No evidence for aberrant expression of amyloid β and phosphorylated tau proteins in herpes simplex virus-infected human neurons *in vivo*

Diana N. Tran¹, Amy T.C.M. Bakx¹, Vera van Dis², Eleonora Aronica³, Robert M. Verdijk² and Werner J.D. Ouwendijk^{1*}

¹Department of Viroscience, Erasmus MC, University Medical Center Rotterdam, the Netherlands; ²Department of Pathology, Erasmus MC, University Medical Center Rotterdam, the Netherlands; ³ Department of (Neuro)Pathology, Amsterdam Neuroscience, Amsterdam University Medical Centers, University of Amsterdam, Amsterdam, the Netherlands.

Running title: Absence of AD pathology in HSE brain

ABSTRACT

Increasing evidence implicates the neurotropic herpes simplex virus 1 (HSV-1) in the pathogenesis of Alzheimer's Disease (AD). However, it is unclear whether previously reported findings in HSV-1 cell culture and animal models can be translated to humans. Here, we analyzed clinical specimens from latently HSV-1 infected individuals and individuals with lytic HSV infection of the brain (herpes simplex encephalitis; HSE). Latent HSV-1 DNA load in trigeminal ganglia was identical between AD patients and controls, and latently HSV-infected neurons did not express amyloid β (A β) or hyperphosphorylated tau (pTau). Some HSE patient brains presented with ageing-related intraneuronal A β accumulations, neurofibrillary tangles (NFT) or extracellular A β plaques, but these were neither restricted to HSV-infected neurons nor brain regions containing virus-infected cells. Analysis of unique brain material from an AD patient with concurrent HSE showed that HSV-infected cells frequently localized close to A β plaques and NFT, but did not exacerbate AD-related pathology in relation to HSV infection. HSE-associated neuroinflammation was not associated with specific A β or pTau phenotypes. Collectively, the data indicate that neither latent nor lytic HSV infection of human neurons *in vivo* is directly associated with aberrant A β or pTau protein expression.

KEYWORDS: Alzheimer's disease, herpes simplex virus, herpes simplex encephalitis, varicella-zoster virus, amyloid β , neurofibrillary tangles

BACKGROUND

Alzheimer's disease (AD) is a progressive neurodegenerative disease that accounts for about 80% of dementia cases (1). Pathological hallmarks of AD are the formation of extracellular amyloid β ($A\beta$) protein plaques and intracellular neurofibrillary tangles (NFT) composed of hyperphosphorylated tau (pTau) protein (2). The deposition of $A\beta$ plaques is an early event in AD pathogenesis, which induces subsequent inflammation, accumulation of NFTs, and ultimately, neuronal dysfunction and cell death (3). Genetic factors largely explain the risks of AD development and appear to be principally involved in $A\beta$ processing and clearance – most notably apolipoprotein $\epsilon 4$ allele (*APOE4*) – and are expressed by CNS-resident immune cells (4). However, an estimated 21 – 42% of lifetime risk on AD is attributed to environmental factors (5). Recent studies demonstrated that $A\beta$ can function as an antimicrobial peptide that protects neurons from bacterial and viral infections (6,7), increasing the evidence that microbial infections – especially herpes simplex virus 1 (HSV-1) – could play a role in the pathogenesis of AD.

Most adults worldwide are infected with the human neurotropic alphaherpesvirus herpes simplex virus 1 (HSV-1). Latent HSV-1 predominantly resides in the somata of pseudounipolar sensory trigeminal ganglion (TG) neurons, which innervate both the orofacial epithelia and the brainstem (8). Periodic HSV-1 reactivation results in virus spread to the oral mucosa and the central nervous system (CNS). Whereas oral HSV-1 shedding can be both asymptomatic and symptomatic (cold sores), symptomatic HSV-1 infection of the CNS is extremely rare and associated with severe morbidity and mortality (herpes simplex encephalitis; HSE) (9,10). HSV-1 DNA is detected in brain of elderly individuals, with a significantly higher frequency in brain of AD patients in which viral DNA was found to co-localize with $A\beta$ plaques (11,12). HSV-1 infection of neuronal cell cultures induces the accumulation of intracellular $A\beta$ and extracellular $A\beta$ aggregates, although variable results were obtained using three-dimensional neuron models (13–17). Additionally, HSV-1 infection induces nuclear accumulation and hyperphosphorylation of tau

protein in neurons *in vitro* (18–20). These AD-related pathological changes were recently found to progressively accumulate with repeated HSV-1 infection and to correlate with cognitive impairment in mice (21); however, humans are the only natural host of HSV-1 and, unlike mice, lytic HSV-1 infection of the CNS is extremely rare, at least in part because human cortical neurons are intrinsically resistant to HSV-1 infection (22). It remains to be determined whether lytic or latent HSV-1 infection of human neurons *in vivo* is directly associated with aberrant A β and pTau expression. Therefore, we investigated the relationship between AD-related neuropathological changes and HSV infection in TG of latently HSV-1 infected individuals and brain of individuals with lytic HSV infection of the brain (HSE).

METHODS

Human clinical specimens. Temporal cortex biopsies from AD patients, and paired human TG and plasma samples from healthy controls, AD patients, and patients with other neurological diseases were obtained from the Netherlands Brain Bank (Netherlands Institute for Neuroscience; Amsterdam, the Netherlands). All donors had provided written informed consent for brain autopsy and the use of material and clinical information for research purposes. All study procedures were performed in compliance with relevant laws in the Netherlands. Institutional guidelines approved by the local ethical committee (VU University Medical Center, Amsterdam, project number 2009/148) and in accordance with the ethical standards of the Declaration of Helsinki. AD brain tissue and part of TG biopsies were formalin-fixed and paraffin-embedded (FFPE) for *in situ* analysis. Additional TG samples were used to generate single cell suspensions (23). FFPE brain samples from patients with traumatic brain injury (TBI), HSE patients and a VZV encephalitis patient were obtained for diagnostic purposes and provided by the BioBanks of the Erasmus MC

(TBI patients) and Amsterdam University Medical Center (HSE and VZV encephalitis). According to the institutional “Opt-Out” system, which is defined by the National “Code of Good Conduct” [Dutch: Code Goed Gebruik, May 2011], these surplus human brain tissues were available for the current study.

DNA extraction, quantitative TaqMan Real-Time PCR (qPCR) and *APOE* genotyping. One-tenth of a TG single cell suspension was used for DNA extraction using the QIAamp DNA Kit (Qiagen). TaqMan qPCR was performed in duplicate on the 7500 Real-Time PCR system using 2X PCR Universal Master Mix (Applied Biosystems) and primer/probe pairs specific for HSV-1 *US4*, VZV *ORF38* and the human single copy gene hydroxymethylbilane synthase (24) (Supplementary Table 1). *APOE* genotyping was performed using allele $\epsilon 2$ -, $\epsilon 3$ - and $\epsilon 4$ -specific primer/probe combinations and TaqMan qPCR, as described (25) (Supplementary Table 1). Results were confirmed by PCR amplification of the *APOE* gene region containing allele-differentiating SNPs rs429358 and rs7412 using PfuUltra II Fusion High-Fidelity DNA Polymerase (Agilent), followed by Sanger sequencing using the BigDye 3.1 Cycle Sequencing Kit on the 3130XL Genetic Analyzer (Applied Biosystems) (25).

Immunohistochemical and immunofluorescent staining. FFPE tissue sections were subjected to heat-induced antigen retrieval using citrate buffer or Trilogy™ (Cell Marque). Immunohistochemical (IHC) staining was performed using the following primary antibodies: monoclonal mouse anti- β -Amyloid (clone 4G8, BioLegend), anti-phosphorylated-Tau (Ser²⁰²/Thr²⁰⁵) (AT8, Invitrogen), and anti-HSV-1 infected cell protein 8 (ICP8) (10A3, Cell Marque). Staining was visualized using biotinylated polyclonal rabbit anti-mouse Ig (Dako) and goat anti-rabbit Ig (Dako), followed horseradish peroxidase-conjugated streptavidin (Dako) and

3-Amino-9-ethylcarbazole substrate. Images were obtained with an Olympus BX51 microscope or by scanning the slides using the Hamamatsu NanoZoomer 2.0 HT.

Immunofluorescent (IF) staining was performed using the following primary antibodies: polyclonal rabbit anti-HSV-1 (cross-reactive with HSV-2; Agilent), anti-Iba1 (Wako) or anti-GFAP (Dako), polyclonal chicken anti-GFAP (Abcam), monoclonal mouse anti-NeuN IgG1 (A60, Sigma-Aldrich), anti-MBP (1.B.645, Santa Cruz Biotechnology), anti-Iba1 (GT10312, Invitrogen), anti- β -Amyloid (4G8, BioLegend), anti-phosphorylated-Tau (Ser²⁰²/Thr²⁰⁵) (AT8, Invitrogen), anti-CD45 (2B11 + PD7/26, Dako), and monoclonal rat anti-CD3 (CD3-12, Abcam). Alexa Fluor® 488 (AF488)-, AF594- and AF647-conjugated polyclonal goat anti-rabbit IgG, anti-mouse IgG1 and IgG2b, goat anti-chicken IgY and goat anti-rat IgG secondary antibodies (Invitrogen) were used. Nuclei were stained with Hoechst 33342 Solution and mounted with ProLong™ Diamond Antifade Mountant (Thermo Fisher Scientific). Images were obtained using a Zeiss LSM700 confocal microscope.

***In situ* hybridization.** Tissue sections were stained for HSV-1 and VZV RNA by *in situ* hybridization (ISH), using the RNAscope® 2.5 HD Kit-RED and probes HSV-1-LAT (Cat No. 315651) and VZV-Pool (Cat No. 400701) (Advanced Cell Diagnostics). Slides were counterstained with hematoxylin (Sigma) and mounted with EcoMount (Biocare Medical).

Statistical analyses. All statistical analyses were performed using GraphPad Prism 8.0.2 (GraphPad Software Inc).

RESULTS

HSV-1 DNA load in TG of AD patient and controls. Previous studies suggest that HSV-1 infection or reactivation, as measured by plasma HSV-1 IgG and IgM levels, is a risk factor for AD development (26). Moreover, clinical HSV-1 reactivation frequency correlates with both *APOE4* carriage (humans) and latent viral DNA load (mice) (27–30). To investigate whether latent HSV-1 DNA load was associated with AD development or *APOE* genotype, we performed qPCR on human TG that were infected with HSV-1 and obtained from AD patients and controls. Additionally, we performed qPCR for the closely related varicella-zoster virus (VZV), because most human TG are co-infected by HSV-1 and VZV (10). HSV-1 and VZV DNA loads were similar between AD patients and controls (Figure 1A). Further, *APOE* genotyping of all analyzed TG specimens demonstrated comparable HSV-1 and VZV DNA levels in *APOE4* carriers and non-carriers (Figure 1B and Supplementary Figure 1), despite the reported effect of *APOE4* on promoting HSV neurovirulence (32,33).

No expression of A β and pTau in latently HSV-infected TG neurons of AD patients and controls. Most individuals acquire HSV-1 infection in their childhood, followed by a lifelong latent infection and frequent (asymptomatic) virus reactivation (34,35). Given that HSV-1 replication leads to the accumulation of A β and increased pTau expression in cultured neurons and in the brains of HSV-infected mice (13,14,18,21,36), we analyzed whether latent HSV-1 infection was associated with A β or pTau expression in TG neurons of AD patients and controls (Supplementary Table 2). Analysis of consecutive TG sections from HSV IgG seropositive (n = 3 AD; n = 5 controls) and HSV IgG seronegative (n = 2 AD) donors did not demonstrate A β , A β plaques or pTau staining in latently HSV-1-infected TG neurons of either AD patients or controls (Figure 1C). Similarly, we did not observe aberrant A β or pTau staining in neurons not infected by HSV-1.

158

159 **Expression of A β and pTau in brain of HSE patients.** We hypothesized that HSV-induced AD-
160 related pathology could be restricted to CNS neurons, rather than peripheral TG neurons, or could
161 be reversible and therefore limited to lytic (productive) virus infection. To test these hypotheses,
162 we acquired rare post-mortem brain specimens from five HSE patients (Table 1), encompassing
163 4 cases of encephalitis caused by HSV-1 and 1 neonatal HSV-2 patient. Brain sections from all
164 patients analyzed contained lytically HSV-infected cells, as evidenced by the detection of
165 intranuclear eosinophilic (Cowdry type A) inclusion bodies, HSV-1 LAT RNA (in all HSV-1 HSE
166 patients) and HSV ICP8 protein (Figure 2A). In all patients, the majority of HSV-infected ICP8^{POS}
167 cells were identified as NeuN^{POS} neurons, with lower frequencies of infected non-neuronal cells,
168 mainly Iba1^{POS} microglia (Figure 2B-C). Thus, brain sections from HSE patients provide a
169 snapshot of lytically HSV-infected CNS neurons in vivo.

170 We analyzed whether lytic HSV infection was associated with increased intracellular
171 production of A β or pTau, or the deposition of extracellular A β plaques in brain sections from HSE
172 patients (Table 1) and, as control for neuronal damage, traumatic brain injury (TBI) (n = 3;
173 Supplementary Table 3) and AD patients (n =3; Supplementary Table 4). TBI-induced neuronal
174 damage is associated with increased production of A β , resulting in its accumulation in axonal
175 spheroids as well as plaques in about 30% of patients (37). Indeed, we observed axonal spheroids
176 in all three TBI patients and increased levels of intracellular A β protein and (diffuse) extracellular
177 A β plaques in two TBI patients (Figure 3A). NFTs and pTau staining were not observed in TBI
178 patients (Figure 3A). Brain sections, containing HSV-infected cells, from two out of five HSE
179 patients did not show A β protein/plaques nor pTau protein/NFTs (Table 1). A β plaques were
180 observed in one of five HSE patients (patient #2), whereas intracellular accumulations of A β
181 protein were detected in three of five HSE patients. NFTs were detected in two of five HSE
182 patients (patient #2 and #5) (Figure 3A and Table 1).

Subsequently, we analyzed the spatial orientation of HSV-infected cells in relation to AD-related pathological changes. Although HSV-infected cells were occasionally observed in close proximity to NFT and/or A β plaques, majority of these AD-related pathological changes were not associated with sites of HSV replication in the brains of HSE patient #2 and #5 (Figure 3B). Similarly, intracellular accumulations of A β protein were widely spread through brain sections of HSV-1 HSE patients, not restricted to areas with HSV-1 infection and also observed in sections without HSV-infected cells (Figure 3B and Supplementary Figure 2). IF staining was performed to investigate potential co-localization between HSV-1 infected cells and plaques/NFT and to assess intracellular levels of A β and pTau in more detail. Again, occasional HSV-infected cells were observed adjacent to A β plaques and NFT, but HSV antigen never co-localized with A β plaques nor NFT (Figure 4A). In HSE patients #2, #4 and #5 abundant intracellular A β protein staining was detected in most HSV-infected neurons, but also in non-infected neurons (Figure 4B). Nuclear pTau staining was observed in some HSV-infected cells in HSE patients containing NFTs (Figure 4C). However, no increased A β or pTau levels were observed in HSV-infected neurons in HSE patients #1 and #3 (Figure 4D).

Finally, we investigated whether the widespread increased levels of intracellular A β protein, in the absence of diffuse A β plaques, in brain of some HSE patients were specific to HSV infection by analyzing brain sections from a case of encephalitis caused by VZV. A 72-year-old, male patient with no known history of dementia presented with VZV encephalitis and died 15 days after onset of disease. Abundant VZV RNA-positive cells were detected (Supplementary Figure 2A), as well as VZV antigen-positive cells, indicative of lytic VZV infection. A β plaques and NFT, as well as intracellular A β protein were observed in hippocampal sections, whereas only widespread prominent intracellular A β protein depositions were observed in medulla oblongata sections (Supplementary Figure 2B). Similar to the HSE brains, we did not observe an association between the spatial orientation of virus-infected cells and AD-related pathological changes

(Supplementary Figure 2C). Overall, these results indicate that lytic α -herpesvirus, especially HSV, infection of human CNS neurons *in vivo* is not consistently associated with immediate aberrant A β or pTau protein expression.

Expression of A β and pTau in brain of an AD patient with concurrent HSE. To investigate how HSV-1 infection interacts with AD-related pathological changes in the AD brain, we obtained rare brain tissue specimens from an AD patient presenting with HSE. Patient was a 63-year-old female diagnosed with AD who developed HSE and died from septic shock resulting from HSE and aspiration pneumonia 1.5 weeks after hospitalization. Analysis of 6 cortical brain regions revealed intermediate AD-related pathological changes, i.e. amyloid Thal phase 5/5 and Braak NFT stage 4/6 (A3B2), consistent with dementia. HSV antigen-positive cells were abundantly detected in insular and temporal cortical tissue sections and less prominent in parietal and occipital cortex sections (Figure 5A). Importantly, HSV-1 infected cells were often found in close proximity to NFT and especially A β plaques (Figure 5B). However, AD-related pathological changes were not different between regions with and without HSV-1 infected cells. HSV-1 infected cells were situated around, but not co-localized with, A β plaques and NFTs (Figure 5C). Similar to HSE patients, increased levels of intracellular A β protein were present in most HSV-infected cells, as well as non-infected cells (Figure 5D). Thus, AD and HSV infection may affect the same brain regions, but we did not observe enhanced AD-related pathology in relation to HSV infection.

Neuroinflammation in HSE patients with and without AD-related pathology. Because neuroinflammation is a major contributor to neurodegeneration in AD (38), we hypothesized that inflammatory cells rather than HSV replication itself could be associated with specific AD-related pathological changes. Brain tissue sections of all HSE patients, including the AD patient with HSE,

indeed demonstrated widespread inflammation and gliosis in comparison to AD patients without viral encephalitis (Figure 6A and Supplementary Table 4). HSE patients and the AD patient with concurrent HSE showed prominent perivascular cuffs, mainly composed of CD45^{POS} CD3^{NEG} Iba1^{NEG} mononuclear cells – most likely macrophages or histiocytes – and lower numbers of CD45^{POS} CD3^{POS} T-cells (Figure 6B). Astrogliosis was observed in the brain parenchyma of all HSE patients, irrespective of Aβ or pTau expression pattern. This was also observed in the AD patient with HSE, but not as extensively in the AD patients (Figure 6C). Notably, while astrocytes interacted with Aβ plaques of all AD patients and HSE patient #2, more prominent GFAP staining was observed in astrocytes embracing Aβ plaques in the AD patient with HSE (Supplementary Figure 4). Additionally, extensive microgliosis was observed in the brain parenchyma of the HSE patients and AD patient with HSE, but not AD patients (Figure 6D). Both amoeboid (Figure 6D) and ramified microglia (Supplementary Figure 5) were observed throughout the tissue sections and in association with Aβ plaques and/or pTau in the AD patient with concurrent HSE and the HSE patients #2 and #5 (Table 2). Microglia were mostly amoeboid in HSE patients #1 and #3, whereas predominantly ramified microglia were present in HSE patient #4 (Figure 6D). Thus, although lytic HSV infection of the CNS induced significant neuroinflammation, the overall pattern of inflammation and gliosis was not associated with specific Aβ or pTau phenotypes.

DISCUSSION

Cumulative evidence suggests that the endemic human neurotropic virus, HSV-1, could play a role in the pathogenesis of AD (14,15,18–21,39). Here, we used clinical specimens from HSV-infected individuals to investigate the relationship between HSV infection and Aβ and pTau expression within human neurons *in vivo*. We report that latent HSV infection is not associated

with A β or pTau expression in TG neurons located in the PNS. Similarly, lytic HSV infection in the CNS was not consistently associated with increased expression of intracellular A β or pTau proteins nor with the deposition of A β plaques. Analysis of unique material from an AD patient with concurrent HSE showed that the same brain regions are often affected by HSV and AD-related pathology, but did not reveal exacerbated A β or pTau production in relation to regions containing virus-infected cells.

Previous studies show that increased HSV IgM plasma levels and possibly anti-HSV IgG avidity can be a measurement of HSV reactivation that correlates with AD development (26,40). One potential explanation is that genetic risk factors associated with AD development, especially *APOE4* and paired immunoglobulin-like type 2 receptor alpha (*PILRA*), directly impact lytic HSV-1 infection. The *APOE4* allele is associated with increased frequency of HSV-1 DNA detection in brain of AD patients and symptomatic HSV-1 reactivation (cold sores) (11,41). By contrast, asymptomatic oral HSV-1 shedding was not affected by *APOE* genotype (41), suggesting that *APOE4* does not directly influence virus reactivation but may play a role in peripheral control of HSV infection. In this study, we did not observe differences in latent HSV-1 DNA load in the TG of AD patients compared to controls or in *APOE4* carriers compared to non-carriers. Collectively, these data indicate that HSV-1 reactivation, rather than HSV-1 infection, is a risk factor for AD development, especially in *APOE4* carriers.

Detailed analysis of rare human HSE brain specimens demonstrated that lytic HSV infection was not associated with increased levels of intraneuronal A β or A β plaque deposition in human neurons *in vivo*. Although we observed accumulation of intraneuronal A β protein in three HSE and one VZV encephalitis patient, A β depositions were widely present in both virus-infected and non-infected neurons and tissue sections. These findings are consistent with the ubiquitous and progressive accumulation of intraneuronal A β in the absence of extracellular A β plaques and NFT that is observed with aging (42). Similarly, we only observed A β plaques in one HSE patient

and locally (hippocampus, but not medulla oblongata) in one VZV encephalitis patient. The elderly age of these two patients, lack of an association between sites of virus replication and A β plaques, and the presence of both senile plaques and NFT suggests that these pathological changes were most likely part of an ongoing development of AD, rather than induced by HSV infection.

HSV particles bind to A β protein and catalyze A β 42 oligomerization, leading to A β aggregates that physically entrap virus particles (7,43). Consistent with the idea of HSV initiating the deposition of A β plaques in the CNS, a previous study used in situ PCR to detect HSV-1 DNA in the brain of AD patients and controls to demonstrate that HSV-1 preferentially co-localizes with A β plaques in AD patients (12). Here, we did not detect viral antigen nor RNA within A β plaques in brain of elderly individuals with HSV-1 or VZV encephalitis. Although HSV-infected cells were often found in close proximity to A β plaques and/or NFT in the brain of the AD patient with HSE, AD-related pathology was not restricted to regions with HSV-infected cells and also in this patient we did not observe HSV antigen or RNA in A β plaques. HSV-1 infection rapidly induces A β plaque deposition in the brain of genetically susceptible AD mouse models, but repeated HSV-1 reactivation and lytic virus replication in brain results in the progressive accumulation of both senile plaques and NFT (21). While HSE is extremely rare in humans, asymptomatic HSV reactivation and viral spread to the CNS, as measured by the presence of viral DNA, occurs frequently (11,12). Our data indicate that lytic HSV infection does not directly induce the formation of A β plaques in the human brain, possibly due to the extensive neuronal cell death observed in HSE patients. Instead, repeated exposure to abortive HSV infections may be required to induce persistent A β plaques or could enhance A β oligomerization in existing plaques.

HSV infection of diverse human neuron models *in vitro* leads to the hyperphosphorylation of tau protein, including Ser²⁰²/Thr²⁰⁵, by cellular cyclin-dependent kinases and relocation of tau to the nucleus (18–20). We report that HSV-infected neurons in brain of human HSE patients occasionally expressed nuclear pTau, but only in individuals presenting with NFT. However, NFTs

were not restricted to areas containing HSV-infected cells in HSE patients and the AD patient with HSE. These findings are in agreement with prior studies in 3xTg-AD mice (prone to develop NFT (44)), in which the degree and residues involved in tau phosphorylation in response to HSV infection varied between brain regions, and progressively increased with repeated viral reactivation events (21). Possibly, the heterogeneity of neurons *in vivo* – including the expression of specific cyclin-dependent kinases – determines their differential susceptibility to HSV-induced aberrant tau phosphorylation and cellular localization.

Specific microglia and astrocyte populations colocalize with A β plaques in brain of AD patients (45–47) and are thought to be involved in both early and advanced stages of AD pathogenesis (48,49). Lytic HSV infection induces robust innate immune responses in brain, involving both microglia and astrocytes, which control ongoing virus replication, but also, cause immunopathology (50). Prominent microgliosis and astrogliosis were present in all HSE patients, but we did not observe overall differences in glia cell morphology or abundance in patients with or without A β plaques and/or NFT. Interestingly, GFAP staining tended to be more abundant in A β plaque-associated astrocytes in brain sections from the patient with combined AD and HSE, compared to patients with either AD or HSE (Supplementary Figure 4). Although these observations suggest that HSV infection may influence reactive astrocyte function in brain of AD patients, more detailed analyses in more patients or experimental animal models are warranted.

In conclusion, we demonstrate that latent and lytic HSV infection of human neurons *in vivo* is not consistently associated with aberrant A β or pTau expression. Importantly, our results do not challenge the increasing epidemiological and functional evidence that implicates a role of HSV-1 infection in the pathogenesis of AD. Human *in vitro* neuron cultures and murine AD models highlight potential mechanisms by which HSV infection can contribute to the initiation, perpetuation, or deterioration of AD. However, our data suggest that the human CNS could be more resilient to HSV-induced AD-related neuropathology than previously anticipated. Future

studies comparing the effects of HSV infection on the healthy ageing brain and AD brain may provide valuable insight into mechanisms by which HSV may affect AD pathogenesis.

ACKNOWLEDGMENTS

We thank Dr. Georges Verjans for critical discussion of the data and Tamana Khemai-Mehraban for technical assistance. Research reported in this publication was in part supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under Award Number R01AI151290 (W.J.D.O.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. This study was funded in part by a Human Disease Model Award 2020 (Erasmus MC).

CONFLICTS OF INTERESTS

The authors have no conflicts of interest to declare.

REFERENCES

1. 2020 Alzheimer's disease facts and figures. *Alzheimer's and Dementia*. 2020;16(3):391–460.
2. Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: Progress and problems on the road to therapeutics. *Science*. 2002;297(5580):353–6.
3. Hardy JA, Higgins GA. Alzheimer's Disease: The Amyloid Alzheimer's disease. *Science*. 1992;256:184–5.
4. Lambert JC, Ibrahim-Verbaas CA, Harold D, Naj AC, Sims R, Bellenguez C, et al. Meta-analysis of 74,046 individuals identifies 11 new susceptibility loci for Alzheimer's disease. *Nature Genetics*. 2013;45(12):1452–8.
5. Barnes DE, Yaffe K. The projected effect of risk factor reduction on Alzheimer's disease prevalence. *The Lancet Neurology*. 2011;10(9):819–28.

- 356 6. Kumar DKV, Choi SH, Washicosky KJ, Eimer WA, Tucker S, Ghofrani J, et al. Amyloid- β Peptide
357 Protects Against Microbial Infection In Mouse and Worm Models of Alzheimer's Disease Deepak.
358 Sci Transl Med. 2016;340(8).
- 359 7. Eimer WA, Kumar D, Kumar V, Shanmugam NKN, Rodriguez AS, Mitchell T, et al. Alzheimer's
360 Disease-Associated β -Amyloid Is Rapidly Seeded by Herpesviridae to Protect against Brain
361 Infection. Neuron. 2018;99(1):56–63.
- 362 8. Smith G. Herpesvirus Transport to the Nervous System and Back Again. Annu Rev Microbiol.
363 2012;66.
- 364 9. Roizman B, Whitley RJ. An inquiry into the molecular basis of HSV latency and reactivation.
365 Annual Review of Microbiology. 2013;67:355–74.
- 366 10. Arvin A, Campadelli-Fiume G, Mocarski E, Moore PS, Roizman B, Whitley R, et al. Human
367 Herpesviruses: Biology, Therapy, and Immunoprophylaxis. Cambridge University Press; 2007.
- 368 11. Itzhaki RF, Lin W, Shang D, Wilcock GK, Faragher B, Jamieson GA. Early reports Herpes simplex
369 virus type 1 in brain and risk of Alzheimer's disease. FASEB J. 1997;349:241–4.
- 370 12. Wozniak MA, Itzhaki RF. Herpes simplex virus type 1 DNA is located within Alzheimer's disease
371 amyloid plaques. J Pathol. 2008;217(September 2008):131–8.
- 372 13. Ill-raga G, Palomer E, Wozniak MA, Ramos-ferna E, Antu C, Tajés M, et al. Activation of PKR
373 Causes Amyloid β -Peptide Accumulation via De-Repression of BACE1 Expression. PLoS ONE.
374 2011;6(6):1–10.
- 375 14. Wozniak MA, Itzhaki RF, Shipley SJ, Dobson CB. Herpes simplex virus infection causes cellular α -
376 amyloid accumulation and secretase upregulation. 2007;429:95–100.
- 377 15. Shipley SJ, Parkin ET, Itzhaki RF, Dobson CB. Herpes simplex virus interferes with amyloid
378 precursor protein processing. BMC Microbiology. 2005;5:1–8.
- 379 16. Abrahamson EE, Zheng W, Muralidaran V, Ikonovic MD, Bloom DC, Nimgaonkar VL, et al.
380 Modeling A β 42 Accumulation in Response to Herpes Simplex Virus 1 Infection: Two Dimensional
381 or Three Dimensional? Journal of Virology. 2020;95(5):1–9.
- 382 17. Cairns DM, Rouleau N, Parker RN, Walsh KG, Gehrke L, Kaplan DL. A 3D human brain-like tissue
383 model of herpes-induced Alzheimer's disease. Science Advances. 2020;6(19):1–14.
- 384 18. Zambrano A, Solisb L, Salvadores N, Cortes M, Lerchundia R, Otthb C. Neuronal Cytoskeletal
385 Dynamic Modification and Neurodegeneration Induced by Infection with Herpes Simplex Virus
386 Type 1. Journal of Alzheimer's Disease. 2008;14:259–69.
- 387 19. Wozniak MA, Frost AL, Itzhaki RF. Alzheimer's disease-specific tau phosphorylation is induced by
388 herpes simplex virus type 1. Journal of Alzheimer's Disease. 2009;16(2):341–50.
- 389 20. Álvarez G, Aldudo J, Alonso M, Santana S, Valdivieso F. Herpes simplex virus type 1 induces
390 nuclear accumulation of hyperphosphorylated tau in neuronal cells. Journal of Neuroscience
391 Research. 2012;90(5):1020–9.

- 392 21. de Chiara G, Piacentini R, Fabiani M, Mastrodonato A, Marcocci ME, Limongi D, et al. Recurrent
393 herpes simplex virus-1 infection induces hallmarks of neurodegeneration and cognitive deficits in
394 mice. *PLoS Pathogens*. 2019;15(3):1–30.
- 395 22. Lafaille FG, Harschnitz O, Lee YS, Zhang P, Hasek ML, Kerner G, et al. Human SNORA31 variations
396 impair cortical neuron-intrinsic immunity to HSV-1 and underlie herpes simplex encephalitis. *nat*
397 *med*. 2019;25(12):1873–84.
- 398 23. Verjans GMGM, Hintzen RQ, van Dun JM, Poot A, Milikan JC, Laman JD, et al. Selective retention
399 of herpes simplex virus-specific T cells in latently infected human trigeminal ganglia. *Proceedings*
400 *of the National Academy of Sciences of the United States of America*. 2007;104(9):3496–501.
- 401 24. van Doornum GJJ, Guldemeester J, Osterhaus ADME, Niesters HGM. Diagnosing herpesvirus
402 infections by real-time amplification and rapid culture. *Journal of Clinical Microbiology*.
403 2003;41(2):576–80.
- 404 25. Zhong L, Xie Y-Z, Cao T-T, Wang Z, Wang T, Li X, et al. A rapid and cost-effective method for
405 genotyping apolipoprotein E gene polymorphism. *Molecular neurodegeneration*. 2016 Jan;11:2.
- 406 26. Lövheim H, Gilthorpe J, Adolfsson R, Nilsson LG, Elgh F. Reactivated herpes simplex infection
407 increases the risk of Alzheimer’s disease. *Alzheimer’s and Dementia*. 2015;11(6):593–9.
- 408 27. Hoshino Y, Pesnicak L, Cohen JI, Straus SE. Rates of Reactivation of Latent Herpes Simplex Virus
409 from Mouse Trigeminal Ganglia Ex Vivo Correlate Directly with Viral Load and Inversely with
410 Number of Infiltrating CD8+ T Cells. *Journal of Virology*. 2007;81(15):8157–64.
- 411 28. Linard M, Letenneur L, Garrigue I, Doize A, Dartigues JF, Helmer C. Interaction between APOE4
412 and herpes simplex virus type 1 in Alzheimer’s disease. *Alzheimer’s and Dementia*.
413 2020;16(1):200–8.
- 414 29. Letenneur L, Pérès K, Fleury H, Garrigue I, Barberger-Gateau P, Helmer C, et al. Seropositivity to
415 Herpes Simplex Virus antibodies and risk of Alzheimer’s disease: A population-based cohort
416 study. *PLoS ONE*. 2008;3(11):1–5.
- 417 30. Lopatko Lindman K, Weidung B, Olsson J, Josefsson M, Kok E, Johansson A, et al. A genetic
418 signature including apolipoprotein Eε4 potentiates the risk of herpes simplex-associated
419 Alzheimer’s disease. *Alzheimer’s and Dementia: Translational Research and Clinical Interventions*.
420 2019;5:697–704.
- 421 31. Lin WR, Casas I, Wilcock GK, Itzhaki RF. Neurotropic viruses and Alzheimer’s disease: A search for
422 varicella zoster virus DNA by the polymerase chain reaction. *Journal of Neurology Neurosurgery*
423 *and Psychiatry*. 1997;62(6):586–9.
- 424 32. Burgos JS, Ramirez C, Sastre I, Valdivieso F. Effect of Apolipoprotein E on the Cerebral Load of
425 Latent Herpes Simplex Virus Type 1 DNA. *Journal of Virology*. 2006;80(11):5383–7.
- 426 33. Burgos JS, Ramirez C, Sastre I, Bullido MJ, Ca FV. ApoE4 is more efficient than E3 in brain access
427 by herpes simplex virus type 1. 2003;14(14):1825–7.

- 428 34. Mark KE, Wald A, Magaret AS, Selke S, Olin L, Huang M-L, et al. Rapidly Cleared Episodes of
429 Herpes Simplex Virus Reactivation in Immunocompetent Adults. *J Infect Dis.* 2008;198(8):1141–9.
- 430 35. Smith JS, Robinson NJ. Age-specific prevalence of infection with herpes simplex virus types 2 and
431 1: A global review. *Journal of Infectious Diseases.* 2002;186:S3–28.
- 432 36. Martin C, Aguila B, Araya P, Vio K, Valdivia S, Zambrano A, et al. Inflammatory and
433 neurodegeneration markers during asymptomatic HSV-1 reactivation. *Journal of Alzheimer's*
434 *disease : JAD.* 2014;39(4):849–59.
- 435 37. Johnson VE, Stewart W, Smith DH. Traumatic brain injury and amyloid- β pathology: a link to
436 Alzheimer's disease? *Nature Reviews Neuroscience* [Internet]. 2010 May 10;11(5):361–70.
437 Available from: <http://www.nature.com/articles/nrn2808>
- 438 38. Kinney JW, Bemiller SM, Murtishaw AS, Leisgang AM, Salazar AM, Lamb BT. Inflammation as a
439 central mechanism in Alzheimer's disease. *Alzheimer's and Dementia: Translational Research and*
440 *Clinical Interventions* [Internet]. 2018;4:575–90. Available from:
441 <https://doi.org/10.1016/j.trci.2018.06.014>
- 442 39. Ashraf GM, Tarasov V v., Makhmutova A, Chubarev VN, Avila-Rodriguez M, Bachurin SO, et al.
443 The Possibility of an Infectious Etiology of Alzheimer Disease. *Molecular Neurobiology.*
444 2019;56(6):4479–91.
- 445 40. Agostini S, Mancuso R, Baglio F, Cabinio M, Hernis A, Costa AS, et al. High avidity HSV-1
446 antibodies correlate with absence of amnesic Mild Cognitive Impairment conversion to
447 Alzheimer's disease. *Brain, Behavior, and Immunity* [Internet]. 2016;58:254–60. Available from:
448 <https://www.sciencedirect.com/science/article/pii/S0889159116303476>
- 449 41. Koelle DM, Magaret A, Warren T, Schellenberg GD, Wald A. APOE genotype is associated with
450 oral herpetic lesions but not genital or oral herpes simplex virus shedding. *BoneSex Transm*
451 *Infect.* 2010;86(3):202–6.
- 452 42. Welikovitch LA, do Carmo S, Maglóczy Z, Szocsics P, Löke J, Freund T, et al. Evidence of
453 intraneuronal A β accumulation preceding tau pathology in the entorhinal cortex. *Acta*
454 *Neuropathologica* [Internet]. 2018;136(6):901–17. Available from:
455 <https://doi.org/10.1007/s00401-018-1922-z>
- 456 43. Ezzat K, Pernemalm M, Pålsson S, Roberts TC, Järver P, Dondalska A, et al. The viral protein
457 corona directs viral pathogenesis and amyloid aggregation. *Nature Communications* [Internet].
458 2019;10(1):1–16. Available from: <http://dx.doi.org/10.1038/s41467-019-10192-2>
- 459 44. Oddo S, Caccamo A, Shepherd JD, Murphy MP, Golde TE, Kaye R, et al. Triple-transgenic model
460 of Alzheimer's Disease with plaques and tangles: Intracellular A β and synaptic dysfunction.
461 *Neuron.* 2003;39(3):409–21.
- 462 45. Habib N, McCabe C, Medina S, Varshavsky M, Kitsberg D, Dvir-Szternfeld R, et al. Disease-
463 associated astrocytes in Alzheimer's disease and aging. *Nature Neuroscience* [Internet].
464 2020;23(6):701–6. Available from: <http://dx.doi.org/10.1038/s41593-020-0624-8>

46. Olah M, Menon V, Habib N, Taga MF, Ma Y, Yung CJ, et al. Single cell RNA sequencing of human microglia uncovers a subset associated with Alzheimer's disease. *Nature Communications* [Internet]. 2020;11(1). Available from: <http://dx.doi.org/10.1038/s41467-020-19737-2>
47. Keren-Shaul H, Spinrad A, Weiner A, Matcovitch-Natan O, Dvir-Szternfeld R, Ulland TK, et al. A Unique Microglia Type Associated with Restricting Development of Alzheimer's Disease. *Cell* [Internet]. 2017;169(7):1276-1290.e17. Available from: <http://dx.doi.org/10.1016/j.cell.2017.05.018>
48. Rodríguez-Arellano JJ, Parpura V, Zorec R, Verkhratsky A. Astrocytes in physiological aging and Alzheimer's disease. *Neuroscience* [Internet]. 2016;323(January):170–82. Available from: <http://dx.doi.org/10.1016/j.neuroscience.2015.01.007>
49. Bates KA, Fonte J, Robertson TA, Martins RN, Harvey AR. Chronic gliosis triggers Alzheimer's disease-like processing of amyloid precursor protein. *Neuroscience*. 2002;113(4):785–96.
50. Mancini M, Vidal SM. Insights into the pathogenesis of herpes simplex encephalitis from mouse models. *Mammalian Genome* [Internet]. 2018;29(7–8):425–45. Available from: <http://dx.doi.org/10.1007/s00335-018-9772-5>

TABLES, FIGURES AND FIGURE LEGENDS

Table 1 HSE patient characteristics and Alzheimer's disease-related pathology

Patient	Age ^a	Gender ^b	Causative Virus	Brain regions analyzed	A β intraneuronal ^c	A β plaques	NFT ^d
1	--	--	HSV-1	Cortex	No	No	No
2	79	F	HSV-1	Hippocampus / Cortex	Yes	Yes	Yes
				Amygdala / Entorhinal Cortex	Yes	Yes	Yes
				Cortex	Yes	Yes	Yes
				Insula	Yes	Yes	Yes
3	0 ^e	F	HSV-2	Cortex	No	No	No
4	81	F	HSV-1	Putamen	No	Yes	No
5	76	M	HSV-1	Hippocampus / Entorhinal Cortex	No	Yes	Yes

^a Age in years; ^b F, female; M, male; ^c intraneuronal A β accumulation; ^d NFT, neurofibrillary tangle; ^e neonatal HSE patient, 2 weeks old

FIGURE 1

Figure 1. HSV-1 infection is not associated with aberrant A β or pTau expression in latently infected human trigeminal ganglia (TG) neurons. (**A**, **B**) HSV-1- and VZV-specific qPCR was performed on DNA extracted from the TG of Alzheimer's disease (AD) patients and control subjects, stratified on disease status (**A**; 20 controls and 10 AD patients) or *APOE4* allele carrier status (**B**; HSV-1: 22 *APOE4*-negative and 8 *APOE4*-positive individuals; VZV: 24 *APOE4*-negative and 13 *APOE4*-positive individuals). Horizontal line: median. (**C**) Sequential TG sections from AD (n = 4) and control (n = 6) subjects were stained for amyloid β protein, HSV-1 latency-associated transcript (LAT) RNA and phosphorylated Tau protein (pTau; Ser²⁰²/Thr²⁰⁵) by immunohistochemistry (IHC) and RNA *in situ* hybridization (ISH). Arrowheads indicate LAT-positive neurons and asterisks indicate lipofuscin granules. Scale bar: 50 μ m.

FIGURE 2

Figure 2. Lytic HSV infection of neurons in brain of herpes simplex virus encephalitis (HSE) patients. (A) Brain sections from HSE patients (Table 1) were stained with hematoxylin and eosin, or stained for HSV-1 LAT RNA by ISH and HSV-1 ICP8 protein by IHC. Boxes indicate area shown at higher magnification in the inset. Scale bar: 50 μm. (B, C) Brain sections of HSE patients were immunofluorescently stained for (B) HSV-1 protein (green), neurons (NeuN; white) and oligodendrocytes (MBP; red), as well as (C) HSV-1 protein (green), microglia (Iba1; white), and astrocytes (GFAP; red). Nuclei were stained with Hoechst-33342 (blue). Representative images are shown for patient #2 (amygdala/entorhinal cortex; Table 2). Scale bar: 20 μm.

FIGURE 3

Figure 3. Lytic HSV infection is not consistently associated with aberrant A β or pTau expression in brain of herpes simplex virus encephalitis (HSE) patients. **(A)** Brain sections from Alzheimer's disease (AD; patient 1, Supplementary Table 4), trauma brain injury (TBI; patient 2, Supplementary Table 3) and HSE patients (Table 1) were stained for A β and pTau (Ser²⁰²/Thr²⁰⁵) by IHC. Filled and open arrowheads indicate mature senile and diffuse A β plaques, respectively. Filled black arrows indicate intracellular A β accumulations. Open arrows indicate neurofibrillary tangles. Boxes indicate area shown at higher magnification in the inset. Scale bar: 50 μ m. **(B)**

519 Consecutive brain sections from HSE patients were stained for A β and pTau (Ser²⁰²/Thr²⁰⁵)
 520 protein by IHC and HSV-1 RNA by ISH. Boxes indicate area shown at a higher magnification.
 521 Scale bars indicate 500 μ m (panels 1), 100 μ m (panels 2) and 50 μ m (panels 3).

522

FIGURE 4

Figure 4. HSV-infected cells do not consistently express aberrant A β or pTau in brain of herpes simplex encephalitis (HSE) patients. Brain sections from HSE patients were immunofluorescently stained for HSV protein (green), A β (red) and pTau (Ser²⁰²/Thr²⁰⁵; white). Nuclei were stained with Hoechst-33342 (blue). Images are shown for HSE patients #2 (A left panel), #5 (A right panel, and C), #4 (B) and #3 (D) (Table 1). White boxes indicate areas shown at higher magnification. Scale bars indicate 10 μ m (A: Amyloid β plaques; and C) and 20 μ m (A right panel, B and D).

FIGURE 5

Figure 5. Detection of HSV-infected cells in close proximity to Aβ plaques in the brain of an Alzheimer's disease patient with herpes simplex encephalitis. **A** Brain section stained for HSV protein (brown) by immunohistochemistry (IHC). Scale bar indicates 50 μm. **B** Consecutive brain sections were stained for Aβ (4G8) and pTau (Ser²⁰², Thr²⁰⁵) by IHC or stained for HSV-1 RNA by in situ hybridization (ISH). Boxes indicate areas shown at higher magnification. Asterisks indicate same blood vessel in consecutive sections. Scale bars indicate 500 μm (low magnification) and 100 μm (high magnification). **C-D** Brain sections were immunofluorescently stained for HSV protein (green), Aβ (red) and pTau (Ser²⁰²/Thr²⁰⁵; white) protein. Nuclei were stained with Hoechst-33342 (blue). Scale bar: 20 μm.

FIGURE 6

Figure 6. Neuroinflammation is similar in herpes simplex encephalitis (HSE) patients with and without Aβ plaques and/or NFT. Brain sections from Alzheimer's disease (AD) patients (n=3, Supplementary Table 4), HSE patients (n=4, Table 1) and an AD with HSE patient (n=1) were stained with (A) hematoxylin and eosin staining or IF stained for: (B) CD3 (green) and GFAP (white) (left panel) or Iba1 (green) and CD45 (white) (right panel); (C) Aβ (red) and GFAP (white); or (D) Aβ (red), Iba1 (green) and CD45 (white). Nuclei were stained with Hoechst-33342 (blue). Images are shown for the AD patient with HSE (B – D), AD patient #1 (C, D; Supplementary Table

554 4) and indicated HSE patients. Boxes indicate areas shown at higher magnification. Scale bar: 50
555 μm .

556

557

SUPPLEMENTARY TABLES AND SUPPLEMENTARY FIGURE LEGENDS

Supplementary Table 1 Primers and probes used in this study

Name	Target	Sequence ^a
HSV-1 US4_Fw	Human herpesvirus 1 <i>US4</i>	TCCTSGTTCCTMACKGCCTCCC
HSV-1 US4_Rv	Human herpesvirus 1 <i>US4</i>	CGTCTGGACCAACCGCCACACAGGT
HSV-1 US4_probe	Human herpesvirus 1 <i>US4</i>	<i>FAM</i> -GCAGICAYACGTAACGCACGCT- <i>BHQ1</i>
VZV ORF38_Fw	Human herpesvirus 3 <i>ORF38</i>	AAGTTCCCCCGTTTCGC
VZV ORF38_Rv	Human herpesvirus 3 <i>ORF38</i>	CCGCAACAACCTGCAGTATATATCGTCTCA
VZV ORF38_probe	Human herpesvirus 3 <i>ORF38</i>	<i>FAM</i> -TGGACTTGAAGTGAACCTAATGAAGC- <i>BHQ1</i>
HMBS_Fw	Homo sapiens <i>HMBS</i>	GCCTGCAGTTTGAAATCAGTG
HMBS_Rv	Homo sapiens <i>HMBS</i>	CGGGACGGGCTTTAGCTA
HMBS_probe	Homo sapiens <i>HMBS</i>	<i>FAM</i> -CGCAGGCACTCGTACTGCTCGCT- <i>BHQ1</i>
ApoE2_Fw	Homo sapiens <i>APOE2</i>	GCGGACATGGAGGACGTGT
ApoE2_Rv	Homo sapiens <i>APOE2</i>	CCTGGTACACTGCCAGGCA
ApoE3_Fw	Homo sapiens <i>APOE3</i>	CGGACATGGAGGACGTGT
ApoE3_Rv	Homo sapiens <i>APOE3</i>	CTGGTACACTGCCAGGCG
ApoE4_Fw	Homo sapiens <i>APOE4</i>	CGGACATGGAGGACGTGC
ApoE4_Rv	Homo sapiens <i>APOE4</i>	CTGGTACACTGCCAGGCG
ApoE_Probe	Homo sapiens <i>APOE</i>	<i>FAM</i> -CAGCTCCTCGGTGCTCTGGC- <i>BHQ1</i>
ApoE_seq1	Homo sapiens <i>APOE</i>	AGCCCTTCTCCCGCCTCCCACTGT
ApoE_seq2	Homo sapiens <i>APOE</i>	CTCCGCCACCTGCTCCTTCACCTCG

^a5' and 3'-modifications of TaqMan probes are indicated. *FAM* 6-Carboxyfluorescein, *BHQ1* black hole quencher 1

Supplementary Table 2 FFPE TG samples used in this study

Subject	Age ^a	Gender ^b	PMI ^c	Cause of death	Neurological disease	HSV-1 status ^d
1	81	F	3:35	Cachexia and dehydration	Alzheimer's disease	negative
2	82	F	5:55	Cardiac arrest	Alzheimer's disease	negative
3	89	F	4:30	Peritonitis	Alzheimer's disease	positive
4	87	F	8:10	Uremia by dehydration	Alzheimer's disease	positive
5	86	F	5:15	Cachexia and dehydration	Alzheimer's disease	positive
6	81	M	8:21	Euthanasia	Parkinson's disease	positive
7	62	F	12:35	Cachexia with pulmonary insufficiency	Multiple sclerosis	positive
8	80	F	9:30	Cardiac arrest	Bipolar disorder	positive
9	103	F	6:35	Dehydration and pneumonia	Tauopathy	positive
10	89	M	6:50	Urosepsis	non-demented control	positive

^aAge in years; ^bF, female; M, male; ^cPMI, post-mortem interval; ^dHSV-1 infection status based on serology and HSV-1 LAT ISH

561

562

Supplementary Table 3 TBI patients used in this study

Subject	Age ^a	Gender ^b	Interval TBI – death
1	84	M	0 days (acute)
2	48	M	7 days
3	13	F	11

^aAge in years; ^bF, female; M, male

563

564

Supplementary Table 4 FFPE AD samples used in this study

Subject	Age ^a	Gender ^b	PMI ^c	Cause of death	Neurological disease	HSV status ^d
1	88	M	4:40	Cachexia and dehydration	Alzheimer's disease	positive
2	74	M	8:00	Dehydration by possible cystitis	Alzheimer's disease	positive
3	86	M	05:10	Cachexia and dehydration	Alzheimer's disease	positive

^aAge in years; ^bF, female; M, male; ^cPMI, post-mortem interval; ^dHSV infection status based on HSV-specific IgG serology data

SUPPLEMENTARY FIGURE 1

Supplementary Fig. 1 Quantification of latent HSV-1 and VZV DNA load in human TG stratified on *APOE* genotype. HSV-1- and VZV-specific qPCR and *APOE* genotyping was performed on DNA extracted from the trigeminal ganglia (TG) of AD patients and controls.

SUPPLEMENTARY FIGURE 2

Supplementary Fig. 2 Intraneuronal accumulation of A β protein is not restricted to areas of HSV-1 infection in brains of HSE patients. Consecutive slides were stained for HSV-1 RNA by ISH and A β by IHC. A β can be seen in areas with HSV-1 RNA (**a**) and in areas without HSV-1 RNA (**b**). Data shown for HSE donor 5. Scale bar: 500 μ m (**a**) and 250 μ m (**b**).

SUPPLEMENTARY FIGURE 3

Supplementary Fig. 3 Lytic VZV infection is not associated with A β plaques or NFT in brain of a VZV encephalitis patient. **a** Brain section stained for VZV RNA by ISH. Box indicates area shown at higher magnification. Open arrowhead indicates examples of VZV RNA-expressing cells. Scale bars indicate 250 μ m (top) and 50 μ m (bottom). **b** Brain

590 sections IHC stained for A β and pTau (Ser²⁰²/Thr²⁰⁵). Scale bars indicate 50 μ m. **c**
 591 Consecutive brain sections were IHC stained for A β and pTau (Ser²⁰²/Thr²⁰⁵) or stained
 592 for HSV-1 RNA by ISH. Boxes indicate areas shown at higher magnification. Filled arrow
 593 indicates intracellular A β protein, open arrow indicates NFT and open arrowhead
 594 indicates VZV RNA-expressing cells. Scale bars indicate 50 μ m (A β , high magnification),
 595 100 μ m (A β , low magnification; pTau high magnification) and 500 μ m (pTau, low
 596 magnification).

597

SUPPLEMENTARY FIGURE 4

Supplementary Fig. 4 Prominent GFAP staining of astrocytes interacting with A β plaques brain of an AD patient with concurrent HSE. IF staining for A β (red), GFAP (white) and nuclei (Hoechst-33342; blue). Scale bar: 50 μ m.

604 **SUPPLEMENTARY FIGURE 5**

605

606 **Supplementary Fig. 5** Microglia morphology and density in the brain of HSE patients

607 and AD patient with HSE. Brain tissue sections were IF stained for Aβ (red), Iba1 (green)

608 and nuclei (Hoechst-33342; blue). Boxes indicate areas shown at higher magnification.

609 Scale bar: 50 μm.

610

611