing enzymes is dysregulated in patients with epilepsy (de Lanerolle and Lee 2005; Eid et al. 2004; van der Hel et al. 2005), and injection of the glutamate receptor agonist kainic acid induces seizures in rodents (Leite et al. 2002). High levels of extracellular glutamate resulting from impaired glutamate uptake by astrocytes may induce seizures by causing depolarization and hyperexcitability of neurons. HHV-6 DNA has been detected in resected tissue from patients with MTLE, and active HHV-6 infection has been localized to astrocytes in primary cell cultures isolated from these patients (Donati et al. 2003). A recent study of primary astrocytes isolated from patients with MTLE demonstrated the presence of HHV-6 viral antigen and mRNA, indicating active infection, and low levels of glutamate transporter mRNA, suggesting HHV-6 may be associated with downregulated transporter expression in vivo (Fotheringham et al. 2007b).

Although HHV-6 infection in the CNS has been associated with neurologic disorders, it is unknown how this virus may contribute to disease pathology. Dysregulated glutamate uptake and transporter expression in HHV-6-infected primary astrocytes constitutes the first report of this virus altering cell function and proposes an intriguing mechanism for the contribution of HHV-6 to the pathology of neurologic diseases like MS and epilepsy.

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