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Herpes simplex virus type 2 infection induces AD-like neurodegeneration markers in human neuroblastoma cells

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ABSTRACT

Herpes simplex virus (HSV) types 1 and 2 are neurotropic viruses that establish lifelong latent infections in neurons. Mounting evidence suggests that HSV-1 infection is involved in the pathogenesis of Alzheimer's disease (AD). The relationships between other herpesvirus infections and events associated with neurodegeneration have not, however, been extensively studied. The present work reports that HSV-2 infection leads to the strong accumulation of hyperphosphorylated tau and the amyloid-β peptides Aβ40 and Aβ42 (all major pathological hallmarks of AD) in human SK-N-MC neuroblastoma cells. Infection is also associated with a marked reduction in the amount of Aβ40 secreted and in the proteolytic fragments of the amyloid-β precursor protein (APP) (secreted APPα and the α-C-terminal fragment). These results indicate that HSV-2 infection inhibits the nonamyloidogenic pathway of APP processing and impairs Aβ secretion in these cells. In addition, HSV-2 induces the accumulation of intracellular autophagic compartments containing Aβ due to a failure in the late stages of autophagy. To our knowledge, this is the first report to show that HSV-2 infection strongly alters the tau phosphorylation state, APP processing, and autophagic process in human neuroblastoma cells, leading to the appearance of AD-like neurodegeneration markers.

1. Introduction

Alzheimer's disease (AD), the single most common cause of dementia, is characterized by massive neuronal damage leading to cerebral atrophy and the loss of cognitive function. The major neuropathological lesions of AD are brain β-amyloidosis and neurofibrillary degeneration; the former is characterized by plaques of extracellular amyloid-β peptide (Aβ) in the brain parenchyma and blood vessels, while the second involves the intracellular accumulation and aggregation of abnormally hyperphosphorylated tau protein (a microtubule-associated protein) in the form of neurofibrillary tangles (Braak and Braak, 1992). Two distinct forms of AD are recognized: familial and sporadic. Familial AD is caused by mutations in the genes for amyloid-β precursor protein (APP), presenilin-1, and presenilin-2. However, most cases of AD (~99%) are of the sporadic type and usually involve people >65 years (Tanzi, 2012).

Sporadic AD is a highly complex disease for which neither the causal agent(s) nor the molecular mechanisms behind its pathogenesis are well known. A growing body of literature suggests, however, that cerebral infections may be risk factors for neurodegenerative disease, and certainly numerous studies have revealed an association between herpes simplex virus type 1 (HSV-1) and AD (Itzhaki, 2014; Piacentini et al., 2014). For example, HSV-1 DNA is present in the brains of a high proportion of people with AD (Jamieson et al., 1991), mainly within amyloid plaques (Wozniak et al., 2009b), the virus has been shown capable of inducing the main hallmarks of AD pathology (Alvarez et al., 2012; Santana et al., 2012b; Wozniak et al., 2007, 2009a), and the analysis of data gathered in genome-wide association studies involving thousands of AD patients and controls identified a set of AD-linked gene variants that might increase brain susceptibility to viral infections, particularly HSV-1 infection (Porcellini et al., 2010). Few studies, however, have focused on the role of other herpesviruses in AD, even though 5—herpes simplex virus type 2 (HSV-2), cytomegalovirus (CMV), human herpes virus 6, varicella-zoster virus and

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Epstein-Barr virus (EBV)—have been detected in the brains of elderly individuals and AD patients (Carbone et al., 2014; Hemling et al., 2003; Lin et al., 2002).

Like HSV-1 (which causes cold sores), HSV-2 (which causes genital herpes) belongs to the alpha-herpesvirus subfamily. These viruses can reach the sensory neurons that innervate the site of primary infection, and establish lifelong latent infections. Reactivation from the latent state causes recurrent disease, which is particularly common in immunocompromised hosts (Steiner and Benninger, 2013). A recent study estimates 57% of US adults to be infected with HSV-1 and 17% with HSV-2 (Koelle and Corey, 2008). It is well known that HSV-2 can infect the brain and cause neurological problems such as encephalitis and meningitis in neonates and encephalitis in adults (Berger and Houff, 2008). HSV-2 DNA, however, is found in the brains of far fewer people than HSV-1 DNA (both in those with AD and age-matched controls) (Lin et al., 2002). There are few comparative studies of HSV-1 and HSV-2 central nervous system infections, but it has traditionally been thought that HSV-2 causes fewer cases of herpes simplex encephalitis in adults than HSV-1 ($\sim$10% compared to $\sim$90%) (Berger and Houff, 2008). However, a recent report suggests that HSV-1 and HSV-2 account for similar numbers (Moon et al., 2014). These discordant findings may result from ethnic or regional differences between the study populations. In addition, the latter study has some limitations—all patients belong to a single center and it is a retrospective study with not well-defined criteria for categorization of neurological diseases. Further studies with well-designed prospective studies in multiple centers and regions are required to confirm that HSV-2 is a major cause of severe encephalitis in adults.

Our group and others have reported HSV-1 infection to modify tau phosphorylation (Alvarez et al., 2012; Wozniak et al., 2009a; Zambrano et al., 2008), APP proteolytic processing (De Chiara et al., 2010; Santana et al., 2012b; Wozniak et al., 2007), and autophagy (Gobeil and Leib, 2012; Santana et al., 2012a), all of which have been associated with the pathogenesis of AD. However, the effect of HSV-2 infection on these processes has not been extensively studied. The present work reveals that, like HSV-1 infection, HSV-2 infection can provoke the appearance of the neurodegeneration markers characteristic of AD in different cell models.

2. Materials and methods

2.1. Drugs, plasmids, and antibodies

Heparin (10 μg/mL), rapamycin (0.2 μg/mL), and bafilomycin A1 (100 nM) were purchased from Sigma. Leupeptin (100 μM) was supplied by Roche. 4′, 6-diamidino-2-phenylindole (DAPI; 5 μg/mL) and ammonium chloride (NH_4Cl; 20 mM) were obtained from Merck.

The GFP—light chain 3 (LC3) expression vector (pGFP-LC3) and the mCherry-GFP-LC3 construct (dtLC3) were kind gifts from T. Yoshimori and N. Mizushima (Kabeya et al., 2000), and T. Johansen (Pankiv et al., 2007), respectively. Rabbit anti-HSV glycoprotein B and D (gB/gD) antibody was kindly provided by E. Tabares. Mouse anti-APP A4 antibody (clone 22C11) was purchased from Millipore (MAB348). Monoclonal anti-human β-amyloid protein [1–17] (clone 6E10) was supplied by Sigma (A1474). Rabbit anti-amyloid precursor protein, C-terminal, antibody, recognizing all APP carboxy-terminal fragments (CTFs), was purchased from Sigma (A8717). Rabbit polyclonal anti-Aβ40 (44348A) and anti-Aβ42 (44–344) unconjugated antibodies, (pS199), (pT205), (pT231), (pS404), and (pS422) tau polyclonal antibodies (44734G; 44738G; 44746G; 44758G, and 44764G) and tau (TAU-5) monoclonal antibody (AHB0042) were purchased from Life Technologies. Two anti-LC3 antibodies were used: rabbit anti-LC3B from Sigma (L7543) and rabbit polyclonal anti-LC3 from MBL (PD014). Human CD63 antibody was obtained from the Developmental Studies Hybridoma Bank, University of Iowa (CD63, clone H5C6). Rabbit serum anti-GFP came from Life Technologies (A-6455). Mouse monoclonal anti-tubulin antibody was supplied by Sigma (T5168). The secondary antibodies used for immunostaining were horseradish peroxidase-coupled antibodies (Vector), or antibodies labeled with Alexa Fluor 488 or 555 dyes (Invitrogen).

2.2. Cell culture

Vero and SK-N-MC (human neuroblastoma) cell lines used in this work were purchased from the American Type Culture Collection. SK-N-MC cells stably expressing human APP (SK-APP) (Recuero et al., 2004) or GFP-LC3 (SK-LC3) (Santana et al., 2012b) were produced in-house. The SK-N-MC cells were grown as monolayers in minimal Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine and 50 μg/mL gentamicin. Vero cells were passaged in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 2-mM glutamine, and 50-μg/mL gentamicin. All cells were grown at 37 °C in a 5% CO_2 atmosphere.

2.3. HSV-2, infection, plaque assays, and viral DNA quantification

HSV-2 strain 333 was propagated on a monolayer of Vero cells as previously described (Carrascosa et al., 1982) and stored at −70 °C. The infectious titers of HSV-2 were determined by plaque assay (Santana et al., 2012b). For infection experiments, cells were grown in 6-well plates (for Western blot assays) or 24-well plates with coverslips (for immunofluorescence assays). When the cells reached 70%–80% confluence they were exposed to HSV-2 at 37 °C for 1 hour. Mock infections were performed using a virus-free suspension. Unbound virus was removed and the cells incubated in complete medium. Times and multiplicities of infection (expressed as plaque-forming units per cell [pfu/cell]) are as indicated in each experiment. Viral DNA levels were quantified by real-time quantitative polymerase chain reaction in the presence of SYBR Green, using primers specific for the overlapping region of *UL18* and *UL19* genes of HSV-2 (5′-GCTCCCCCCCGTGGA-3′ and 5′-AGGATTTCGCGCAGGTGAT-3′), employing an ABI Prism 7900HT SD system (Applied Biosystems). The quantification of human genomic DNA was performed using an Assay-on-Demand probe specific for the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene (Applied Biosystems; item nu Hs99999905_m1). The quantification results were calculated as viral DNA copy numbers per ng of genomic DNA.

2.4. Immunofluorescence analysis

Cells grown on coverslips were fixed in 4% formaldehyde and incubated with the appropriate primary and secondary antibodies. DAPI was added 10 minutes before the end of the procedure to visualize the nuclei. GFP-LC3 and dtLC3 imaging was performed in the same way but without antibody incubations. All cells were examined using a Zeiss Axiovert 200 fluorescence microscope or a Zeiss LSM710 vertical confocal microscope equipped with a 63× oil-immersion objective. Pictures were taken with a Spot RT slider digital camera (Diagnostic) using Metamorph 5.07 (Universal Imaging Corporation) or ZEN 2012 (Carl Zeiss Microscopy GmbH) imaging system software, and processed using Adobe Photoshop CS4.

2.5. Western blot analysis

For Western blot assays, cells were treated with lysis buffer (50-mM Tris-HCl pH 7.6, 300-mM NaCl, and 0.5% Triton X-100) containing a protease inhibitor cocktail (Complete Mini, Roche) and incubated for 30 minutes at 4 °C. For tau analysis, cells were lysed in

radioimmunoprecipitation assay buffer (10-mM Tris-HCl pH 7.5, 50-mM NaCl, 0.2% sodium deoxycholate, 1% Nonidet P-40%, and 0.1% sodium dodecyl sulfate) containing protease and phosphatase inhibitor cocktails (PhosSTOP, Roche), and incubated for 30 minutes at 4 °C. Lysates were centrifuged at 13,000*g* for 15 minutes at 4 °C. For endogenous LC3 analysis, lysates were prepared as described elsewhere (Santana et al., 2012b). Determinations of protein content were performed using the bicinchoninic acid assay (Pierce). For secreted APP analysis, the conditioned medium was treated with a protease inhibitor cocktail and concentrated 10 fold. Cell lysates and medium were mixed with Laemmli buffer, sonicated, and heated for 5 minutes at 100 °C. After electrophoretic separation, the gels were blotted onto a nitrocellulose membrane and stained with the appropriate antibodies. A peroxidase-coupled antibody was used as a secondary antibody. Detection by enhanced chemiluminescence was performed using ECL Western blotting detection reagents (Amersham Biosciences) according to the manufacturer's instructions.

2.6. Measurement of secreted Aβ

SK-APP cells were infected with HSV-2 and the medium later assayed for human Aβ40 using a commercial sandwich enzyme-linked immmunosorbent assay kit (Life Technologies) as described elsewhere (Santana et al., 2012b).

2.7. Secretase activity assays

α- and β-secretase activity was determined using commercial kits (R&D Systems) according to the manufacturer's instructions, as described elsewhere (Santana et al., 2012b).

2.8. Statistical analysis

Graph values are expressed as mean ± standard error of the mean. Differences between groups were analyzed using the 2-tailed Student *t* test. Significance was recorded at $p < 0.05$ (*), $p < 0.01$(**), and $p < 0.001$(***). Before analysis, the largest and the smallest variances were tested for homogeneity using the F-test.

3. Results

3.1. HSV-2 induces the accumulation of hyperphosphorylated tau

Before all else, the HSV-2 infection parameters for the SK-N-MC cells were optimized. In this cell model, HSV-2 establishes a lytic replication cycle resulting in cell lysis ~20–22 hours after infection. In this work, all infections were performed at a moi of 10 pfu/cell because this viral dose guarantees that almost all cells become infected, as revealed by confocal images of HSV-2-infected cells, visualized via the use of an antibody that recognizes glycoproteins B and D of the virus (Supplementary Fig. 1).

Immunofluorescence analysis was used to determine whether HSV-2 infection modifies the phosphorylation state of tau in SK-N-MC cells. For this, the Tau-5 antibody, which reacts with a phosphorylation-independent epitope, and the phosphorylation-sensitive antibodies ser199, thr205, thr231, ser404, and ser422 were used. In noninfected cells, phosphorylation-sensitive antibodies stained the cytoplasm only weakly (Fig. 1A). It should be noted that, except for the ser404 epitope, stronger immunoreactivity was observed in mitotic cells (Supplementary Fig. 2); this is consistent with the results of other authors (Delobel et al., 2002) who reported that the abnormal tau-phosphorylation characteristic of AD also occurs during mitosis. When the cells were exposed to HSV-2, all the tested epitopes revealed an accumulation of phosphorylated tau (Fig. 1A). Western blot analysis was used to quantify the effects of HSV-2 infection on tau phosphorylation (Fig. 1B). As suggested by the immunofluorescence experiments, a significant increase of phosphorylated tau levels was observed in all the examined epitopes compared to noninfected cells (Fig. 1C). However, the infected cells actually showed a small reduction in total tau levels, ruling out the possibility that the increase in phosphorylated tau induced by HSV-2 was due to an increase in tau content.

HSV-1 infection has previously been shown to induce the intranuclear accumulation of hyperphosphorylated tau at viral replication sites (Alvarez et al., 2012). In the present work, when the staining patterns of the phosphorylation-sensitive antibodies and the nuclear counterstain DAPI in HSV-2-infected cells were examined by confocal microscopy, the structures stained by the ser199, thr231, and ser404 antibodies were seen to fill the large, dark holes that appear in the nuclei of infected cells (Supplementary Fig. 3). Numerous reports have revealed that dark areas of DAPI staining coincide with the location of the viral replication centers (Calle et al., 2008; Wilcock and Lane, 1991). These data therefore indicate that some of the hyperphosphorylated tau accumulates within the nuclei of HSV-2-infected cells.

3.2. HSV-2 modifies the levels of Aβ and APP proteolytic fragments

Tests were next performed to determine whether HSV-2 also modulates APP processing. It has previously been shown that HSV-1

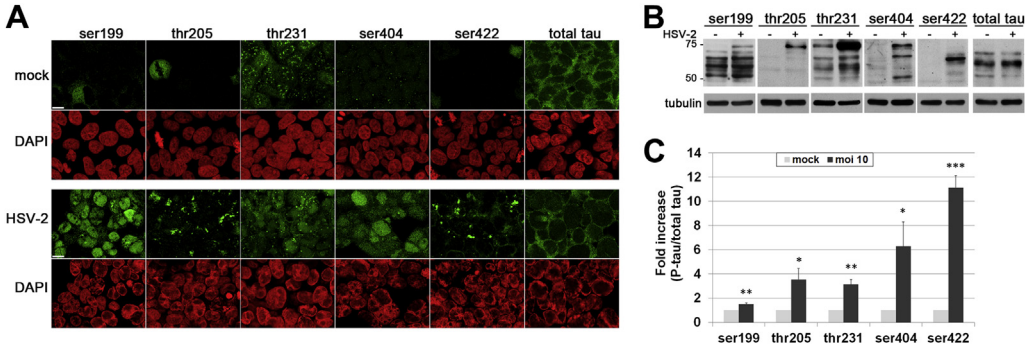

Fig. 1. HSV-2 infection leads to increased tau phosphorylation. SK-N-MC cells were exposed to HSV-2 at 10 pfu/cell for 18 hours and tau phosphorylation was assessed using the phosphorylation-sensitive antibodies ser199, thr205, thr231, ser404, and ser422. An antibody that recognizes total tau (Tau5) was also used. (A) Immunofluorescence images of mock and HSV-2-infected cells. DAPI-stained nuclei are shown. Scale bar: 10 μm. (B) Western blot analysis of endogenous tau levels. A tubulin blot is shown as a control to ensure equal loading. The blots are representative of 3 independent experiments. (C) Phosphorylated tau levels normalized by total tau were quantified by densitometric analysis. The ratio of phospho-tau to total tau in HSV-2-infected cells with respect to noninfected cells is represented. Data are the mean ± standard error of the mean of 3 independent experiments (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Abbreviations: DAPI, 4′, 6-diamidino-2-phenylindole; HSV-2, herpes simplex virus type 2.

causes the accumulation of intracellular Aβ and to intensely inhibit its secretion (Santana et al., 2012b). As a first step, the Aβ levels in the SK-N-MC cell line overexpressing the human APP protein (SK-APP) were examined by immunofluorescence. Intracellular Aβ was undetectable in noninfected cells at both test times (Fig. 2A). In contrast, HSV-2 infection induced a strong elevation of both the main intracellular species of Aβ peptide at 18 hours postinfection (hpi). In fact, this increase began at least at 6 hpi (Fig. 2B), indicating that HSV-2 induces Aβ accumulation early in infection. Similar results were obtained in Vero cells (Supplementary Fig. 4), another cell type in which HSV-1 has been reported to increase intracellular Aβ levels (Wozniak et al., 2011). The Aβ40 content of the extracellular medium of infected and noninfected SK-APP cells was then determined by enzyme-linked immmunosorbent assay. When these cells were infected with HSV-2, the Aβ40 levels fell drastically (by >4 fold). Neither Aβ42 nor intracellular Aβ was detectable by this assay. To confirm that the reduction in Aβ40 was provoked by HSV-2 infection, the cells were treated with heparin, an inhibitor of herpesvirus entry; this completely stopped the reduction in Aβ40 previously recorded (Fig. 2C). Heparin treatment drastically reduced the levels of HSV-2 DNA demonstrating that heparin efficiently blocked viral replication in SK-APP cells (>99%; Fig. 2D), thus ensuring that the effect of heparin on Aβ secretion was mediated by inhibition of HSV-2 infection.

To investigate how HSV-2 infection impacts APP processing, the activities of α- and β-secretases were recorded using specific fluorescent activity assays. Analysis of HSV-2-infected cells revealed their α-secretase activity to be reduced by 40% compared to noninfected cells. In contrast, no changes were recorded in β-secretase activity after HSV-2 infection (Fig. 3A). Consistent with these data, when the generation of products resulting from α-secretase activity was examined by Western blotting, HSV-2 infection led to a dramatic reduction in secreted sAPPα (Fig. 3B) and α-CTF (Fig. 3C). No significant differences were detected in APP expression in the cells after infection with HSV-2 (Fig. 3D), ruling out that the alteration of the different APP proteolytic products was due to any change in the actual amount of APP available.

Taken together, these findings are consistent with those obtained in HSV-1-infected cells and suggest that infection with herpesviruses strongly modifies APP processing and Aβ levels.

3.3. Inhibition of autophagic flux in HSV-2-infected cells

HSV-1 has been reported to strongly alter autophagy, a major degradative pathway of the lysosomal system known to be much impaired in AD. The microtubule-associated protein 1 LC3 is synthesized as a precursor (proLC3) which is proteolytically processed into the cytosolic LC3-I isoform. On activation of autophagy, LC3-I is modified into the phosphatidylethanolamine-conjugated form, LC3-II, which specifically binds to autophagic membranes. Changes in LC3 provide a very useful marker of autophagy and can be monitored by immunofluorescence and Western blotting (Klionsky et al., 2012). To examine the effects of HSV-2 infection on autophagy, the endogenous LC3 levels of SK-N-MC cells were determined. Immunofluorescence

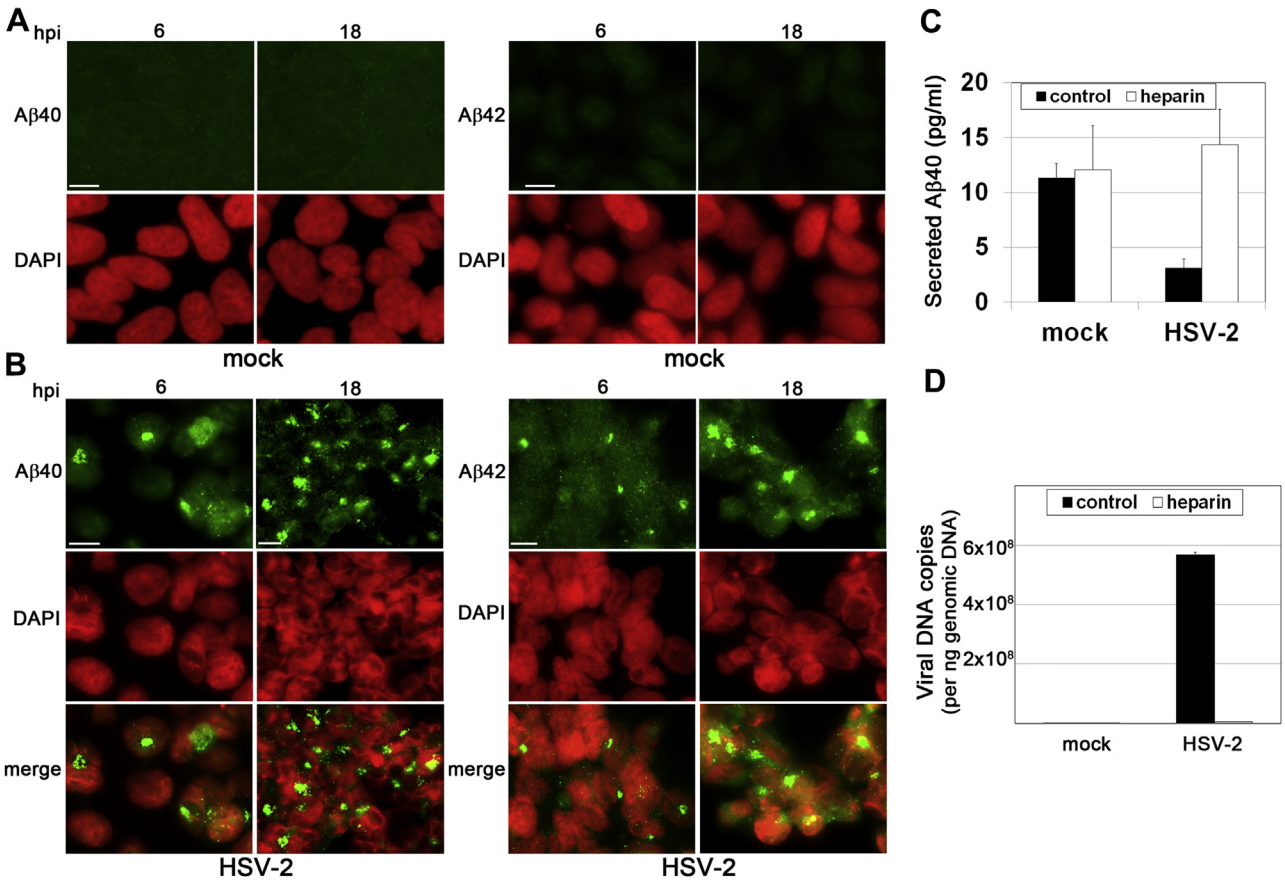

Fig. 2. Effects of HSV-2 infection on Aβ levels. SK-APP cells were mock-infected (A) or infected with HSV-2 at 10 pfu/cell for 6 and 18 hours (B), and Aβ40 and Aβ42 immunoreactivities analyzed by immunofluorescence. Images of DAPI-stained nuclei are shown. Scale bar: 10 μm. (C) Quantitative analysis by enzyme-linked immunosorbent assay of extracellular Aβ40 levels in conditioned medium from mock and HSV-2-infected SK-APP cultures (10 pfu/cell) after 18 hpi in the presence or absence of heparin. (D) The effect of heparin on viral DNA levels was analyzed by real-time quantitative polymerase chain reaction. In (C) and (D), data are the mean ± standard deviation for 3 samples from a representative experiment of 3 performed. Abbreviations: Aβ, amyloid beta; DAPI, 4′, 6-diamidino-2-phenylindole; hpi, hours post infection; HSV-2, herpes simplex virus type 2.

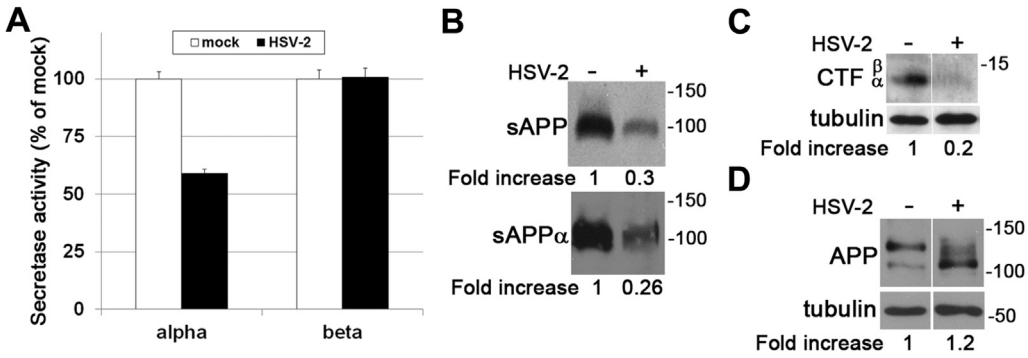

Fig. 3. HSV-2 infection inhibits the nonamyloidogenic pathway of APP processing. SK-APP cells were mock-infected or exposed to HSV-2 at 10 pfu/cell for 18 hours in all panels. (A) Analysis of α- and β-secretase activities by fluorogenic assays. The relative secretase activities were calculated and normalized by the amount of protein in the lysates in comparison with that seen for mock-infected cells. Data are the mean ± standard deviation for 3 samples from a representative experiment of 3 performed. (B) Secreted APP (sAPP) levels in conditioned medium in which SK-APP cells were cultured were analyzed by Western blotting using 2 different anti-APP antibodies: 22c11 (upper panel) and 6E10 (specifically recognizes sAPPα; lower panel). (C) Analysis of α-CTF levels by Western blotting using an antibody that recognizes all CTFs of APP (A8717). The β-CTF fragment was undetectable in these conditions. (D) APP levels in lysates were examined by Western blotting using the 22c11 antibody. Tubulin blots were performed as a loading control. The ratio of the analyzed proteins in HSV-2-infected cells with respect to mock-infected cells is shown at the bottom of the blots. Abbreviations: APP, amyloid-β precursor protein; CTF, carboxy-terminal fragment; HSV-2, herpes simplex virus type 2.

analysis showed HSV-2 to induce a strong increase in LC3 immunoreactivity, suggesting an accumulation of autophagic compartments in infected cells (Fig. 4A). To better quantify the effects of infection, the lipidation of LC3 was monitored by Western blotting. Consistent with the results obtained for the immunofluorescence experiments, endogenous LC3-II levels were markedly increased in cells exposed to HSV-2 at 18 hpi (Fig. 4B). In addition, the distribution of LC3 in SK-N-MC cells stably expressing the GFP-LC3 fusion protein (SK-LC3) was studied by fluorescence microscopy. In noninfected cells, few GFP-LC3 spots were observed. In contrast, GFP-LC3-positive structures accumulated in HSV-2-infected cells (Supplementary Fig. 5A). When the GFP-LC3 levels were analyzed by Western blotting, HSV-2 was seen to provoke a strong accumulation of the autophagy-associated isoform GFP-LC3-II (Supplementary Fig. 5B). These results were reproduced in Vero cells (which are also susceptible to HSV infection) (Supplementary Fig. 6A and B).

Increased LC3-II levels could stem from the overactivation of autophagy or reduced autophagic turnover due to defects in the late stages of autophagic degradation. Determining LC3-II levels in the presence of the lysosomal inhibitors leupeptin (a lysosomal protease

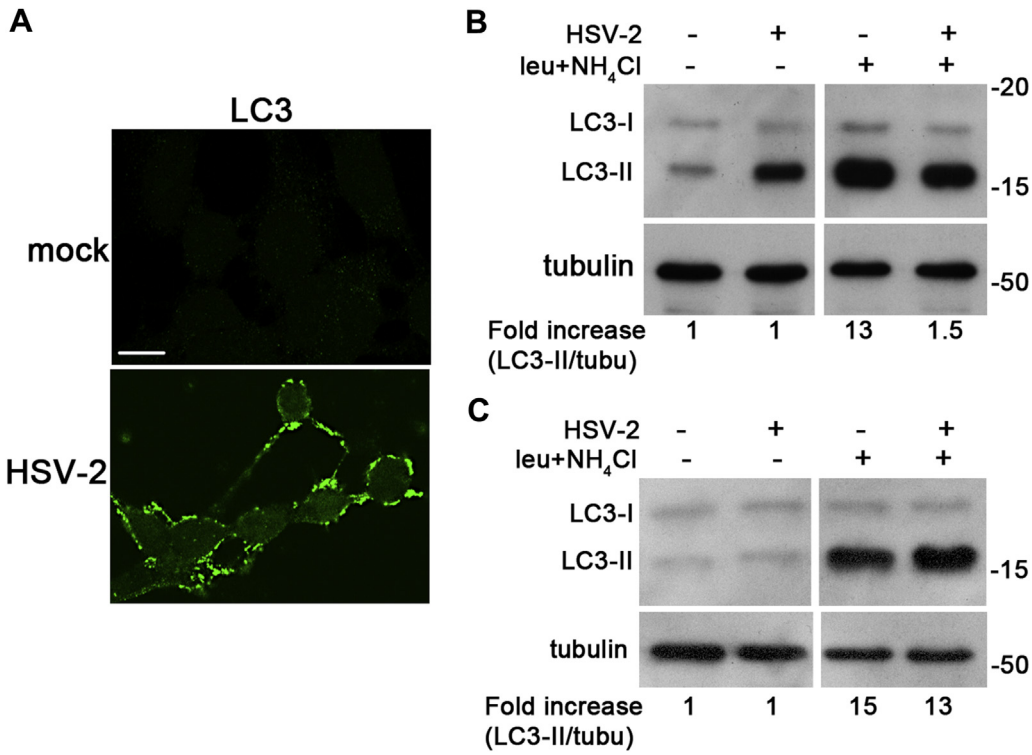

Fig. 4. HSV-2 infection induces the accumulation of autophagosomes in SK-N-MC cells. In all panels, cells were infected with HSV-2 at 10 pfu/cell. (A) Mock-infected cells or cells infected with HSV-2 for 18 hours were analyzed by immunofluorescence using an anti-LC3 antibody. Scale bar: 10 μm. (B and C) Western blot analysis of endogenous LC3 levels in HSV-2-infected cells after 18 (B) or 8 hours (C) in the absence or presence of the lysosomal inhibitors leupeptin (leu) and ammonium chloride (NH_4Cl). An antibody specific for LC3 protein was used. Tubulin blots are shown as a control of equal loading. The ratio of LC3-II to tubulin is shown at the bottom of the blots. All the blots are representative of 4 independent experiments. Abbreviations: LC3, light chain 3; HSV-2, herpes simplex virus type 2.

inhibitor) and ammonium chloride (a lysosomotropic agent that neutralizes the acidic lysosomal pH) by Western blotting is a well-established means of measuring autophagic flux. With the lysosomal inhibitor treatment, endogenous LC3-II levels dramatically increased in noninfected SK-N-MC cells (>10 fold) indicating a functional lysosomal degradation of LC3-II. However, when cells were exposed to HSV-2, a modest increase in LC3-II was recorded in the presence of lysosomal inhibitors (1.5 fold) during the late stages of infection (18 hpi), suggesting autophagosome turnover becomes blocked in HSV-2-infected cells (Fig. 4B). When the effect of lysosomal inhibitors was analyzed in SK-LC3 cells, similar results were obtained (Supplementary Fig. 5B). These findings, which were also reproduced in Vero cells (Supplementary Fig. 6B), indicate that only some of the LC3-II molecules are degraded by lysosomes in HSV-2-infected cells, resulting in the accumulation of autophagosomes. In contrast, when these experiments were performed in the early stages of infection (8 hpi) in SK-N-MC (Fig. 4C) and Vero (Supplementary Fig. 6C) cells, treatment with lysosomal inhibitors caused a similar increase in endogenous LC3-II levels in both noninfected and HSV-2-infected cells, indicating that HSV-2-infected cells experience unaltered autophagic flux early in infection.

The tandem fluorescent-tagged LC3 construct—the mCherry-GFP-LC3 reporter (dtLC3)—was used to confirm the inhibition of autophagic flux in HSV-2-infected cells. The acidic lysosomal environment quenches GFP fluorescence but not mCherry fluorescence. Therefore, when autophagosomes fuse with lysosomes, GFP fluorescence from dtLC3 is lost and only red fluorescence is recorded. In noninfected SK-N-MC cells, and cells treated with rapamycin to induce autophagy, yellow and red vesicles appeared, revealing the formation of autophagosomes and autolysosomes and thus demonstrating functional autophagy. A significant increase in the number of vesicles was seen in rapamycin-treated cells, consistent with the activation of autophagy induced by the drug. In contrast, a marked increase in the number and proportion of yellow puncta was observed in cells infected with HSV-2 at 18 hpi compared with nontreated cells. These effects are analogous to the changes produced by lysosomal alkalinization induced by bafilomycin A1 (Fig. 5) and are indicative of accumulation of autophagosomes, probably caused by a failure in the autophagosome-lysosome fusion process.

Taken together, these results indicate that HSV-2 blocks autophagic flux in the late stages of the viral replication cycle.

3.4. HSV-2 induces the accumulation of Aβ in autophagosomes

To further investigate the relationship between autophagosome accumulation and alterations in APP processing, the intracellular distribution of Aβ was monitored in HSV-2-infected cells by confocal microscopy. To this end, SK-APP cells were stained for Aβ, along with markers for autophagic compartments (LC3), and late endosomes and/or lysosomes (CD63). Aβ and LC3 immunoreactivities were almost undetectable in noninfected cells, whereas CD63 showed a characteristic punctate cytosolic pattern (Fig. 6A). Analysis of the confocal images revealed Aβ structures to colocalize with endogenous LC3 in cells exposed to HSV-2, indicating that Aβ was located in autophagic compartments (Fig. 6B). The colocalization of

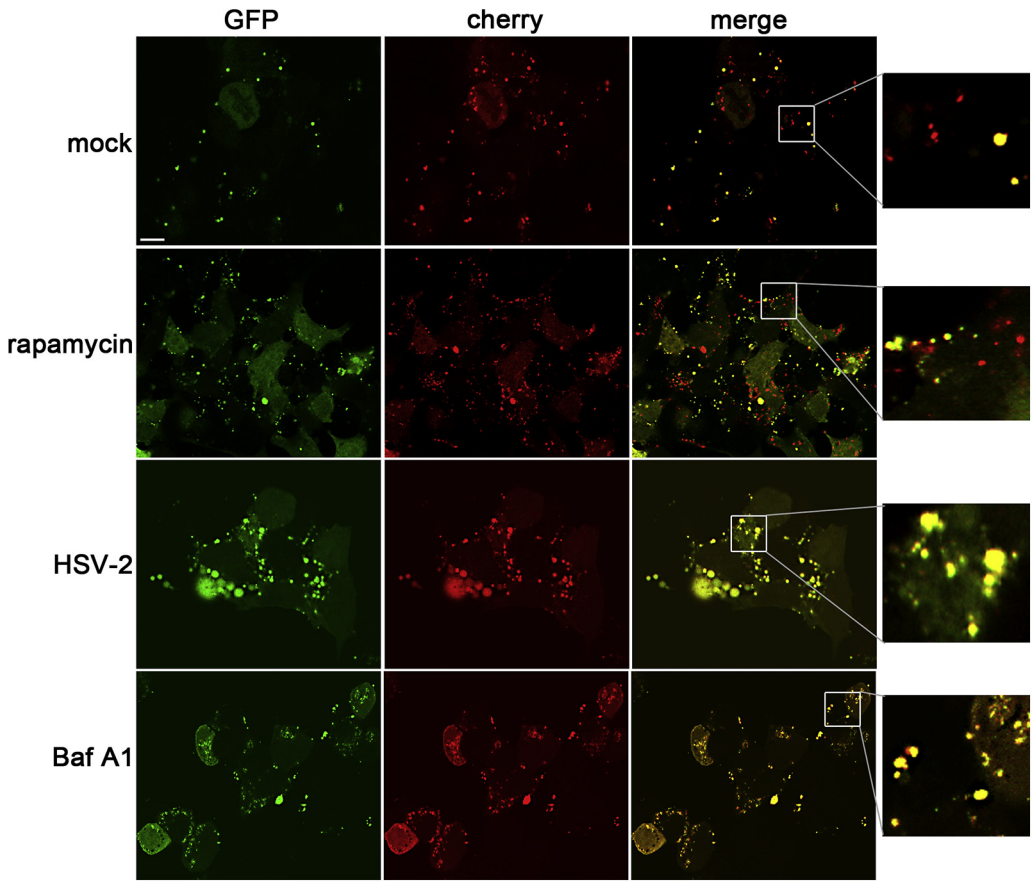

Fig. 5. Impairment of autophagic flux in HSV-2-infected cells. SK-N-MC cells were transiently transfected with mCherry-GFP-LC3 reporter and infected with HSV-2 at 10 pfu/cell, or treated with bafilomycin A1 (Baf A1) or rapamycin. Confocal microscopy images were taken after 18 hours of treatment. The insets in the merged panels show the colocalization of green and red signals under the different conditions assayed. Scale bar: 10 μm. Abbreviation: HSV-2, herpes simplex virus type 2.

Aβ with the lysosomal marker CD63 was then monitored. Lysosomes are the destiny organelles of the autophagic pathway where the vesicular contents are degraded. When SK-APP cells were infected with HSV-2, no colocalization of CD63 with either Aβ isoform was detected (Fig. 6C).

Taken together, these results show that Aβ does not reach the lysosomal compartment resulting in the accumulation of Aβ in autophagosomes, confirming a failure in autophagic flux in HSV-2-infected cells.

4. Discussion

AD is a multifactorial disorder that apparently involves different etiopathogenic mechanisms. One of the factors involved may be infectious agents that are able to colonize the brain and thus escape the immune response. Infection by different members of the herpesvirus family may be a risk factor for AD. These viruses are highly prevalent in the human population and the infection rate increases with age, they can infect neurons, and they establish life-long latent infections that cannot be removed by the immune system. Most of the studies have focused on the relationship between HSV-1 and AD but with nearly 70% genetic homology and a very similar transcription map, HSV-2 is a closely related virus (Aguilar et al., 2006), and it would not be surprising to discover that HSV-2 infection has effects similar to HSV-1 infection on processes whose modification has been related to neurodegeneration. Certainly it is well known that HSV-2 can infect the brain and that it is associated with neurological alterations (Steiner and Benninger, 2013), but its impact on the appearance of AD-like neurodegeneration markers has not been studied in depth. In this report, HSV-2 infection is

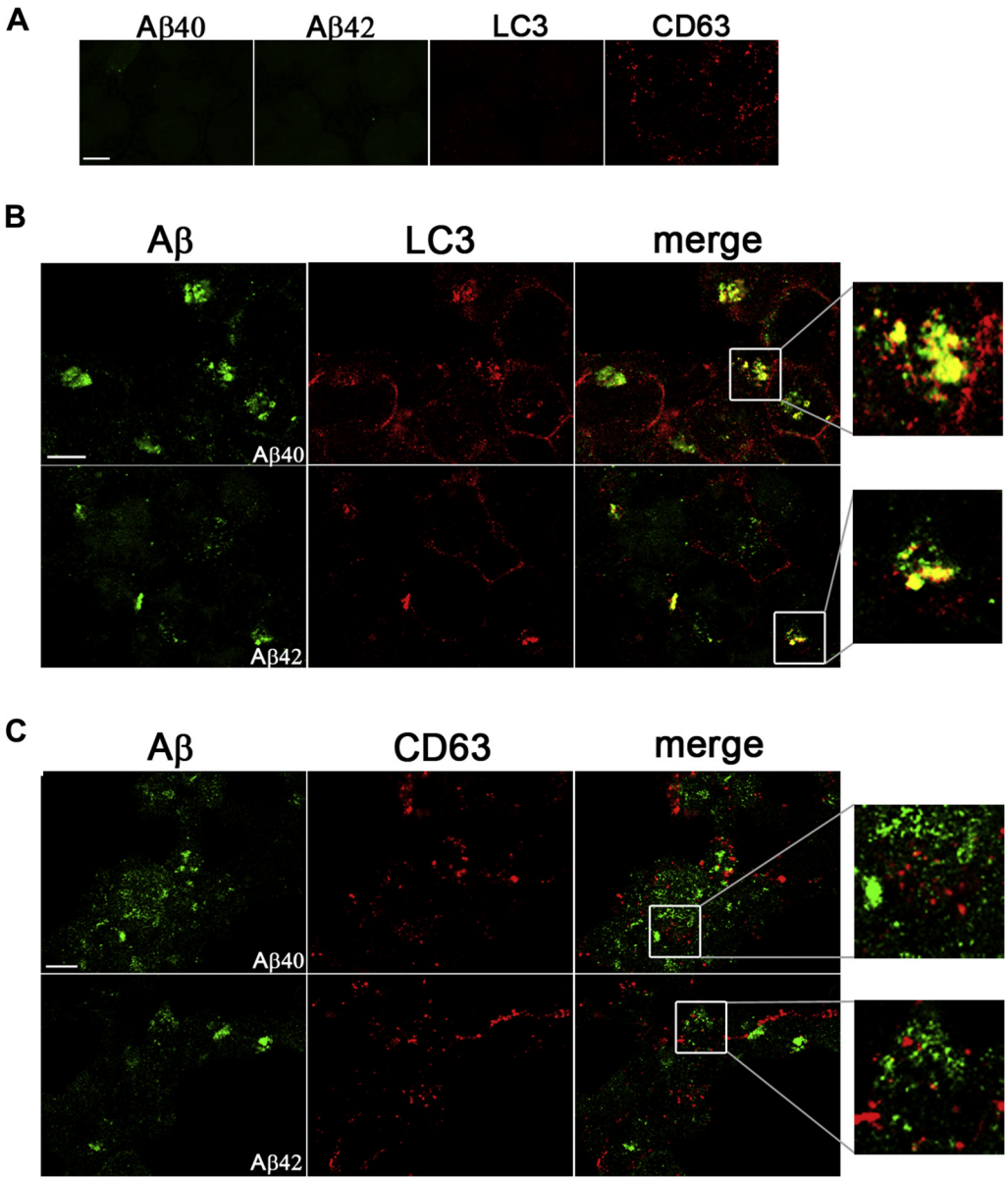

Fig. 6. Intracellular Aβ colocalizes with endogenous LC3 in herpes simplex virus type 2 (HSV-2)-infected cells. SK-APP cells were exposed to HSV-2 at 10 pfu/cell for 18 hours and then examined by confocal microscopy. (A) Mock-infected cells were immunostained for Aβ, LC3, and CD63 proteins. Aβ and LC3 signals are undetectable in these conditions. (B) Confocal images of HSV-2-infected cells obtained with anti-Aβ and anti-LC3 antibodies. The insets in the merged panels show the colocalization of green and red signals. (C) Confocal images of cells exposed to HSV-2 showing the staining pattern of Aβ40, Aβ42, and CD63 proteins. The insets in the merged panels show the absence of colocalization of green and red signals. Scale bar: 10 μm. Abbreviations: Aβ, amyloid beta; LC3, light chain 3.

shown to cause the main pathological alterations found in AD brains, supporting the hypothesis of an active role for this and perhaps other herpesviruses in AD.

Neurofibrillary degeneration is a major neuropathological hallmark of AD. It results from the aggregation of abnormally phosphorylated tau protein as paired helical filaments (PHFs). Tau is found in the adult brain in 6 isoforms, generated by alternative splicing, ranging from 48 to 68 kDa in molecular weight (Goedert and Jakes, 1990). In the present work, a complex band pattern characteristic of the expression of all 6 tau isoforms was obtained in SK-N-MC cells. In cell cultures exposed to HSV-2, all 5 examined epitopes reported an increase in phosphorylated tau levels, and the band profile of phospho-tau species became very complex. The present findings are similar to those reported in AD and other tauopathies, in which a band profile of phospho-tau strongly dependent on the phospho-specific tau antibody used is reported (Puig et al., 2005; Santpere et al., 2006). Importantly, all the sites examined in the present work are reported to be hyperphosphorylated in PHF tau in AD brains (Lovestone and Reynolds, 1997), which supports the role of herpesviruses in promoting aberrant tau phosphorylation in AD. In addition, confocal analysis showed that some of the phosphorylated tau species induced by HSV-2 accumulate in the nucleus. Consistent with these results, previous work performed at our laboratory has shown hyperphosphorylated tau to accumulate in the viral replication compartments in the nucleus of HSV-1-infected cells (Alvarez et al., 2012). The effect of nuclear accumulation of phosphorylated tau induced by HSV might not be related to tangle formation. However, tau phosphorylation may determine the recruitment of tau to the nucleus, or may alter tau activity to promote the viral replication and/or transcription processes in neuronal cells, with the consequence of neurodegeneration. Alternatively, cellular proteins may be targeted to damaged viral DNA that arises during replication (Mohni et al., 2010). In this respect, a role of tau in neuronal DNA protection has been recently described (Sultan et al., 2011). Further studies are required to examine the role of nuclear phosphorylated tau in herpesvirus infections. In addition, HSV-2 also increases tau phosphorylation in the cytoplasm of infected cells. The accumulation of hyperphosphorylated tau in the cell body could contribute to the generation of PHFs and, therefore, to the neurodegeneration characteristic of AD. The functional significance of these differential phosphorylation events is unclear but suggests that tau protein is a relevant target of HSV-2 infection and may play a functional role in the infection process.

It is well established that the buildup of intracellular Aβ is one of the earliest events in the pathogenesis of AD (LaFerla et al., 2007). HSV-2 infection was found to increase intracellular Aβ levels and to intensely inhibit Aβ secretion. Aβ accumulation has also been shown to be induced by other brain pathogens related to AD, supporting the infectious hypothesis (Miklossy, 2011). Recent work has shown that Aβ may act as a defensive molecule of the innate immune system, with activity against several bacteria and yeasts (Soscia et al., 2010), influenza A virus (White et al., 2014), and HSV-1 (Bourgade et al., 2014). This role is consistent with the idea that viral infections are associated with the etiology and pathogenesis of AD. Herpesviruses and other infectious agents might trigger an overproduction of Aβ peptides, which might contribute to amyloid plaque formation and the progression of AD.

β-secretase is the initiating and rate-limiting enzyme in the production of Aβ (Cai et al., 2001). There are numerous reports that an increase in the activity of β-secretase leads to the accumulation of intracellular Aβ, contributing to AD pathogenesis. In the present work, no significant changes were seen in β-secretase activity in HSV-2-infected cells. However, HSV-2 induced a potent inhibition of α-secretase activity along with a strong reduction in the levels of the proteolytic products α-CTF and sAPPα. This indicates a downregulation of the nonamyloidogenic pathway of APP processing in infected cells. Finally, the accumulation of intracellular Aβ in LC3-positive compartments strongly suggests a failure in Aβ degradation in the autophagic compartments induced by HSV-2. To test this hypothesis, the effects of HSV-2 on autophagy were analyzed and it was found that HSV-2 induced a marked increase in the levels of the autophagosome-associated LC3-II isoform, resulting in the accumulation of autophagic compartments in infected cells. Autophagy defects might play a role in neurodegenerative disorders, especially in AD. Indeed, large accumulations of autophagic compartments in AD brains have been reported (Nixon et al., 2005), indicating impaired clearance of autophagosomes rather than any strong induction of autophagy (Nixon and Yang, 2011). In line with these findings, the strong accumulation of autophagosomes induced by HSV-2 seems to be due to a failure in the autophagic flux. This in turn seems to be provoked by a defect in autophagosome-lysosome fusion. Interference with the late stages of autophagy might therefore enhance the accumulation of Aβ in autophagosomes in infected cells. Our group has previously reported that HSV-1 induces similar alterations in APP processing and autophagy pathways (Santana et al., 2012a, 2012b). Taken together, the present findings suggest that the intracellular accumulation of Aβ caused by HSV-2 infection is produced by the inhibition of Aβ secretion and the failure of Aβ degradation by lysosomes. The inhibition of the nonamyloidogenic pathway might also contribute to this accumulation.

Numerous experimental findings suggest the implication of HSV-1 infection in AD pathogenesis [see recent reviews by Itzhaki, 2014; Piacentini et al., 2014] and there is growing evidence that other herpesviruses might be involved. The presence of IgM anti-HSV antibodies (recognizing HSV-1 and HSV-2 antigens) in serum—a marker of HSV reactivation—was found to be correlated with an increased risk of developing AD (Letenneur et al., 2008; Lovheim et al., 2014). Recently, EBV and human herpes virus 6 DNA positivity, along with CMV and EBV IgG plasma levels, were also associated with cognitive decline and progression to AD in the elderly (Carbone et al., 2014). Finally, several studies have shown the viral load of different herpesvirus to be associated with cognitive impairment in elderly individuals with vascular risk (Katan et al., 2013; Strandberg et al., 2003, 2005), and a recent study showed that accumulated infections, including those of HSV-1 and CMV, might be associated with AD (Bu et al., 2014). It has been widely demonstrated that inflammation is strongly associated with neurodegeneration and cognitive impairment (Schott and Revesz, 2013). It may be that successive cycles of latency-reactivation of these viruses trigger systemic immune responses and induce inflammatory processes that may subsequently produce cognitive decline. Moreover, when these viruses reach the brain, they may also induce neuroinflammation, which would contribute to neuronal death. In addition, during chronic exposure to HSV-1 and/or HSV-2 infection, virally-induced Aβ and hyperphosphorylated tau might accumulate and persist in brain tissue, triggering a cascade of events leading to neurodegeneration. Consistent with this hypothesis, asymptomatic reactivation episodes promoting neuroinflammation and neurodegenerative markers have been reported in a mouse model of latent infection of HSV-1 (Martin et al., 2014). Finally, HSV-2 infection might potentiate the brain damage caused by HSV-1 or another pathogenic agent, perhaps by enhancing reactivation in the brain. However, the mechanisms responsible for the association between herpesvirus infections and AD remain unclear.

In summary, the present observations show that infection with HSV-2, like HSV-1, induces the main AD-like neurodegeneration markers in human neuroblastoma cells, including tau hyperphosphorylation, anomalies in APP proteolytic processing

leading to intracellular Aβ accumulation, and impairment of autophagy. These findings strongly support the idea that herpesviruses are involved in the pathogenesis of sporadic AD.

Disclosure statement

Soraya Santana is currently employed by the commercial company BioPharma Division, Neuron Bio, Granada, Spain. The other authors declare no actual or potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2015.06.014.

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