PhD in Public Health and infectious Disease

XXX Cycle

Multiple Herpes Simplex Virus-1 (HSV-1) reactivations induce neurodegenerative and oxidative damages in mouse brains

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1. INTRODUCTION

1.1 Herpesviridae family and Herpes Simplex Virus type 1 (HSV-1)

The Herpesviridae family includes over 200 members, capable to cause diseases in animals and humans, and among them are nine currently known to cause disease in humans (Roizman and Knipe 2001). The name herpes comes from the Latin herpes which, in turn, comes from the Greek word "herpein" which means to creep and reflects the creeping or spreading nature of the skin lesions caused by these viruses. Once a patient has been infected by herpes virus, this infection remains for life. Indeed, the initial infection (i.e., primary infection) is usually followed by latency with subsequent life-long periodic reactivations. The human herpesvirus (HHV) are grouped into three subfamilies, based on the genome structure, tissue tropism, cytopathologic effect, site of latent infection, pathogenesis, and manifestations of the disease (Murray et al 2015). These subfamilies are: 1) *Alphaherpesvirinae*, that includes herpes simplex virus types 1 and 2 (HSV-1 and HSV-2, respectively) and Varicellovirus-varicella-zoster virus (VZV), infecting a wide range of humans. These viruses are characterized by a short life-cycle, ability to rapidly spread between cells and to efficiently lyse infected cells, and the establishment of latent infections in sensory ganglia; 2) *Betaherpesvirinae*, including Cytomegalovirus-human cytomegalovirus (CMV) and Roseoloviruses including Human Herpesvirus types 6A, 6B and 7 (HHV-6A, HHV-6B and HHV-7), have a restricted host range and a long replicative cycle with slow progression of infection in cell cultures. They may establish a latent infection in glandular tissues, lymphoreticular cells, the kidneys, and other tissue; 3) *Gammaherpesvirinae*, including Lymphocryptovirus-Epstein-Barr virus (EBV) and Rhadinovirus-human herpesvirus type 8 (HHV-8), able to establish latent infections mainly in lymphoblastoid cells. Some may produce lytic infections in epithelioid and fibroblastic cells and have oncogenic potential (Barton et al 2001, Kukhanova et al 2014).

The alpha Herpesvirus HSV-1 is a highly contagious virus, that is common and endemic throughout the world, with a seroprevalence of about 60 to 85% in the adult population (Johnson et al 1989) and it is characterized by specific biological and molecular features.

1.1.1 HSV-1 Structure

HSV-1 is an enveloped virus with a linear double-stranded DNA genome enclosed by an icosahedral capsid composed of 162 capsomeres. The virion is spherical with 186 nm in

diameter with glycoprotein spikes anchored to the envelope (Arduino and Porter 2008). The genome consists of two covalently linked components, designated as L (long) and S (short), each formed by unique sequences (UL e US, respectively) flanked by regions of repeated and inverted sequences that facilitate replication of the genome (Murray et al 2015). The capsid is composed by viral proteins VP5, VP19C, VP23, VP24, VP26, and the protein encoded by the UL6 gene (De Silva and Bowers 2009). Between the capsid and the envelope there is an amorphous layer, called tegument, composed of more than 20 proteins that regulate virus lifecycle. Among them the main abundant and essential proteins are:

- α-trans- inducing factor (α-TIF), known as VP16, involved in transcription of immediate early genes (IE);
- the virion host shut-off protein (VHS) or VP22, responsible for degrading the mRNA of the host cell in the cytoplasm;
- VP12 which may play a role in DNA release at the nuclear pore during viral entry.

The envelope consists of a lipid bilayer and anchors approximately 11 viral glycoproteins, four of which (gB, gD, gH, and gL) are essential for virus entry into cells (Arduino and Porter 2008).

1.1.2 Life-cycle

HSV-1 infection is initiated by virus adsorption to host cell membrane through the binding of viral glycoproteins to at least three different classes of cell-surface receptors. Among them, heparan sulphate (HS) is considered the main HSV-1 binding receptor, having a key role in adherence of viral glycoproteins C (gC) and B (gB) on the surface of the target cell (Spear et al 2004). The interaction of gC and gB with HS receptor is labile until the participation of gD in the process. Several cellular receptors for gD are known as herpesvirus entry mediator (HVEM), such as nectin 1 and 2, and a specific form of HS called 3-O-sulfated heparan sulphate (3-OS HS). When gD interacts with one of these receptors, its polypeptide chain undergoes a conformational change enabling the interaction with the viral heterodimer gH/gL. The resulting complex exposes the fusion domains gH/gL that, together with the fusion domain of gB, allow fusion of the viral envelope with the plasma membrane of the target cell (Everett 2014).

After penetration into the cytoplasm, the nucleocapsid is transported via cytoskeleton microtubules to a nuclear membrane pore, that allows viral DNA release in the nucleus. In parallel, tegument viral protein VP16 binds a cellular protein called host cell factor (HCF-1) in

the cytoplasm, and together they interact with the octamer-binding protein (Oct-1). This neo-formed complex is able to enter in the cell nucleus and, forming an activator complex that associates to viral DNA, allows to start the processes of viral genome transcription and replication (Roizman and Whitley 2013). These processes involve the regulated expression - through sequential and coordinated steps- of three sets of viral genes: α or immediate early (IE), β or early (E), and γ or late (L). Initially, the viral DNA is transcribed by the host cell RNA polymerase II, and IE genes are the first genes transcribed. In detail, six IE proteins are synthesized in the early stages (synthesis peak at 3h p.i.) of a productive infection: ICP0, ICP4, ICP22, ICP27, ICP47, and a protein encoded by the US1.5 gene. Then, α proteins return to the nucleus to activate transcription and translation of the β genes in early proteins or β polypeptides (synthesis peak at 6h p.i.), including DNA polymerase and thymidine kinase, that are responsible for the synthesis of viral nucleic acid. As soon as β proteins are expressed, several cellular proteins migrate to the nucleus and gather to form the replication complex in the pre-replicative sites where the synthesis of viral DNA begins (Knipe and Cliffe 2008). Then, the γ gene transcription and translation start resulting in late protein expression, such as tegument and capsid proteins and glycoprotein spikes. Then capsid assembly and DNA packaging occur, followed by capsid budding from the inner nuclear membrane into the perinuclear space. The neo-particles then bud through the outer nuclear membrane via membrane fusion, and are released into the cytoplasm, where they capsids associate with the membranes of Golgi vesicles. These are plenty of tegument and envelope proteins that become assembled around the capsids. Finally, these vesicles bud out of cytoplasmic membrane thus releasing the mature virus particles from the cell (Abaitua et al 2009).

1.1.3 Latency and Reactivation

HSV-1 is a neurotropic virus that establishes latency within sensory neurons. Following primary infection, the virus replicates productively within mucosal epithelial cells and enters in sensory neurons via nerve termini. The virus is then transported through retrograde axonal transport to neuronal cell body where latency can be established. This results in the lifelong retention of the HSV-1 genome in a silent state and episomal form in the nucleus, characterized by repression of almost all viral genes. Only one region, encoding the latency-associated transcripts (LATs), remains active during latency. These are a set of co-linear RNAs transcribed from a locus within the repeat regions flanking the

unique long region of the viral genome (Stevens et al 1987). Their transcription leads to the production of an 8.3 kb primary transcript, which is then spliced to produce an unusually stable 2.0 kb intron, which is further spliced to produce an additional stable 1.5 kb intron (Zabolotny et al 1997). LAT role in controlling virus latency has yet to be fully understood, but several evidences support their involvement in the downregulation of viral gene required for lytic replication, such as the IE ICP0. Furthermore, HSV-1 encodes several microRNA (miRNAs) that cooperate with LATs in the regulation of viral life-cycle. Periodically, the silent viral genome responds to cellular signals that provoke the resumption of viral gene expression and in turn the reactivation of virus normal lytic cycle, resulting in the generation of new viral progeny that are transported axonally back to the mucosal tissues.

1.1.3.1 Establishment and regulation of latency

Latency establishment in sensory neurons has thought to be the result of a failure in initiation/activation of IE gene expression (Preston et al 2000, Efstathiou & Preston, 2005). Consistent with this view, several studies showed that virus mutants in one or more IE genes or VP16 have an impairment in starting lytic cycle (Dobson et al 1990, Steiner et al 1990, Sedarati et al 1993, Marshall et al 2000), whereas they are able to establish latent infection. In addition, the formation VP16-induced complex (VP16, HCF-1 and Oct-1) required for efficient lytic gene expression is thought to be severely impaired in sensory neurons, due to restrictions in the availability of all its members: VP16 was suggested as not efficiently transported along axons, so that insufficient amount reach the neuronal cell body (Kristie et al 1999); HCF-1 was exclusively detected in the cytoplasm in sensory neurons (Kolb & Kristie 2008), thus being unavailable to participate in the formation of a VP16-induced complex; Oct-1 expression levels were found downregulated in neuronal cells (Lakin et al 1995).

Another possible mechanism that may account for HSV-1 latency involves the immune system cells. In particular, Theil and colleagues (2003) detected non-cytotoxic CD8+T cells around HSV-1 infected sensory neurons even when HSV-1 were in latent form. Moreover, Knickelbein and colleagues (2008) demonstrated that CD8+ T cells can completely inhibit HSV-1 reactivation in mice through both IFN-γ and Granzyme B secretion. In particular, IFN-γ blocks HSV-1 gene expression cascade, whereas Granzyme B is able to trigger the degradation of viral ICP4 that is essential for further viral gene

expression. Furthermore, Ramachandran and colleagues (2010) provided evidence that specific non-cytotoxic CD8+T cells recognize gB expressed at low levels on HSV-1-infected neurons during latency phase, thus inhibiting virus reactivation at a very late stage of viral life-cycle. Since gB is a late viral gene normally expressed during the last stage of viral gene cascade, its detection during HSV-1 latent infection indicates that also IE and early genes have been likely expressed and that $CD8^+$ T cells succeeded in inhibiting HSV-1 reactivation without blocking viral gene expression.

1.1.3.2 Reactivation from latency

Several stimuli are known to induce HSV-1 reactivation. In humans, exposure to UV light, emotional stress, fever, tissue damage and immune suppression are known inducers of virus reactivation. Several agents have also been described as able to reactivate virus in cultured neurons, such as: NGF deprivation (Wilcox and Johnson, 1987), the histone deacetylase inhibitor trichostatin A (Arthur et al 2001), forskolin (Danaher et al 1999), inducible cyclic AMP early repressor (Colgin et al 2001), capsaicin (Hunsperger & Wilcox, 2003), caspase-3 activator C2-ceramide (Hunsperger and Wilcox, 2003), transient hyperthermia (Moriya et al 1994) or dexamethesone (Halford et al 1996).

Miller and colleagues reported in an *in vitro* neuronal model that VP16 is an essential factor for stress-induced HSV-1 reactivation (Miller et al 2006). Some years later, Sawell's group validate the importance of VP16 in HSV-1 reactivation in *in vivo* model demonstrating that VP16 is required at the first stage in the transition from the latent to the lytic cycle, and its absence not allows latent viral genome to reactivate and enter in lytic cycle (Thompson et al 2009). They hypothesize that stress induces de novo production of VP16 and this could coordinate active IE genes to start the lytic transcriptional program. All these were unexpected findings since it is known that VP16 is a late protein that is optimally expressed only after the onset of viral DNA replication.

Other data have also shown that HCF-1 recruits the lysine-specific demethylase (LSD1) to viral IE promoters in order to reverse repressive histone methylation marks, and in turn facilitate both viral gene expression and reactivation from latency (Liang et al., 2009).

Establishment of latency as well as reactivation are critical phase of HSV-1 life cycle and it is not surprising that they are highly regulated. Unfortunately, the mechanism beyond HSV-1 reactivation is not well understood and studies are ongoing to better explain this main viral process.

1.1.4 Epidemiology

Primary HSV-1 oropharyngeal infection occurs mostly early in life and is most often asymptomatic. In less developed countries HSV-1 seroconversion occurs early in life, at age of 5 years in around one out of three children, and by 70–80% within adolescence. More than 3.7 billion people under the age of 50, approximately 67%, are infected by HSV-1. The prevalence of HSV-1 infection worldwide has increased over the last several decades making it an important problem of public health (WHO 2015, Looker et al 2015).

1.1.5 Clinical Manifestation

HSV-1 disease ranges from the usual case of mild illness, no discernible in most individuals, to sporadic, severe, and life-threatening disease in a few infants, children, and adults.

Generally, the primary HSV-1 infection occurs in oropharyngeal mucosa. The infection may have a great variety of symptoms, but in the most cases is fully asymptomatic. The symptomatic cases typically affect the lips, tongue, gingiva, buccal mucosa, and palate and it may cause fever, throat ulcers, ulcerative and vesicular lesions, gingivostomatitis and edema (Arduino and Porter 2008).

HSV-1, mainly in young people, can also causes genital herpes, due to the practice of oral sex. Genital herpes is characterized by ulcerative vesicles in the penis and in the cervix, vulva, vagina, or perineum (Mertz 1993). Other manifestations are keratoconjunctivitis, cutaneous lesions by cuts or abrasions on the skin, paronychia/whitlow (fingers), glossitis, herpes gladiatorum (body), and erythema multiform, a rare skin rash on the back of arms and hand (Arduino and Porter 2008). Encephalitis is one of the complications following primary infection and is associated with a high mortality rate and neurologic sequelae among survivors (Jouan et al 2015, Kennedy et al 2002). In the developed world HSV-1 accounts for a large proportion of Herpes Simplex Encephalitis (HSE) (Singh et al 2015, Whitley et al 1977). HSE is estimated to affect at least 1 in 500,000 individuals per year (Whitley et al 2006) and some studies suggest an incidence rate of 5.9 cases per 100,000 born (Kropp et al 2006) and approximately 50% of individuals who develop HSE are over 50 years of age (Whitley et al 2002).

Confirmation of Herpes Simplex Encephalitis (HSE) is usually based on the detection of anti-HSV-1 antibody in the serum or in cerebrospinal fluid (CSF), or by detection of viral antigen or genetic material in the same specimen.

1.1.6 HSV-1 in Central Nervous System (CNS)

The mechanism used by HSV-1 to reach, infect CNS and cause encephalitis has not been definitively established. The neuropathological aspects of HSE consist in necrosis and inflammation of brain tissues, especially at the orbitofrontal and temporal lobes with involvement of insular cortex (Kennedy and Chaudhury, 2002). The clinical features of HSE are nonspecific, in fact the disease may cause an influenza-like illness or bring symptoms like fever, headache, memory loss or altered consciousness that goes unnoticed and passes within a few days.

Many studies reported mild forms of HSV-1 encephalitis. These patients with mild encephalitis usually have less severe symptomatology and good prognoses when compared to patients with severe diffuse HSE. Klapper and colleagues (1984) suggest that sub-acute HSV-1 encephalitis is not so rare, but often it is missed sub-clinical presentation of the disease (Marton et al 1995). Interestingly, DeBiasi et al (2002) detected viral genes in CSF from a higher percentage of patients than that accounting for HSE, thus providing evidence of mild and asyntomatic HSE in humans. Moreover, some studies detected HSV-1 genome in post-mortem brain from patients died of non-neurological diseases (i.e., frontal and temporal lobe brain from immunosuppressed leukemia patients) and suggested that virus reactivations occur easily in brains of immunosuppressed patients (Saldanha et al 1986).

Additionally, Lewandowski and colleagues (2002) in brain from HSV-1 infected mouse detected TK gene, using PCR technique, in the absence of infectivity, 30-40 days after primary infection, indicating that HSV-1 may establish a latent infection in brain tissue as well as in trigeminal ganglion.

Overall these findings support the idea that HSV-1 may easily reaches CNS where it can establish active or latent infection.

1.2 Alzheimer's Disease

Alzheimer's disease (AD) is a chronic and progressive neurodegenerative disorder and the most common neurodegenerative disorder in the industrialized world. In 1906, Aloise Alzheimer, a German Psychiatrist, identified and presented the first case of a fifty-year-old woman called Auguste Deter which suffered by early senile dementia, loss of memory and temporary vegetative states.

In the early stages of AD, the most commonly recognized symptom is the inability to

acquire new memories, such as difficulty in recalling recently observed facts. Gradually, bodily functions are lost, ultimately leading to death. Individual prognosis is difficult to assess, and the duration of the disease varies. Neurodegeneration associated with AD develops for an indeterminate period before becoming clinically apparent, and it can progress undiagnosed for years. In fact, the cause and progression of AD are not well understood. When AD is suspected, the diagnosis is usually confirmed by behavioral assessments and cognitive tests, often followed by a brain scan. Indeed, subjects with AD typically show patterns of atrophy involving the medial temporal lobe, particularly the hippocampus and entorhinal cortex, and the posterior cingulate, precuneus and the tempo-parietal neocortex, with concurrent expansion of the ventricles.

1.2.1 Epidemiology

It is estimated that 46.8 million of people worldwide currently suffer from AD (WHO, World Alzheimer Report 2015). This number is expected to double by 2030 and more than triple by 2050. Dementia affects people in all countries; with more than half living in low- and middle-income countries and by 2050 this is likely to rise to more than 70%.
The regional distribution of AD cases is 22.9 million (49% of the total) in Asia, 10.5 million (22%) in Europe, 9.4 million (20%) in the Americas, and 4 million (9%) in Africa. Compared to 2012 estimates, these values represent an increased proportion of new cases arising in Asia, the Americas and Africa, while the proportion arising in Europe has fallen (WHO, World Alzheimer Report 2015). In Italy, AD patients are almost 1 million and the annual incidence is going to increase because of the progressive increase of the mean age and life expectancy in our population. Moreover, AD is more prevalent in women than in men, likely because of differences in life expectancy between the genders (Vest et al. 2012).

1.2.2 Risk Factors for AD

- *Age*

  The principal risk factor for AD is age, indeed the incidence of AD increases exponentially with increasing age, doubling with every 6.3-year increase in age. Most patients develop AD after the age of 65 years old. The risk of developing AD reaches 50% for individuals beyond age 85.

- *Role of environment*

Several studies indicate a role for environmental effects on AD development. In a recent review Mayeux and colleagues (2012) summarized the role of diet, activities, or diseases that potentially play a role in the onset of AD. Diabetes, hypertension, smoking, obesity, and dyslipidemia have all been found to increase risk as well a history of brain trauma, cerebrovascular disease, and vasculopathies.

- *Genetics of AD*

  The most AD cases are not inherited although some genes may act as risk factors. The current thinking is that AD can be divided in sporadic/late onset and familial/early onset (FAD). Usually, FAD patients develop symptoms before 60 years of age and the disease may appear among persons between ages 30 and 40. Different gene polymorphisms are involved in FAD pathogenesis. The most important genes are: PSEN1, PSEN2 APP, and APOE (Tanzi et al 2012); among them the presence of the APOE-ε4 allele is considered a common genetic risk factor also for the sporadic form of AD (Chartier-Harlinet al 1994). APOE gene is polymorphic, with three common alleles (ε2, ε3, ε4), and in studies of the general population six genotypes are observed: 3/3, 4/3, 3/2, 4/4, 4/2, and 2/2. The three major alleles of the APOE locus, ε2, ε3, and ε4, correspond to combinations of two amino acid changes at residues 112 and 158 (ε2: Cys112/Cys158; ε3: Cys112/Arg158; ε4: Arg112/Arg158). APOE ε 4/4 increases the AD risk more than about 12-fold, and APOE ε3/4 about 3-fold when compared with the APOE ε 3/3 genotype (Koffie et al 2012). In contrast, the APOE ε2 allele exerts "protective" effects (Corder et al. 1994). Interestingly, imaging studies and biochemical analyses on post-mortem AD brains provide evidence linking synaptic toxicity, Aβ oligomers and APOEe4, suggesting that the presence of this allele exacerbates Aβ toxicity and accelerate AD progression (Koffie et al 2012). However, the mechanistic link between apo APOE isoform and their roles in AD remains to be clarified.

1.2.3 Pathogenesis

Alois Alzheimer firstly described histopathological features of August Deter's disease in his autopsy case report

described "a peculiar disease of the cerebral cortex", the clinical and neuropathological features of August Deter. He noted that brain's August showed a thinner cerebral cortex usually seen in elderly people as well as two further abnormalities in the brain: amyloid plaques which are extracellular deposits of a neurotoxic substance and neurofibrillary

tangles which represent changes in the cytoskeleton of nerve cells which are often associated with cell death.

1.2.3.1 Amyloid beta (Aβ) aggregates

The current view of AD pathogenetic mechanisms describes amyloid deposition and neuritic plaques formation as key events leading to neurodegeneration and cognitive impairment. Amyloid plaques consist mainly of aggregates of A□s, that are peptides mainly of 40-42 amino acids generated by atypical proteolytic cleavage of amyloid precursor protein (APP). APP is a trans-membrane glycoprotein widely expressed, produced by the endoplasmic reticulum and involved in the neuronal and dendritic growth and synapses formation (Steuble et al. 2012). The metabolic cleavage of APP involves three different enzymes called α, β and γ secretase, that work in combination. Sequential processing by the α and γ-secretases result in a large N-terminal peptide called soluble APPα (sAPPα) and a smaller 3 kDa peptide called P3. Proteolytic cleavage by α secretase prevents Aβ release and results in the so called non-amyloidogenic pathway (Thinakaran et al. 2008). On the contrary, when both β and γ secretases process APP, Aβ release occurs. In detail, the β-secretase cut produces a large N-terminal peptide called soluble APPβ (sAPPβ), as well as a smaller C-terminal fragment called CTFβ.Then, γ-secretase cuts CTFβ and Aβ peptides, that are the upstream 40-42 amino acid peptides defined by the β- and γ-secretase cuts (Aβ40 and Aβ42, respectively). Aß40 is the major form of secreted Aβ, although Aß42 aggregates are thought to seed extraneuronally more readily during the early stages of plaque formation (Jarrett et al 1993). Aβ degradation is mainly mediated by enzymes, including neprilysin and insulin degrading enzyme (IDE). Although AD plaques are extracellular deposits of Aβ aggregates, it was wide demonstrated that accumulation of Aβ42 begins intraneuronally both in transgenic AD mouse models (Lord et al 2006, Zerbinatti et al 2006) and in AD patients (Busciglio et al 2002, Ohyagi et al 2005). Interestingly, intraneuronal Aβ peptide accumulation appears before plaques formation but together with cognitive impairments, suggesting that intraneuronal Aβ peptide accumulation is one of the earliest events of AD pathogenesis (Gouras et al 2010). Moreover, under pathological conditions, Aβ can form different aggregate species, which include amyloid fibrils, protofibrils, annular structures, and smaller oligomeric species (Selkoe 2008, Caughey et al 2003). Oligomers of Aβ peptides can organize into dimers, trimers, tetramers, and higher forms, and increasing

evidence suggests that Aβ42 oligomers are pathogenic, especially in AD. A study by Cleary and colleagues (2005) showed that infusion of Aβ dimers and trimers into rodent brain induced cognitive impairments (Cleary et al 2005). These reports suggest that Aβ42 oligomers may be pathogenically important in AD, although the determination of which precise species may be the most involved is technically challenging and controversial. Moreover, Aβ42 was widely demonstrated to induce a redox imbalance through ROS production, protein oxidation, lipid peroxidation both in in vitro and in in vivo model (Behl et al 1997, Butterfield et al 2013).

1.2.3.2 Neurofibrillary tangles

Neurofibrillary tangles (NFTs), composed mainly by hyperphosphorylated tau, are an intracellular hallmark of AD. Tau is a microtubule-associated protein (MAP) crucial for microtubule assembly and stabilization, and is mainly expressed in neuronal axon (Wang et al 2016). It has more than 30 phosphorylation sites that may be targets for intracellular kinases. When tau is abnormally hyperphosphorylated, it loses the capability to bind microtubules, resulting in their destabilization and in turn cytoskeleton collapse, and in protein progressive aggregation in fibrils, paired helical filaments (PHF) and finally NFTs (Santacruz et al 2005). Thus, tau phosphorylation is a key event in neurodegeneration and is mainly determined by a balance between the activation of various tau protein kinases and phosphatases. The kinases responsible for tau phosphorylation are grouped into three classes: (1) proline-directed protein kinases (PDPK), including glycogen synthase kinase-3 (GSK3), cyclin-dependent protein kinase-5 (CDK5), and mitogen activated protein kinases (MAPK) (e.g. p38, Erk1/2 and JNK1/2/3); (2) non-PDPK, including tau-tubulin kinase 1/2 (e.g. casein kinase 1α/1δ/1ε/2), dual specificity tyrosine- phosphorylation-regulated kinase 1A/2, microtubule affinity regulating kinases, phosphorylase kinase, cAMP-dependent protein kinase A (PKA), PKB/Akt, protein kinase C, protein kinase N, and Ca2+/calmodulin-dependent protein kinase II (CaM kinase II); and (3) tyrosine protein kinases, including Src family kinase (SFK) members (e.g. Src, Lck, Syk, and Fyn), and c-Abelson kinase or Abl related gene kinase. Phosphatases responsible for tau dephosphorylation are also usually classified into three classes according to the structure of their catalytic site and their sensitivity to inhibitors. These groups are: (1) phosphoprotein phosphatase (PPP), (2) metal-dependent protein phosphatase, and (3) protein tyrosine phosphatase (PTP).

Tau pathology has been considered for decades to be a downstream effect of Aβ in the progression of AD (Hardy et al 1991). However, tau can also form aggregates in the absence of Aβ, directly leading to synaptic impairment (Cowan et al 2013). Previous studies have shown that hyperphosphorylated tau colocalizes with Aβ in synaptic terminals in brain from postmortem AD patients as well as from transgenic mouse models of AD (Fein et al 2008). The reduction in total synapse number is also correlated with increased p-tau and Aβ levels (Takahashi et al 2010). Some evidence suggested that truncation of tau protein at the aspartic acid421 site (Asp421) by caspase3 precedes its hyperphosphorylation and may be necessary for the assembly of tau into filaments in AD and other tauopathies (Canu et al 1998, Gamblin et al 2003, Rissman et al 2004). Other in vitro studies showed that this cleavage can increase the polymerization rate of tau (Gamblin et al 2003, Rissman et al 2004, Fasulo et al 2000). In AD-affected brains, tau truncated at Asp421 has been found in NFTs (GAmbiln et al 2003, Chung et al 2001), and their amount significantly correlated with the increase of clinical symptoms (Wischik et al 1988, Chung et al 2001). Interestingly, this truncation was reported as toxic and capable of inducing morphologic and functional alterations when expressed in several neuronal and non-neuronal cell models. Moreover, it was recently demonstrated that a small number of NFT composed of Asp421-truncated tau coexisted in neurons undergoing apoptosis (Garcia-Sierra et al 2011). In addition, growing evidence demonstrated that Aβ oligomers can elicit tau hyperphosphorylation and seed tau oligomerization both in vitro and in vivo (Castillo-Carranza et al 2015). Both Aβ and NFT aggregates can be easily cleared by two specific mechanisms used by the cell to eliminate misfolded protein: autophagy and unfolded protein response (UPR). Interestingly, both abnormal UPR and impaired autophagy have been implicated in the development of AD. Associated with the abnormal marked accumulation of Aβs and the deposition of NFTs, the brain of patients suffering AD present a significant degree of oxidative damage (Melov et al 2007, Marques et al 2003, Cai et al 2011).

1.2.3.3 Oxidative Stress

Several lines of evidence support the hypothesis that oxidative stress play a detrimental role in the pathogenesis of AD leading to the damage of vital cellular elements such as nucleic acids, lipids and proteins (Butterfield et al 2014, Butterfield et al 2010, Mecocci et al 1994). Oxidative stress is a condition where reactive oxygen species (ROS) and reactive

nitrogen species (RNS) production overcome the cellular antioxidant system. Similarly, oxidative stress result from the accumulation of oxidized/damaged macromolecules that are not efficiently removed and renewed. A major protective system of the cell are antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutaredoxins, thioredoxins and catalase, as well as non-enzymatic antioxidant factors. A reduction or a loss of function of the antioxidant enzymes, as indexed by decreased specific activity, has been reported in AD (Chang et al 2014). Senile plaques and Aβ aggregates can induce inflammatory responses and oxidative stress. Oxidative stress has been associated with abnormal tau phosphorylation and aggregation, and with the formation of NFTs which in turn induce neuron death, and this can result in further inflammation and oxidative stress. On the other hand, inflammation and oxidative stress can enhance further Aβ deposition, resulting in a feedback loop (Zhang et al 1997).

Proteins are highly susceptible to oxidative damage that inevitably affects secondary and tertiary structures resulting in irreversible modification of protein shape and, consequently, function. These modifications include dissociation of subunits, unfolding, exposure of hydrophobic residues and aggregation (Dean et al 1997). The pathologic aggregation of protein can lead to fibril formation and insolubility (Markesbery et al 1999).

There are four major indices of protein oxidation that are detectable by standard methods: protein carbonylation, protein nitration, protein bound-lipid peroxidation products and protein glycoxidation. Protein carbonyl groups are generated from the direct oxidation by ROS of various side chains of amino acid residues (Lys, Arg, Pro, Thr, His and others). Protein tyrosine nitration (3-NT) is a well-characterized chemical modification and is normally considered an irreversible form of protein damage, and therefore a robust biomarker of nitrosative stress (Beckman et al 2002). Protein nitration occurs following formation of peroxynitrite ($ONOO^-$) from superoxide ($O2^-$) and nitric oxide (NO). Peroxynitrite, in the presence of CO2 covalently modify tyrosine residues to produce 3-NT. Intriguingly, the addition of a large nitro group in the 3′ position causes steric constraints on the tyrosine residues which in turn affects its phosphorylation, via tyrosine kinases, and therefore can compromise several signaling mechanisms (Schopfer et al 2003). Lipid peroxidation is one of the main events causing redox imbalance and subsequent buildup of oxidative damage within the cell. Lipid peroxidation is able to directly damage membranes, because ROS attack polyunsaturated fatty acids (PUFA) in the lipid bilayer. Neuronal membranes are particularly rich in PUFAs, that are broken down and release elevated levels of reactive electrophilic aldehydes, able to covalently

bind proteins by forming adducts with specific amino acids (Perluigi et al 2012). According to a number of factors, such as acyl chain length and degree of unsaturation, the lipid hydroperoxide that is formed by reaction of a carbon radical with oxygen, can form malondialdehyde (MDA), 4-hydroxy-2- trans-nonenal (HNE) and acrolein, besides other products of lipid peroxidation. Among these, HNE is a highly reactive and is primarily produced in the brain via lipid peroxidation of arachidonic acid, a highly abundant PUFA component of neuronal membranes. Since the brain membrane phospholipids are highly composed by PUFA, this organ is particularly vulnerable to free radical attacks. Furthermore, oxidation of the brain can affect DNA, producing strand breaks, sister chromatid exchange, DNA-protein crosslinking, and base modification. Thus, the overproduction of ROS resulting in oxidative stress may have a deleterious effect and can be an important mediator of damage to cell structures in various disease states and aging (Huang et al 2016). Butterfield and Markesbery's group showed that oxidative stress in brain was associated with AD. They highlighted, indeed that brains in subjects with AD compared to controls are under significant oxidative stress, evinced by several markers, among them elevated indices of protein oxidation (protein carbonyls and 3-NT), HNE level and other oxidative markers (Sultana et al 2006, Butterfield et al 2004, Markesbery et al 1997, Castegna et al 2002).

1.2.3.4 Inflammation

The hypothesis that inflammation may participate in AD pathogenesis has debated for 20 years, but its role remains uncleared.

Inflammatory response is a very complex process; slightly regulated, that involves the synthesis and the release of numerous factors such as cytokines, inflammatory mediators, histamine, prostaglandin and some hormones (McGeer et al 1998). Epidemiological investigations also support the notion that inflammation may play a significant role in AD. In fact, head injuries in early adulthood may be associated with increased risk of AD in late life, and the routine use of non-steroid anti-inflammatory drugs is associated with a decreased incidence of AD (Plassman et al. 2000). Inflammation is associated with the degeneration of brain areas; in fact, senile plaques in AD brains are associated with reactive astrocytes and activated microglial cells and cytokines and acute phase proteins are over expressed in microglia and astrocytes surrounding neuropathological lesions in AD brains. Inflammatory factors, such as cytokines, chemokines, complement components

and acute phase proteins co localize as secondary components in neuritic or senile plaques or are over-produced in AD brains, and activated microglia surround senile plaques and areas of neurodegeneration (Wang et al 2015). There is accumulating evidence that Aβ peptide may promote or exacerbate inflammation by inducing glial cells to release immune mediators. In AD brain, moreover, inflammatory response appears to be altered: levels of cytokines such as TNF, IL-1, IL-6, IL-8 IL-10 and some interferons seem to be elevated (Wang et al 2015).

Inflammation clearly occurs in pathologically vulnerable regions of the AD brain, and it does so with the full complexity of local peripheral inflammatory responses. On the other hand, several other investigations have shown increased blood levels of some cytokines, such as IL-1β and IL-6, and acute phase proteins as α-1-antichymotrypsin (ACT) both in brain homogenates and in peripheral blood from AD patients (Licastro et al. 2000, Licastro et al. 2003). These findings suggested that an important, but still largely unknown, interplay between brain and peripheral immune responses existed in the diseases

Although inflammation, Aβ peptide accumulation, tau hyperphosphorylation and oxidative stress are surely involved in the pathophysiological process of AD, the etiology of AD remains uncertain, and several environmental risk factors have been proposed. Among them, a growing body of evidence support the role of infectious agents especially those able to reach end establish a chronic infection in the brain (De Chiara et al 2012, Piacentini et al 2014).

*1.3 **HSV-1 and AD***

1.3.1 Pioneer studies

In 1982, Melvin Ball hypothesized that HSV-1 played a key role in AD pathogenesis. The first evidence suggesting the involvement of HSV-1 in AD is based on the observation that people surviving to HSE showed some clinical signs AD-like (i.e., memory loss and cognitive impairment) and that brain regions primarily affected in HSE (limbic system, frontal and temporal cortices) were the same regions compromised in AD. Thus, he proposed that latent HSV-1 reactivating from the trigeminal ganglia, where it establishes a

latent infection, may ascend along nerve and affect brain tissues causing damages AD-like (Ball 1982).

This hypothesis was supported by subsequent studies showing the presence of HSV-1 in brain areas (temporal cortex and hippocampus) most affected by AD (Jamieson et al 1992; Mori 2004; Rodriguez 2005). In particular, Jamieson et al. using PCR to detect HSV-1 DNA in autoptic brains found that latent virus is present in a high proportion (70–100%) of sporadic AD and elderly normal brains (Jamieson et al 1991). Interestingly, the virus was found in very low percentage of brains from young people, most likely because their immune response was stronger than those of elderly and thus more able to efficiently control HSV-1 latency. Another explanation may be that the Blood Brain Barrier (BBB) could be more damaged in old people and this could facilitate HSV-1 entry in the brain (Jamieson et al 1992).

Additional evidence for HSV-1 involvement in AD were based on possible synergisms with APOE-ε4 allele. Indeed, a significantly increased risk for sporadic AD is associated with the presence of both HSV-1 DNA in brain and carriage of the APOE-ε4 allele (Itzhaki et al 1997; Itzhaki et al 1998). Interestingly, Burgos and colleagues, by infecting transgenic mouse models expressing different APOE isoforms, measured the highest HSV-1 titer in brain tissues from APOEe4 transgenic mice, thus demonstrating that HSV-1 infection is stronger in brain carrying APOEe4 allele (Burgos et al 2003). The mechanisms involved in this link are not completely defined, but the most plausible hypothesis is based on the evidence that HSV-1 and apoE compete for the same receptor (i.e., heparan sulfate proteoglycans, HSPG) to enter into cells (Itzhaki et al 2006). Differences in the strength of apoE-HSPG association correlate with the different APOE allele, indeed APO-ε4 exhibits less affinity for HSPG than the other APOE isoforms. Thus, in APOe4 carriers HSV-1would find more available receptor for entering into host cell (Burgos et al 2003).
A growing body of evidence demonstrated the possible link between HSV-1 cerebral infection and AD analyzing different aspects of this potential relationship.

1.3.2 Epidemiological and Immunological studies

By following up a large group of old patients (512) for 14 years, Letenneur and colleagues studied, the risk of developing AD according to the presence of serum anti-HSV-1 IgG,

marker of life-long infection, and IgM, marker of primary infection and/or virus reactivation, antibodies. He saw that the subjects who were IgM-positive showed a significantly higher risk of developing AD whereas no significant increased risk was found in IgG-positive subjects. In addition, among the IgM-positive subjects, only a really few percentage were IgG-negative, that supports recent HSV-1 reactivation rather than primary infection occurring in most of the IgM-positive subjects (Letenneur et al 2008). Similar results were obtained in another study involving a larger amount (3432) of elderly patients with a mean follow-up time of 11.3 years. Increased serum levels of anti-HSV-1 IgM antibodies were associated with increased risk of developing AD by a factor of 2 (Lovheim et al 2015). In the same line is the study of Kobayashi and colleagues, that demonstrates a strong correlation between HSV-1 reactivation and the risk for AD. These authors measured the avidity index of anti-HSV-1 IgG antibodies as an indicator of HSV-1 reactivation in the blood from g patients with amnestic mild cognitive impairment (MCI, a cognitive disorder that may result in AD), AD, and healthy controls. They showed that MCI patients had a higher anti-HSV-1 IgG antibody avidity index than AD patients or healthy controls, suggesting that HSV-1 reactivations occur more frequently in the MCI group than in the other groups, and that they may concur to the conversion of MCI in AD (Kobayashi et al 2013).

More recently, Mancuso and colleagues (2014) showed that higher levels of HSV-1 IgG antibodies inversely correlate with AD-related damages in AD brain cortex, and suggest that they may play a protective role in the early stages of the disease, thus underlining again the link between HSV-1-damaging effects and AD (Mancuso et al 2014).

1.3.3 HSV-1, APP cleavage and Aβ accumulation

Several studies demonstrated that HSV-1 also affects APP processing. Itzhaki's group found that in neuronal cells HSV-1 induces APP cleavage with the production of a 55-kDa fragments starting from 6 h post infection and the concomitant reduction of band intensity relative to full-length APP (Shipley et al 2005). They also showed that HSV-1 induce in human neuroblastoma cells (SH-SY5Y) Aβ42 and Aβ40 production, and increased amounts of enzymes involved in the cleavage of APP, such as Beta-site AβPP-Cleaving Enzyme (BACE-1, β secretase) and nicastrin (a component of the gamma secretase enzyme) (Wozniak et al 2007). In vivo studies also support HSV-1 as causative in the formation of Aβ. Indeed, mouse infected with HSV-1 showed in brain tissues an increase

in Aβ42 five days post-intranasal infection when compared to uninfected controls (Wozniak et al 2007). Later, the same group also showed that HSV-1-DNA colocalizes with Aβ in AD amyloid plaques (Wozniak et al 2009), but any mechaniscistic explanation was provided by these studies. One possible explanation came out from an *in silico* study showing a structural sequence homology between Aβ 34-42 and viral gB (Cribbs et al 2000). These authors, demonstrating that gB fragment accelerated *in vitro* the formation of Aβ fibrils, hypothesized that this viral protein is involved in Aβ aggregation serving as "core". More recently, Cheng and colleagues (2011) demonstrated that HSV-1 capsid proteins interact with APP to allow the migration of new viral particles inside infected cells and that this interaction interferes with normal APP transport and localization within neurons, facilitating its amyloidogenic processing. Our group provided further demonstration on HSV-1 involvement in APP amyloidogenic processing, showing that in cultured neuronal cells HSV-1 induces the amyloidogenic APP cleavage, with production of several APP fragments including Aβ (De Chiara et al., 2010; Piacentini et al., 2011). In particular, De Chiara et al showed that (2010) HSV-1 infection in SH-SY5Y human neuroblastoma cells and rat cortical neurons induces multiple cleavages of APP, which result in the intra- and extra-cellular accumulation of several APP-fragments with neurotoxic potential. Among them: 1) APP fragments of 45 and 35kDa (APP-F45 and APP-F35) including Aβ portion, APP-F35 was further identified as Aβ nonamer ; 2) N-terminal APP fragments that are secreted extracellularly; 3) intracellular C-terminal APP fragments, including APP intracellular domain (AICD), whose effects on the transcription of neurotoxic genes were further investigated (Civitelli et al 2016); and 4) Aβ40 and Aβ42 in the form of monomers and small oligomers (dimers and trimers), whose effects on synaptic function where further investigated (Piacentini et al, 2015). This multiple cleavage of APP is produced in part by known components of the amyloidogenic APP processing pathway, i.e., host-cell β-secretase, γ-secretase, and caspase-3-like enzymes, and in part by other cellular or viral enzymes not yet identified. APP cleavage was also triggered by a GSK-3b-dependent phosphorylation on APP (at Thr688) in the early phase of the infection (Piacentini et al 2011 and 2015). This is a key event critically involved in APP processing and Aβ formation (Pierrot et al., 2006). Moreover, the APP fragments secreted in the extracellular space following HSV-1 infection were found capable to induce apoptosis in the neighbouring cells (De Chiara et al., 2010).

Overall these evidences suggest that HSV-1 may induce neurodegeneration through the induction of APP amyloidogenic processing.

1.3.4 HSV-1 and Tau hyperphosphorylation

Many papers Results from in vitro and in vivo studies showed that HSV-1 (Zambrano et al 2008; Wozniak et al., 2009 Santana et al 2012, Alvarez et al 2012) (Martin et al 2014) is able to induce an increased hyperphosphorylation of tau protein, a microtubule associated protein (MAP) involved in the pathogenesis of AD and other neurodegenerative disorders. In addition, an augmented form of caspase-3-cleaved tau, considered a marker of early neurodegenerative processes, was observed following HSV-1 infection in neuronal cells. Thus, both tau hyperphosphorylation and its cleavage induced by HSV-1 may contribute to neurodegeneration. HSV1 can trigger abnormal tau phosphorylation either by increasing the activity of the relevant kinases (i.e. GSK3β and PKA) and by decreasing that of the main cellular phosphatases (Pelech 1995).

In brain from AD patients abnormal phosphorylated tau is thought to be a consequence of inappropriate stimulation of cell to re-entry into cell cycle (Butterfield et al 2006; Nagy et al 1997; Vincent et al 1998). Interestingly, cell cycle anomalies are another process linking AD and HSV1. Notably, HSV-1 infection increases the activity of cdc2 kinase (Advani et al 2000; Schang et al 2003), a cell cycle enzyme not normally expressed in resting neurons. This enzyme is also upregulated in AD and the mitotic regulatory complex cdc2/cyclin B1 is aberrantly expressed and activated only in cells containing the mitosis-specific phosphoproteins that are incorporated into NFT (Vincent et al 1998). Another reason why HSV1 causes tau phosphorylation might relate on the fact that tau is a MAP and it is known that HSV-1 uses microtubules to travel along axons and so the virus might exploit this machinery for its own use. Phosphorylation of tau prevents its binding to microtubules, so by causing this phenomenon, the virus would make the microtubules available to interact with its own proteins such as the protein encoded by UL21, which is a functional homologue of tau (Takakuwa et all 2001).

1.3.5 HSV-1 and oxidative stress

Oxidative damage provides an alternative mechanism underling HSV-1-induced neuronal

injury. Experiments performed in human neuronal cells showed that oxidative stress produced may enhance the neurodegenerative effects provoked by HSV-1 (Santana et al 2013). A paper from our group found that antioxidant compounds prevent the formation of Aβ oligomers induced by HSV-1 in SH-SY5Y human neuroblastoma cells, suggesting that oxidative stress is involved in this process (De Chiara et al 2010). In addition, alterations in the redox state have been associated with the appearance of the characteristic pathological anomalies that accumulate in AD brains. Moreover, other studies have shown that pro-oxidant agents can increase the production of Aβ (Paola et al 2000, Tamagno et al 2005) and a recent report has revealed that antioxidant therapy interrupts the progression of amyloid and tau pathology in a mouse model of AD (Clausen et al 2012). Furthermore, several kinases involved in tau phosphorylation belong to the stress activated protein kinase family known to be activated during oxidative stress (Ferrer 2004).

Papers from our group demonstrated that glutathione (GSH), main cellular antioxidant, inhibit HSV-1 replication both in vitro (Palamara et al 1995) and in vivo (Nucci et al 2000) experimental models. Accordingly, the work by Kavouras demonstrated that HSV-1 in P19N neuronal cells induced an increased in ROS and lipid peroxidation levels. Moreover, they showed that HSV-1 replication is inhibited by the antioxidant compound ebselen, suggesting that HSV-1–induced oxidative stress is required for efficient viral replication in neural cells (Kavouras et al 2007). In addition, Arimoto and colleagues showed that hydrogen peroxide promotes the release of HSV-1 from epithelial cells, increasing the amount of cell-free virus without reducing levels of cell-associated virus (Arimoto et al 2006). In contrast Santana and colleagues recently demonstrated that oxidative stress reduces HSV-1 replication. However, they show that oxidative stress strongly increases not only the neurodegeneration markers induced by HSV-1 (i.e., Aβ accumulation) but also the impairment in autophagy observed in HSV-1-infected cells (Santana et al 2013). These authors suggested that the increase in oxidative stress condition concomitant with aging may enhance the neurodegeneration induced by HSV-1 infection.

Taken together, all these studies outline an interesting link between oxidative stress and HSV-1 infection and support the hypothesis that HSV-1-induced modification of intraneuronal redox state may concur to neurodegeneration events typical of AD pathogenesis.

2. AIM

While HSV-1 replicative cycle has been extensively studied, very little is known about the effects of repeated HSV-1 spreading in the brain occurring following multiple viral reactivations. There is even a lack of knowledge on long term damages, potentially cumulating in the brain, derived from the multiple viral interactions with intracellular pathways involved in several neuronal functions. Indeed, although an increasing number of papers highlighted a role of HSV-1 in AD pathogenesis, a clear demonstration of the cause-effect relationship between HSV-1 reactivations in the brain and accumulation of AD molecular hallmarks has to be demonstrated yet.

On the basis of these considerations, the present study was aimed at evaluating, in a mouse model of recurrent HSV-1 infection, whether multiple virus reactivations trigger in mouse brain the accumulation of AD-like alterations, as signs of neurodegeneration (abnormal cleavage of APP, and hyperphosphorylation/aggregation of tau protein), as well as oxidative damages.

3. MATERIALS AND METHODS

3.1 Ethics Statement

Mice were purchased from Harlan Laboratories. All the experimental protocols used in the present study were in compliance with the European Guide for the Care and Use of Laboratory Animals and institutional guidelines and with the Italian legislation on animal experimentation (Decreto legislativo n. 26/2014, Direttiva UE 63/2010).

3.2 Primary cortical neuron cultures

Primary cultures of cortical neurons were obtained from brains from E16-18 BALB/c mouse embryos, as previously described (Piacentini et al 2015). Briefly, after removing brains and freed of meninges, cortices were dissected and incubated for 10 min at 37 °C in trypsin-EDTA (0.025%/0.01% w/v), and the tissues were mechanically dissociated at room temperature (RT) with a fire-polished Pasteur pipette. The cell suspension was harvested and centrifuged at 1200 rpm for 8 min. The pellet was suspended in Minimum Essential Medium (MEM, Biochrom) containing, 5% fetal bovine serum, 5% horse serum, 1% glutamine (2 mM), 1% penicillin-streptomycin-neomycin antibiotic mixture (PSN), and glucose (25 mM). Cells were plated at a density of 10^6 cells on 6 well-plates, precoated with poly-L-lysine (0.1 mg/ml; Sigma). Twenty-four hours later, the culture medium was replaced with Neurobasal medium (Gibco) containing 2% B-27 (Invitrogen), 0.5% glutamine (2 mM), and 1% PSN. After 72 h, this medium was replaced with a glutamine-free version of the same medium, and the cells were grown for 7-10 days before carrying-out experiments.

3.3 Virus production and titration

HSV-1 production was performed as previously reported (Fabiani et al 2017) on monolayers of kidney epithelial VERO cells cultivated in 75 cm^2 tissue culture flasks and infected with HSV-1 strain F (a wild-type strain) at a multiplicity of infection (m.o.i.) of 0.01. After 48 h at 37°C, HSV-1-infected cells were harvested, and following 3 cycles of freeze-and-thaw, cell debris were removed with low-speed centrifugation. Virus titer in the supernatant was measured by standard plaque assay (Killington et al 1991). In this study the virus had a titer of $5x10^8$ pfu/ml. Similarly, Mock solution was prepared from not infected VERO cells.

3.4 Intracellular reactive oxygen species (ROS) assay in mouse primary cortical neurons

Levels of intracellular ROS were measured by detecting 2',7'-Dichlorofluorescin diacetate (DCFH-DA; Sigma; St. Louis, MO) oxidation. Mouse cortical neurons were seeded in 6-well plates and were infected with HSV-1 (3 m.o.i.). At specific time points, cells were washed and incubated with media containing DCFH-DA (10 μM) for 30 min. Afterwards, cells were excited at 485 nm and DCFH-DA fluorescence read at 530 nm emission by a fluorimetry reader. Each sample was run in triplicate and sample means were normalized to their respective controls (% of control).

3.5 Mouse model of recurrent HSV-1 infection

6-7 week old Female BALB/c mice were HSV-1 infected (and named hereafter HSV1-M) by snout abrasion inoculation of HSV-1 suspension (equivalent to 10^6 pfu), following anesthesia with intraperitoneally injection of Ketamine (80mg/Kg) + Xylazine (6mg/Kg). As controls, a set of mice was Mock-infected with 2 □l of conditioned medium from uninfected VERO cells (and named hereafter CONTROL-M). HSV1-M and CONTROL-M were marked and kept in separated cages. All animals were daily and individually examined for the appearance of neurological signs, temperature, feedings habits, weight and posture during the first weeks post infection (p.i.). When latent infection was established (6 weeks p.i.) HSV1-M, and CONTROL-M were individually subject to hyperthermia (40-42°C for 15': thermal stress =TS) to reactivate latent virus, according to protocols from Huang et al (2011) and Sawtell et al (1992). TS was repeated at 6-8 weeks of interval for 7 times during mouse life. Following the 3rd and 7th TSs a set of animals per group sacrificed and was analyzed as described below.

3.6 PCR analysis of viral genes

Nucleic acids were extracted from mouse brains by one step method (AllPrep DNA/RNA/Protein Mini Kit, Qiagen) according to manufacturer's protocols, and their concentrations were assessed. by spectrophotometrically. PCR was performed with the iTaq ™ DNA Polymerase on a iQ5 Real-Time PCR Detection system (Bio-Rad), according to the manufacturer's recommendations. Briefly, total RNA was reverse transcribed in cDNA using Iscript cDNA Synthesis Kit 89 (Bio-Rad) according to the manufacturer's recommendations, and then used for ICP4 amplification. Total, DNA was used for amplification of TK, and β-actin gene was used as an endogenous control for the

PCR reaction. The PCR reaction (94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 57 °C/55 °C for 30 s, and 72 °C for 30 s) was performed with the following primers:

TK forward: AGCGTCTTGTCATTGGCGAA
TK reverse TTTTCTGCTCCAGGCGGACT
ICP4 forward GGCGGGAAGTTGTGGACTGG
ICP4 reverse CAGGTTGTTGCCGTTTATTGCG

3.7 Immunofluorescence analysis

Once anesthetized by intraperitoneal injection of a cocktail of Ketamine (100mg/Kg), Xylazine (10 mg/Kg), mice were perfused transcardially with PBS (pH 7.4) and then with 4% paraformaldehyde (PFA). The removed brains were then fixed for 24h at 4°C in PFA, followed by cryoprotection first in 15% sucrose and then in 30% sucrose in PBS. Using a vibratome, 30/40 μm coronal slices were cut and stored at -20°C in cryoprotectant solution (50 mM sodium phosphate buffer, pH 7.4, containing 15% glucose and 30% ethylene glycol) until immunohistochemical evaluation. Slices were washed with PBS, permeabilized with 0.5% Triton X-100 in PBS for 15 min at r.t.(22°C), incubated for 90 min with 10% horse serum and 0.2% Triton X-100 in PBS to block unspecific binding sites, and then incubated overnight at 4°C with specific primary antibodies diluted in 5% horse serum,0.2% Triton X-100 PBS. In detail, we used the following primary antibody dilutions: 1: 2000 anti-Aβ40/42 antibody (AB5076 Millipore); 1:300 anti-NeuN antibody (MAB377, Millipore); 1:2500 anti phosphoThr205-tau antibody (ab4841 Abcam); 1:100anti ICP4 antibody (sc-69809 Santa Cruz). Slice were then washed 3 times in PBS, and incubated for 90 min at r.t with specific secondary antibodies coupled to Alexa 488 or Alexa 546 (1:1000, Invitrogen). After 3 washes in PBS, slices were stained with DAPI (1:500 dilution in PBS) for 20 min at r.t., washed briefly and then mounted on coverslip with mounting medium (ProLong Gold Anti-fade reagent, Invitrogen). Images were acquired at 63× magnification with a confocal laser scanning system (Leica TCS-SP2) and an oil-immersion objective (N.A. 1.4). For some images, additional 2×magnification was applied. Quantifications of immunofluorescence were carried out in the maximum projection images from the acquired confocal stacks after having traced regions of interest (ROIs) in specific brain areas of the slices (somatosensory neocortex, and DG and CA1 regions of the hippocampus).

3.8 Thioflavin S staining of amyloid plaques

Following the permeabilization step, some slices were stained with a solution of 0.5% Thioflavin S (ThS, Sigma-Aldrich) in 50% filtered MeOH (8 min at r.t), washed 2 times (5 min each) in in 70% MeOH, 1 time (5 min) in 0.1 M phosphate buffer, and finally 1time (15 min) in PBS. Brain sections were then mounted on coverslip with ProLong Gold Anti-fade reagent (Invitrogen). Images were acquired as described for immunofluorescence analysis.

3.9 Western Blotting

Brain tissues were homogenized on ice with RIPA buffer (20 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing protease and phosphatase inhibitors, separated from unlysed cells by centrifugation at 15000 x g at 4°C for 20 min, and supernatants quantified for total protein concentrations by Micro BCA method. Equal amount of proteins was separated on SDS-PAGE, then blotted onto nitrocellulose membrane (Amersham Protran 0.45 μm, Hybond ECL, GE Healthcare 0.22 μm). Membranes were then blocked for 3-5 h in 10 % not fat milk in 0.1 % Triton X-100 TBS (T-TBS) and incubated overnight at 4°C with suitable dilutions of specific primary antibody (in 5% not fat milk in T-TBS), such as anti-APP A4 antibody N-term (MAB348-100UL clone 22C11, Millipore), anti-APP antibody C-term (A8717, Sigma-Aldrich), anti-phospho Tau (phospho T205 ab 4841, Abcam), anti-phospho Tau (phospho T396 sc1018, Santa Cruz), anti-phospho Tau (phospho T212 and S214 AT100, Thermo Fisher Scientific), anti-actin (A2228, Sigma-Aldrich) or anti-tubulin (T6074 Sigma-Aldrich), anti-HNE (HNE-13M, Alpha Diagnostics, San Antonio, TX), anti-nitrotyrosine (N0409, Sigma-Aldrich) antibodies. For carbonylated proteins detection, after blocking, the membranes were equilibrate in 20% methanol, incubated in 2 N HCl and finally derivatized with 0.5 mM 2,4-dinitrophenylhydrazone (DNP). Finally, DNP-protein adducts were detected on the nitrocellulose using a primary anti-DNP antibody (Millipore). Following 3 washes in T-TBS, membranes were incubated with horseradish peroxidase-conjugated antibodies (Jackson ImmunoResearch Laboratories, 1:5000 in T-TBS). For normalization of phosphorylated tau, membranes were then stripped and reported with anti-tau antibody (tau5 Ab-2, NeoMarkers). Enhanced chemiluminescence reaction (Amersham Biosciences) were used to detect specific immunosignals. Densitometric analysis was performed using Quantity One software (BioRad):

3.10 2D electrophoresis

Brain samples proteins (200 µg) were precipitated in 15% trichloroacetic acid for 10 min in ice. Subsequently, each sample was centrifuged at 10 000 g for 5 min and precipitates were washed three times in ice-cold ethanol-ethyl acetate 1:1 solution. The final pellets were dissolved in 200µl rehydration buffer (8 M urea, 20 mM dithiothreitol (DTT), 2.0% (w/v) Chaps, 0.2% Bio-Lyte, 2M thiourea, and bromophenol blue). First-dimension electrophoresis (isoelectric focusing) was performed with ReadyStrip IPG Strips (11 cm, pH 3–10; Bio-Rad, Hercules, CA, USA) at 300V for 2h linearly, 500V for 2h linearly, 1000V for 2h linearly, 8000 V for 8 h linearly, and 8000 V for 10 h rapidly. All the above processes were carried out at room temperature. After the first-dimension run the strips were equilibrated two times, first for 10 min in 50 mM Tris–HCl (pH 6.8) containing 6 M urea, 1% (w/v) sodium dodecyl sulphate (SDS), 30% (v/v) glycerol, and 0.5% DTT and again for another 10 min in the same buffer containing 4.5% iodoacetamide in place of DTT. The second dimension was performed using 12% precast Criterion gels. The gels were incubated in fixing solution (7% acetic acid, 10% methanol) for 45 min, then, stained for 2 h with SYPRO Ruby Gel Stain (Bio-Rad) and finally de-stained overnight in deionized water.

3.11 2D Western blot

2D gels were then blotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) and HNE-protein adducts were detected on the membranes. Briefly, membranes were blocked for 1 h with 3% albumin in T-TBS, incubated with the primary Mouse Monoclonal anti-HNE antibody (1:500; Alpha Diagnostic, San Antonio, TX, USA) for 2h at room temperature. After washing with T-TBS (3 X 5 min), membranes were further incubated at room temperature for 1 h with the secondary alkaline phosphatase-conjugated anti-mouse antibody (1:5000). Membranes were then washed three times with T-TBS and developed using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium solution (BCIP/NBT).

3.12 Image analysis

2D gels and 2D blots were analysed by PD-Quest 2D Analysis (7.2.0 version; Bio-Rad, Hercules, CA, USA). PD-Quest spot-detection software allows the comparison of 2D gels as well as 2D blots. Powerful auto-matching algorithms quickly and accurately match gels or blots and sophisticated statistical analysis tools identify experimentally significant spots.

The intensity value for each spot from an individual gel is normalized using the average mode of background subtraction. This intensity is afterward compared between groups using statistical analysis. Statistical significance was assessed using a two-tailed Student's t-test. P values.

3.13 Trypsin digestion and protein identification by mass spectrometry

Protein spots identified statistically different from controls after PD-Quest analysis were digested in-gel by trypsin. Spots of interest were excised from gel, washed with 0.1 M NH_4HCO_3 at room temperature for 15 min then incubated wit acetonitrile for 15min. This solvent mixture was then removed, and gel pieces were dried. The protein spots were then incubated with 20 ml of 20 mM DTT in 0.1 M NH_4HCO_3 at 56 °C for 45 min. The DTT solution was removed and replaced with 20 ml of 55 mM iodoacetamide in 0.1 M NH_4HCO_3. The solution was then incubated at room temperature for 30 min. The iodoacetamide was removed and replaced with 0.2 ml of 50 mM NH_4HCO_3 at room temperature for 15 min then incubated with acetonitrile for 15 min and then the gel spots were dried for 30 min. The gel pieces were rehydrated with 20 ng/ml modified trypsin (Promega, Madison) in 50 mM 67 NH_4HCO_3 and incubated overnight at 37 °C in a shaking incubator. Protein spots of interest were excised and subjected to in-gel trypsin digestion, and the resulting tryptic peptides were analyzed with MALDI ToF. MALDI-ToF MS analyses were performed in a Voyager-DE STR instrument (Applied Biosystems, Framingham, MA, USA) equipped with a 337 nm nitrogen laser and operating in reflector mode. Mass data were obtained by accumulating several spectra from laser shots with an accelerating voltage of 20 kV. Two tryptic autolytic peptides were used for the internal calibration (m/z 842.5100 and m/z 2807.3145).

Data were analysed by MoverZ program, according to default parameters. Identification by peptide mass fingerprint (PMF), with the mono-isotopic mass list, after exclusion of expected contaminant mass values by Peak Erazor program, was performed using the Mascot search engine (v. 2.3) against human SwissProt database. Up to one missed cleavage, 50 ppm measurement tolerance, oxidation at methionine (variable modification) and carbamidomethylation at cysteine (fixed modification) were considered. Identifications were validated when the probability-based Mowse protein score was significant according to Mascot.

3.14 Statistical analysis

Statistical comparisons were performed with GraphPad 6.0 (Prism) software by using Student's *t*-test or one-way analysis of variance (ANOVA) when appropriate. Data are presented as means $\pm$ Standard Deviation (SD) or as mean $\pm$ standard error of the mean (SEM) when appropriate. The level of significance was set at 0.05.

4. RESULTS

4.1 ***Mouse model of recurrent HSV-1 infection and experimental design***

To study whether repeated HSV-1 reactivations, as those occurring in humans over life, may contribute to the accumulation of the neurodegenerative and oxidative damages, that characterize AD, we first established a mouse model of recurrent HSV-1 infection. The experimental protocol was set up on the basis of preliminary experiments in which we inoculated in anesthetized mice different HSV-1 doses (F strain, 10^5-10^8 plaque forming unit, pfu range) using different inoculation procedures (snout abrasion and intraperitoneal injection) to determine the most efficient conditions that promote virus spreading into CNS and avoid the occurrence of virus-induced encephalitis with seizures or paralysis. With HSV-1 at 1×10^6 pfu, we observed a low reasonable mortality within the 10-month protocol schedule and the mortality was mainly related to thermal stress induction or age-related events (data not shown). Therefore, for the experimental model we inoculated in anesthetized 6-8-week-old female BALB/c mice via snout abrasion HSV-1 with 1×10^6, pfu or Mock solution.

Following the establishment of viral latency (obtained 6 weeks after virus inoculation), HSV-1-infected mice (HSV1-M), and Mock-infected mice (CONTROL-M) as controls, were subjected to hyperthermia to induce the reactivation of latent virus (Huang et al 2011). Virus reactivation by thermal stress (TS) was repeated up to 7 times at 6-8 week intervals, in order to induce multiple HSV-1 reactivations over mouse life, thus mimicking what usually occurs in humans. Under these experimental conditions, HSV1-M and CONTROL-M did not show any difference in body weight over life (Fig. 1A), nor signs of virus-induced encephalitis. Conversely, signs of recurrent infections were observed following both primary infection and TSs (Fig. 1B). The presence of the virus in the animal brains was verified by PCR amplification of the viral TK gene on DNA extracted from their brain tissue (Fig. 1C), whereas virus reactivation was checked by RT-PCR amplification of mRNA for ICP4, the main transcriptional regulatory protein of HSV-1, in brain of mice sacrificed 24 h after TS (Fig 1D). Both these analyses were repeated in sets of mice sacrificed following TSs: we identified viral TK gene in a high percentage of HSV-1-M (79%, N=29), indicating that in most HSV1-M the virus actually reached the brain, and ICP4 mRNA in 61% of the analyzed TK^+ brains (N=1). For the further analyses

we considered only HSV-1 infected mice with brain TK+ and ICP4+. As expected, these viral genes were not found in none of the analyzed CONTROL-M.

We, therefore, characterized this mouse model of recurrent HSV-1 infection at molecular levels. In particular, we checked the presence of AD hallmarks in the mouse brain after the 3th and the 7th TS (i.e., at 6 and 13 months of age, respectively) and the presence of oxidative stress markers only in brains of mice sacrificed after the 7th TS.

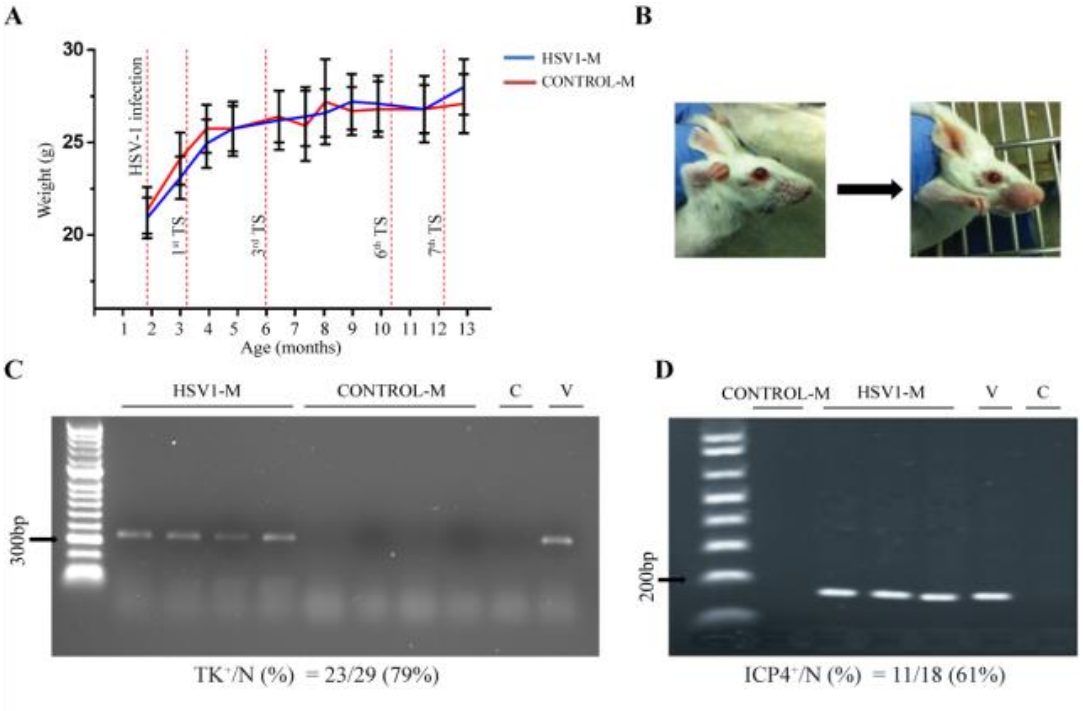

Figure 1. Mouse model of recurrent HSV-1 infections. HSV-1 or Mock solution were inoculated via snout abrasion on 6-8 week-old female BALB/c mice. Six weeks later, mice were subjected to thermal stress (TS) to induce virus reactivation. TS was repeated up to 7 times over mouse life. A) Body weight assessed for HSV-1-infected and Mock-infected in function of age (months); red lines indicate the time points of primary infection and selected TSs. B) photo of a representative mouse showing typical signs of herpes labialis. C) and D) Representative image of TK PCR amplification (C) and RT-PCR amplification of ICP4 mRNA (D) in 4 animal brains for experimental group, C= negative control of PCR reaction V= TK or ICP4 amplification in lysed HSV-1 as positive control. The numbers of studied brains and the percentages of TK$^+$ and ICP4$^+$ brains are shown under panels C and D.

*4.2 **Recurrent HSV-1 infections induce the main AD hallmarks in mice***

On the basis of previous papers from our laboratory (De Chiara et al, 2010; Piacentini et al, 2011) and others (Alvarez et al 2012) demonstrating that HSV-1 infection in *in vitro* neuronal cultures induces Aβ hyperproduction and tau hyperphosphorylation, we checked whether *in vivo* recurrent HSV-1 infection produced the same molecular hallmarks of AD. To this aim, we first sacrificed HSV1-M and CONTROL-M after the 3rd TS (N=5 per

group), and then after and the 7th TS (N=7 per group) and analyzed their cortices and hippocampi by Western blotting (WB) and immunofluorescence (IF).

4.2.1 Aβ and CTF.

Firstly, we studied whether recurrent HSV-1 infections induce APP amyloidogenic processing by analyzing the presence of APP amyloidogenic fragments in mouse brain homogenates. In particular, we analyzed the expression pattern of APP and its C-terminal fragments (CTFs) by WB. Results in Figure 2A show a significant increase in CTF levels together with slight but not significant decrease in the levels of APP full length in neocortical homogenates from 6-month-old HSV1-M undergone 3 TSs. On the contrary, no significant differences in APP full length and CTFs levels were found in hippocampi from the same mice (Figure 2B). Interestingly, in 13-month-old mice undergone 7 TSs we found an increase in CTFs accumulation, that resulted significant in hippocampi when compared to matched CONTROL-M, together with a small, but significant, APP downregulation in neocortical homogenates (Fig. 2B). These data suggest that APP processing was triggered by virus reactivations and that in hippocampus the number of virus reactivations influence more strongly CTF accumulation.

Next, we employed IF analysis of brain slices to check the presence of Aβ. In particular, we stained brain slices with anti-Aβ40/42 antibody, that specifically recognizes these two peptide isoforms without cross-reacting with the parental protein APP, and focused on specific brain regions such as somatosensory neocortex, dentate gyrus and CA1 regions of hippocampus. Results in Figure 3A-B evidenced a significant accumulation of Aβ in different brain sections from HSV1-M starting from the 3rd TS, indicating that the virus induced the amyloidogenic processing of APP. Aβ accumulation significantly increased in HSV1-M undergone 7 TSs, as compared to matched CONTROL-M (Fig.3C). We also checked the presence of extraneuronal deposits of Aβ in amyloid plaques, by staining brain slices with Thioflavin-S, the commonly used stain for amyloid plaque detection. In particular, we checked the presence of amyloid plaque deposition only in older mice (13 month of age after the 7th TS) according to reported data on transgenic mouse models of AD demonstrating an evident accumulation of amyloid plaques in cortical region and hippocampus starting from 12 months of age (Oddo et al 2003). Results in Fig 3D show a

higher number and size of e amyloid plaque in HSV1-M as compared to CONTROL-M. These data suggest that HSV-1 reactivations promote accumulation of amyloidogenic APP fragments and their deposition in amyloid plaques, that increased with time and number of TSs.

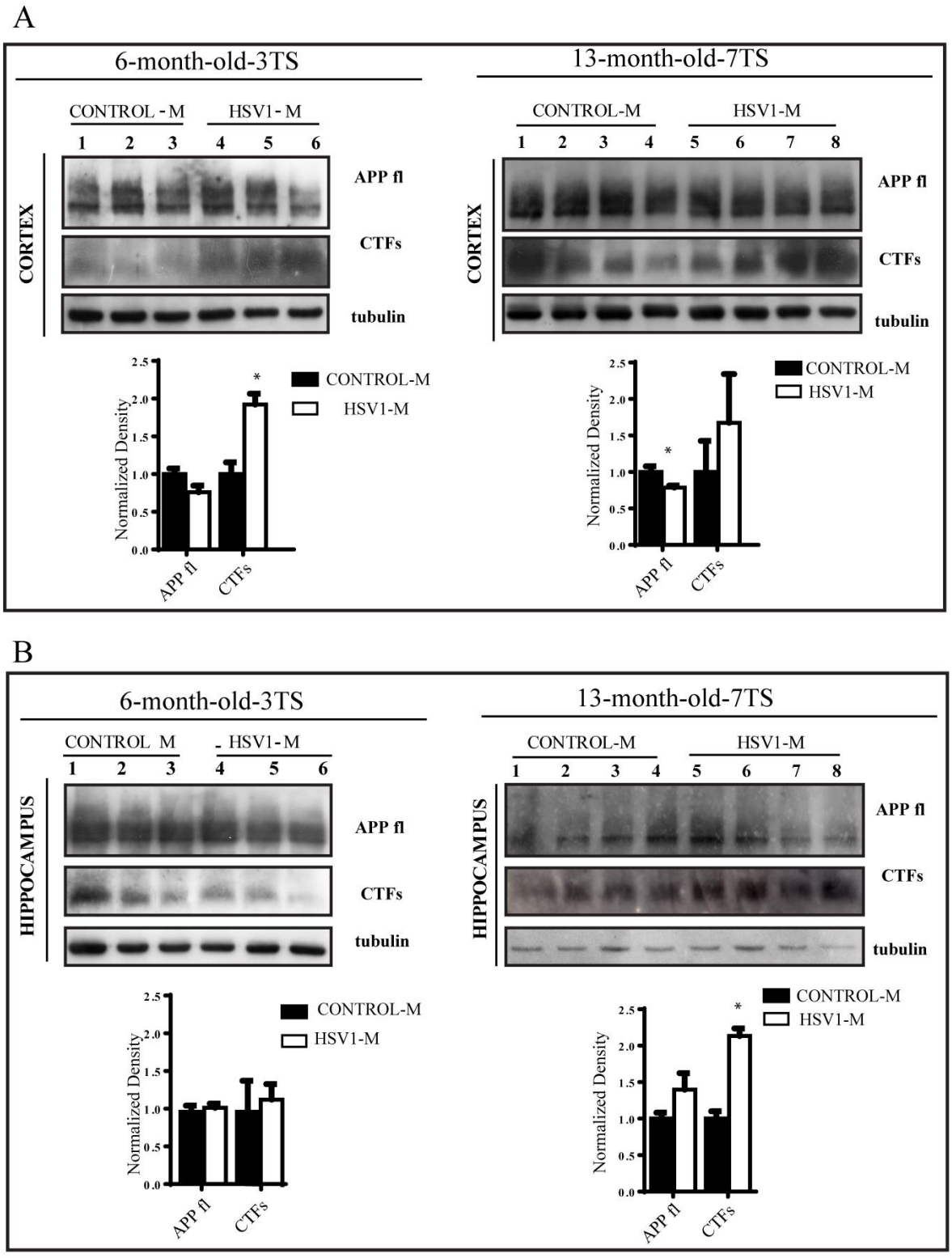

Figure 2. HSV-1 reactivations induce APP processing and CTF accumulation. APP full length (APP fl) and CTF levels in neocortex (A) and hippocampus (B) homogenates from CONTROL-M and HSV1-M sacrificed following 3 TSs or 7 TSs. Tubulin expression level was used as sample loading control. Graphs show the densitometric analysis of the immunoreactive bands normalized to tubulin: values represent the normalized fold changes in protein levels from HSV-M with respect to CONTROL-M (mean $\pm$ SEM,); * $p < 0.05$ vs CONTROL-M.

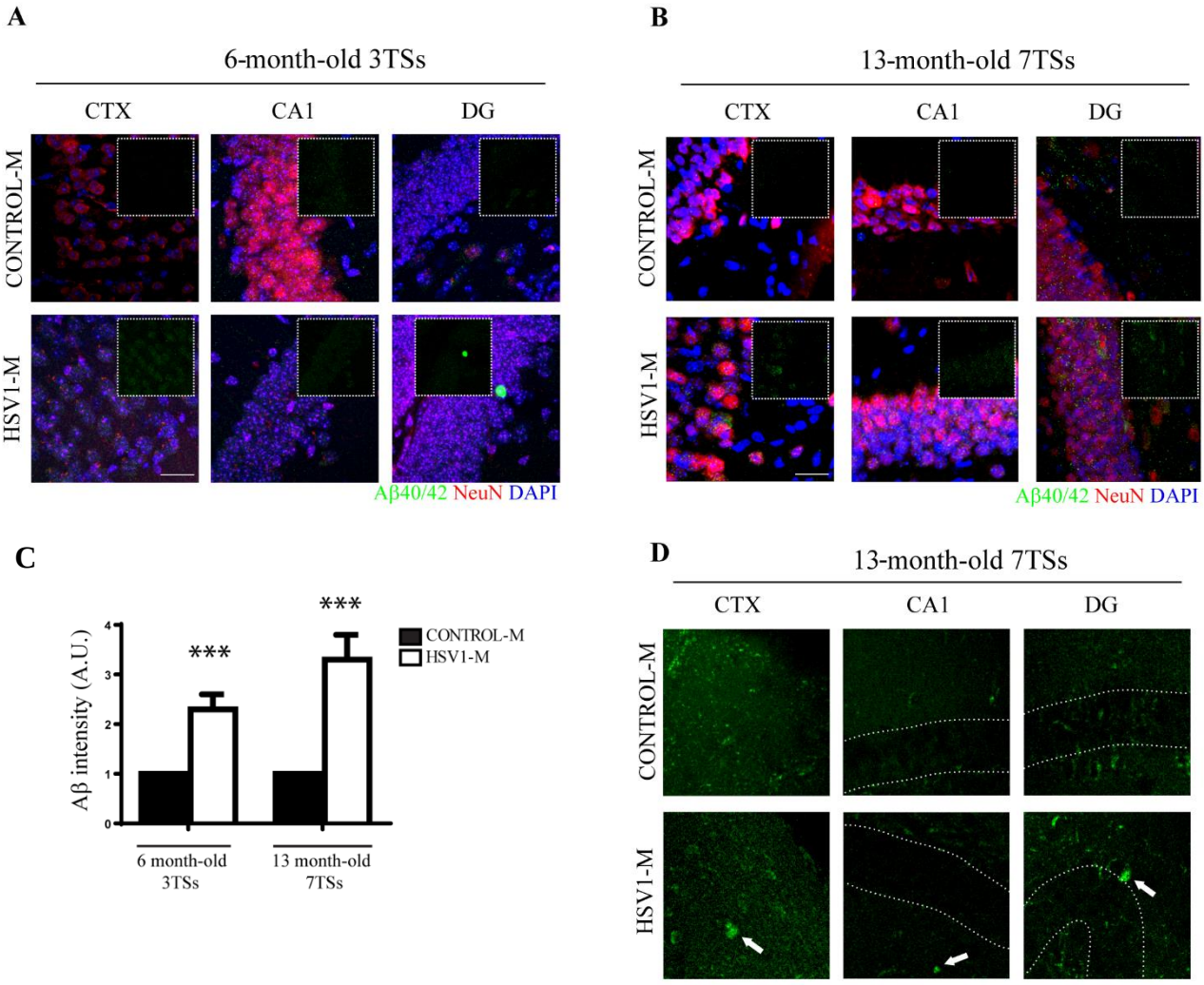

Figure 3. HSV-1 reactivations induce Aβ accumulation and deposition in amyloid plaques. Confocal immunofluorescence analysis of coronal brain slices from HSV1-M and CONTROL-M undergone 3 TSs (A) and 7 TSs (B), stained for Aβ40/42 peptides (green). Neurons were identified by their immunoreactivity for anti-NeuN antibody (red). Cell nuclei were stained with DAPI (blue). Panels show representative images from somatosensory neocortex (CTX) and hippocampal CA1 or dentate gyrus (DG) regions. Insets show Aβ fluorescence only. C) Bar graphs showing mean A fluorescence intensity in whole slices as fold change with respect to CONTROL-M. Mean ± SEM, ***p<0.0005. D) Thioflavin-S (ThS) staining (green) in brain slices from mice undergone 7 TSs. Arrowheads indicate plaques, dotted lines delimitate pyramidal neuron layer in CA1 and granule cell layer in DG. Scalebars: 35 µm.

4.2.2 Tau hyperphosphorylation and cleavage

Then we studied the expression pattern of hyperphosphorylated tau in the same mouse brain tissues (cortices and hippocampi). To this aim, we first employed WB with different antibodies that specifically recognize phosphorylated sites of tau protein known to be associated to neurodegeneration, as threonine 205 (Thr205), serine 396 (Ser396), and

double phosphorylation in threonine 212 and in serine 214 (Thr212 Ser214). Results in Figure 4A-B show an increase in phosphoThr205-tau (pT205) and double phosphoThr212Ser214-tau (pT212S214) only in brain homogenates from 13-month-old mice undergone 7 TSs, as compared with matched CONTROL-M, whereas no significant changed in tau phosphorylation were found in younger mice. We also found an increase in a high-weight molecular band (MW>100 KD) recognized by AT100 antibody that detects paired helical filaments (PHF), suggesting that HSV-1-inducing tau hyperphosphorylation at Thr212Ser214 may promote its aggregation. On the contrary, we did not find significant differences in phosphoSer396-tau (pS396) between HSV1-M and CONTROL-M. To further study the timing of tau phosphorylation, we then performed IF staining for pT205 in mouse brain slices, detecting an earlier (post 3TSs) increase in HSV1-M, that was much evident in mice subjected to 7 TSs (Fig.5). Furthermore, WB analysis also revealed that HSV-1 multiple reactivations in the brain induced a caspase-3-mediated cleavage of tau at aspartate 421, as revealed by the significant increase in TauC3 fragments in hippocampal tissues from 13-month-old HSV1-M undergone 7 TSs (Fig.4B). This is considered a marker of early neurodegeneration and was previously observed both *in vitro* and *in vivo* model of productive HSV-1 infection (Martin et al 2014; Gamblin et al 2003).

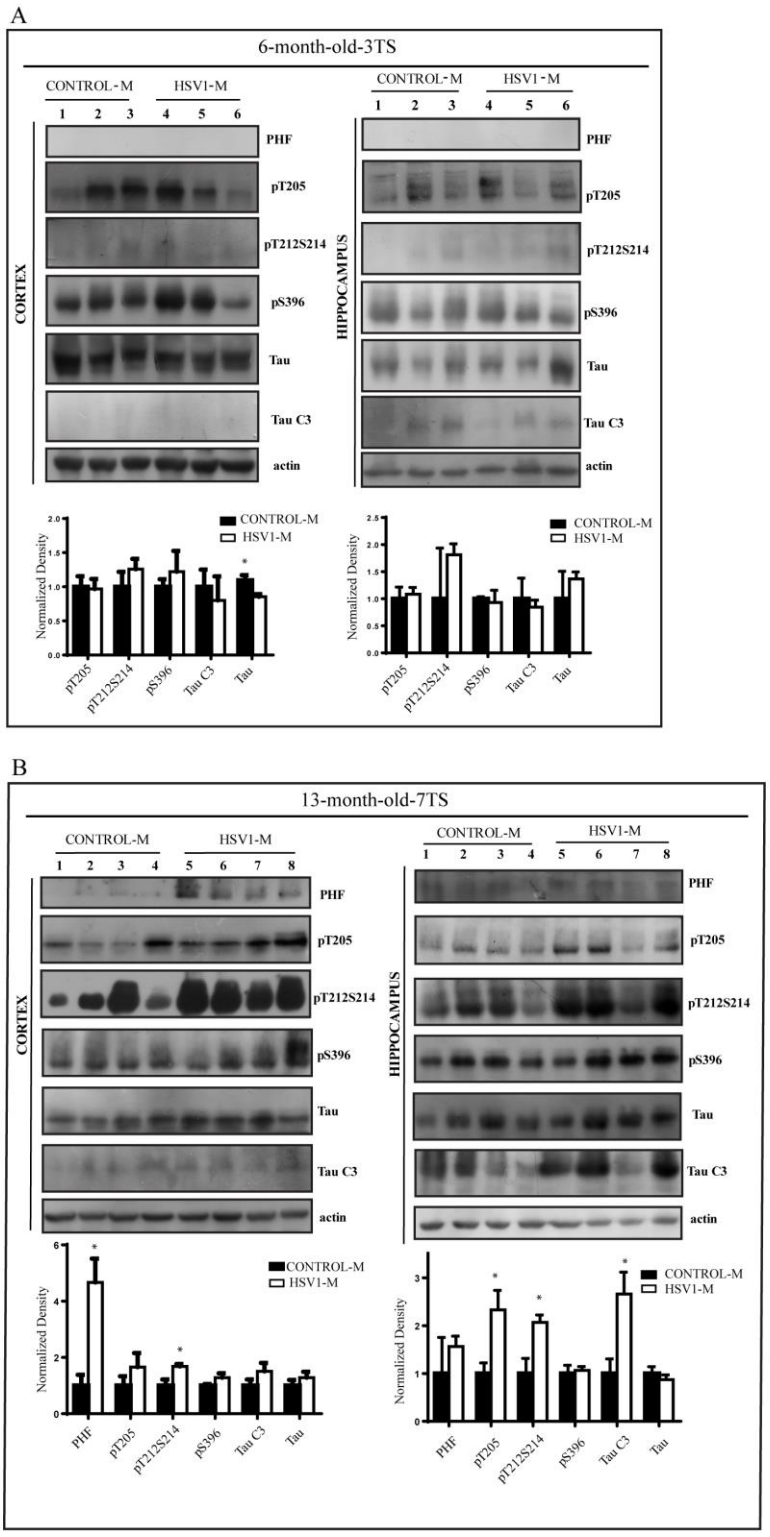

Figure 4. Multiple HSV-1 reactivations induce tau phosphorylation, cleavage and aggregation. Levels of phospho-tau and of its cleaved fragment TauC3 were investigated by the aid of specific antibodies in neocortex and hippocampus homogenates from CONTROL-M and HSV1-M sacrificed following 3 TSs (A) or 7 TSs (B). Actin expression level was used as sample loading control. Densitometric analysis of immunoreactive signals normalized to tau (for pTau) or actin (for tau and TauC3) are shown in the graphs: values represent the normalized fold changes in protein levels from HSV1-M with respect to CONTROL-M (mean $\pm$ SEM); * $p < 0.05$.

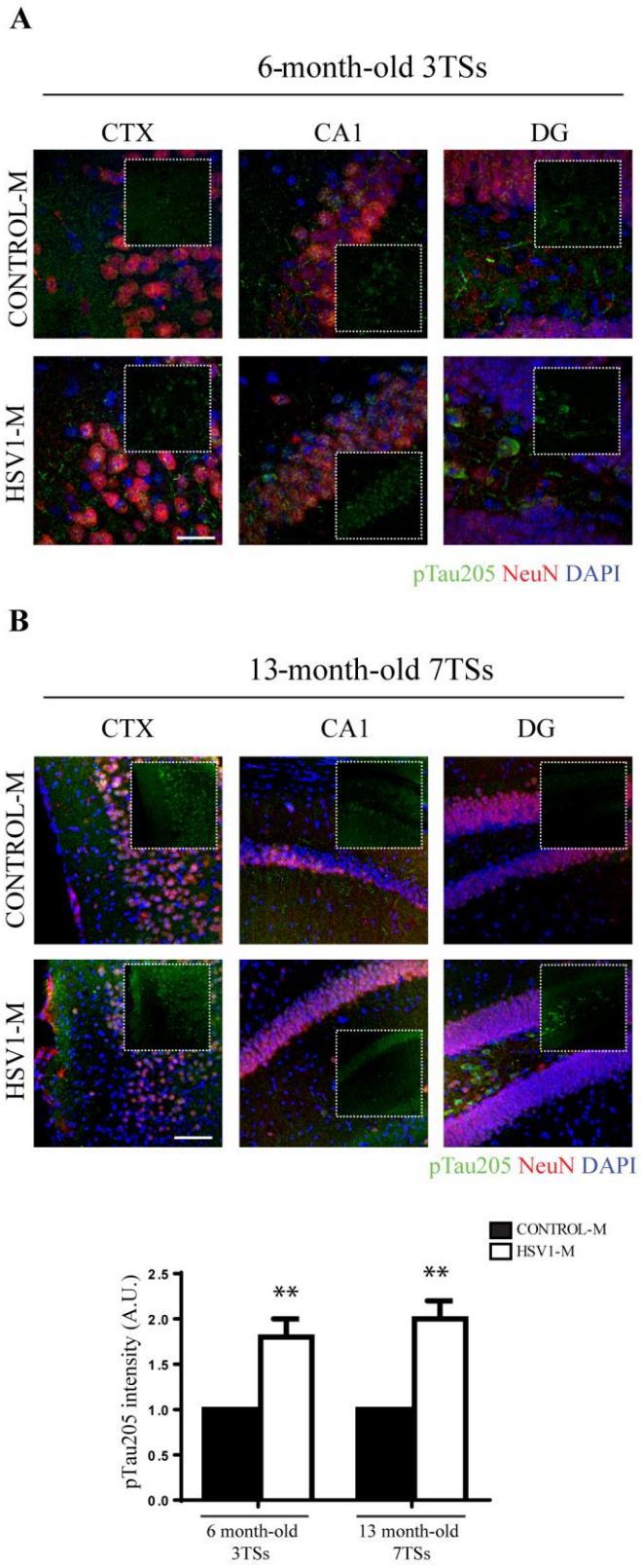

Figure 5. HSV-1 reactivations induce increased levels of pT205, in brain mouse.
A) and B) Representative Confocal immunohistochemical fluorescence images of coronal brain slices from HSV1-M and CONTROL-M sacrificed post 3 TSs (A) and 7 TSs (B) stained with a specific antibody direct against tau phosphorylation at Thr205 (pT205) (green) and anti-NeuN antibody (red), used to identify neuron nuclei. Cell nuclei were stained with DAPI (blue). Panels show representative images from somatosensory neocortex (CTX), hippocampal CA1, and dentate gyrus (DG) regions. Insets show pT205 fluorescence only. C) Bar graphs showing mean pT205 fluorescence (± SEM) in whole slice. Scal bar 35 µm (A), 75 µm (B); ** $p<0.005$

4.3 ***Oxidative damages induced by recurrent HSV-1 infections in mice***

4.3.1 Oxidative modifications of proteins and lipids

Considering that brain tissues are characterized by a progressive increment of oxidative stress conditions within the development of AD (Sayre et al 2008), we investigated whether recurrent HSV-1 infections in mouse brain, besides promoting Aβ accumulation and tau hyperphosphorylation, may induce also oxidative damages. Since it is known that both Aβ accumulation and tau hyperphosphorylation are associated to oxidative stress (Butterfield et al 2013; Liu et al 2015), we decided to analyze the presence of oxidative damages in brain tissue from the mice underwent 7 TSs, where we mainly detected AD hallmark accumulation. Hence, we evaluated by WB 4-Hydroxynonenal (HNE), Nitrosylated (3-NT) and Carbonylated Proteins (CP) levels in cortical lysates from HSV-1M (N=6) compared to CONTROL-M (N=6) sacrificed after 7 TSs at 13 months of age. Results in Figure 7 show a significant increase in all of these oxidative damage markers (HNE, 3-NT and CP) in HSV1-M respect to CONTROL-M, indicating that recurrent HSV-1 infection triggers oxidative damage accumulation.

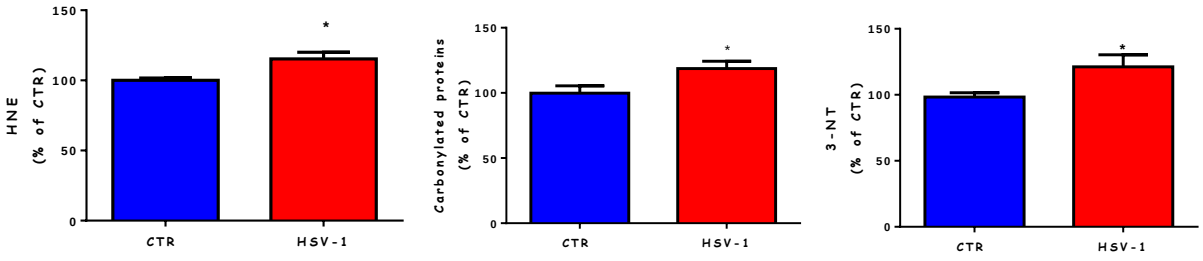

Figure 7. HSV-1 reactivations in the brain induce oxidative damages accumulation.
WB quantification of 4-Hydroxynonenal (HNE), nitrosylated (3-NT) and carbonylated proteins in cortical lysates from HSV1-M or CONTROL-M sacrificed after 7 TSs at 13 months of age. Densitometric analysis of oxidative modifications observed in HSV-1 (N = 6) are shown in the graph as percentage vs CTR (N = 6). Error bars represent SD, (* $p<0.05$).

4.3.2 HNE-proteomic profile

To further investigate the oxidative potential of recurrent HSV-1 infections, we decided to employ redox proteomic analysis focusing on HNE modifications according to results from preliminary experiments in *in vitro* model of HSV-1 infection. In particular, in primary culture of cortical neurons, once we evaluated ROS level by fluorimetry to confirm the

involvement of oxidative stress in HSV-1 infection, we checked HNE, 3-NT and CP levels in cell pellets harvested 1, 2, 3, 4, 5 and 6 h after HSV-1 (1 MOI) or Mock infection by WB. Results showed in Figure 8 indicate that HSV-1 infection induces a significant increase in ROS intracellular level 4 h p.i. and increased intracellular levels of HNE 4 and 6 h p.i., whereas it does not affect 3-NT and CP levels.

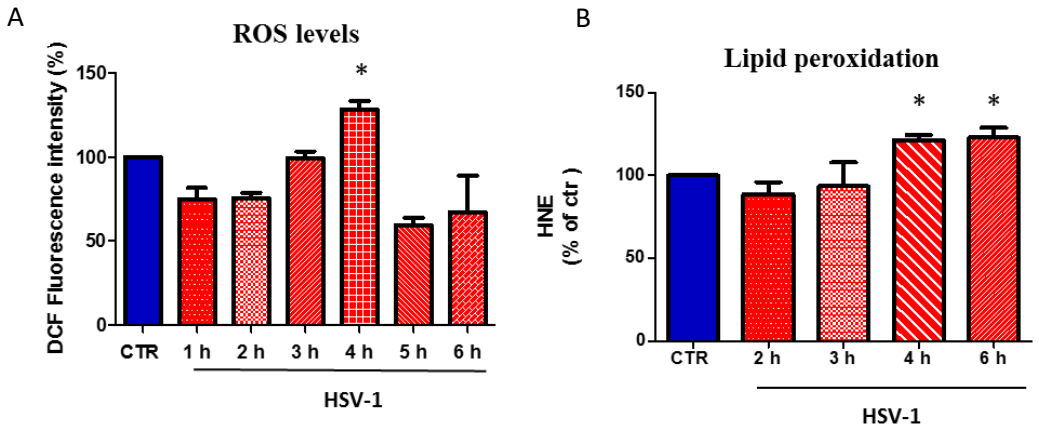

Figure 8. HSV-1 in vitro infection on primary culture of cortical neurons induce oxidative stress. ROS (A) and HNE (B) levels in cortical neurons at the indicated time post HSV-1 infection (p.i.) or Mock-infection (CTR), detected by DCF fluorescence assay and by WB, respectively.

Thus, since HNE levels and HNE-proteins adducts are known to be increased in AD brains (Hardas et al 2013; Butterfield et al 2006), we used redox proteomic approach to identify protein targets of HNE peroxidation in brain from 13-month-old HSV1-M undergone 7 TSs. To this aim, HSV1-M and CONTROL-M cortices were analysed by coupling immunochemical detection of 4-HNE-bound proteins with 2-D polyacrylamide gel electrophoresis and mass spectrometry (MS) analysis. First, cortical homogenates from 6 animals per group were separated by 2-D polyacrylamide gel electrophoresis and total protein spots detected by SYPRO Ruby staining (a representative gel in Figure 9A). Then each 2-D gel was blotted on nitrocellulose membrane where HNE-modified proteins were revealed by anti-HNE antibody staining (representative 2D WB in Figure 9B). HNE-bound protein levels were calculated by dividing the densitometry of HNE immunoreactivity band on the membrane by the densitometry of its corresponding protein spot on the gel. Comparing the densitometric intensities of individual spot of each HSV-1M samples (N=6) with those measured in CONTROL-M samples (N=6), we identified thirteen HNE-modified spots whose levels were significantly modulated (both upwards and downwards) in the cortex from HSV-1-M (see highlighted and numbered spots in Figure 9A).

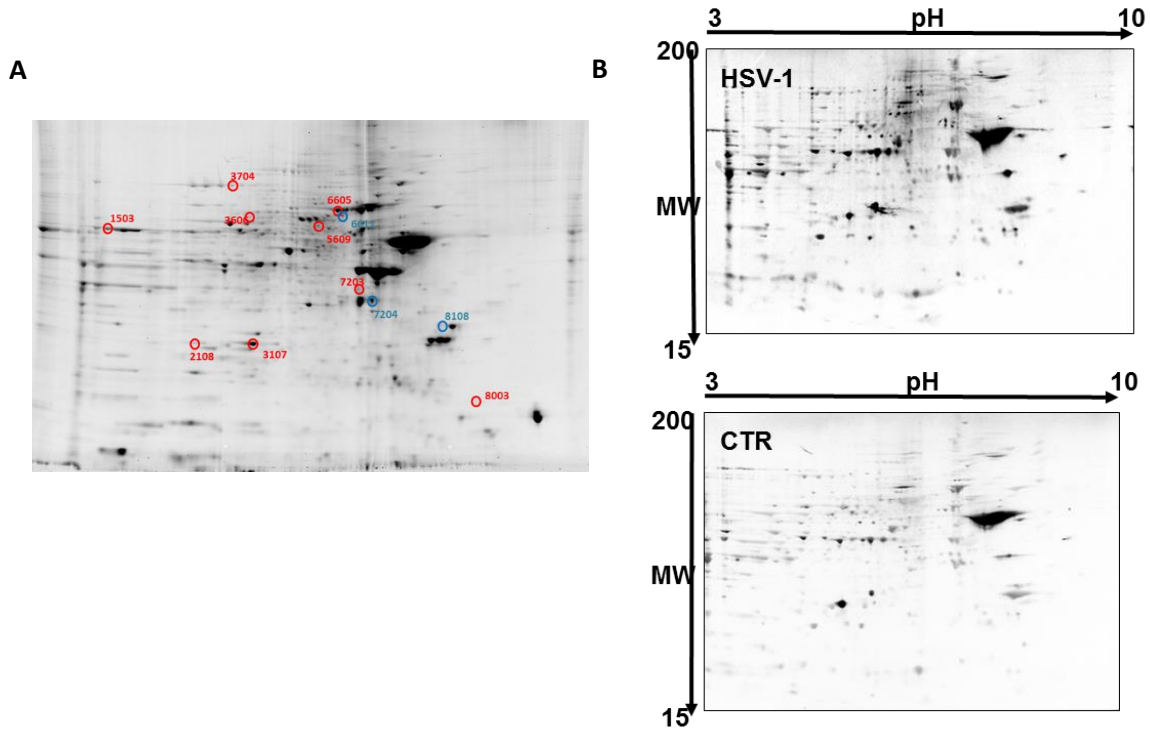

Figure 9. HNE proteomic brain profile in *in vivo* model of recurrent HSV-1 reactivation. A) Representative total protein spots detected by SYPRO Ruby staining in 2D gel from HSV1-M sample. Numbered spots indicate all the HNE-modified proteins with significant altered levels in HSV1-M with respect to CONTROL-M (red = upwards; blue = downwards, see also Table 1). B) Representative 2D-Map-blot proteomic profiles of HNE-oxidized proteins in cortical lysates from HSV1-M and CONTROL-M sacrificed after 7 TSs at 13 months of age.

These spots were further analyzed by MS (MALDI TOF) to identify the corresponding proteins (Table 1). Interestingly, all these proteins are involved in important cellular processes, such as energy metabolism, (DHPR, PGAM1, IDH3A, ATPA) protein folding (GRP78, S23IP), degradation process (AREL1, LSM11) and cell structure (DPYL2, GBB1, MYH10, OCLN, TCTP) suggesting that their oxidative modification may affect brain physiology. Among them, we focus our attention on GRP78 (BiP) that is a key protein in UPR response, a pathway involved in ER stress, and whose alterations are also linked to AD pathogenesis. In particular, GRP78 has been reported to be involved in Aβ clearance in microglia and in tau phosphorylation (Kakimura et al 2011, Liu et al 2012 Chu et al 2014).

WB analysis of brain homogenates revealed that its expression was significantly increased in brain homogenates from HSV1-M as compared to matched CONTROL-M (Fig. 10), suggesting that recurrent HSV-1 infection induce ER stress

Table 1. Protein with increased HNE modification found modulate in HSV1-M vs CONTROL-M identified by redox proteomics analyses.

Spot N°	Protein	Uniprot N°	FOLD	Function
2108	Dihydropteridine reductase (DHPR)	Q8BVI4	1.5 ↑	Tetrahydrobiopterin biosynthesis
3107	Phosphoglycerate mutase 1 (PGAM1)	Q9DBJ1	1.9 ↑	Glycolysis
7203	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial (IDH3A)	Q9D6R2	4.5 ↑	TCA cycle
1503	ATP synthase subunit alpha, mitochondrial (ATPA)	Q03265	5.6 ↑	ATP Synthesis
1503	78 kDa glucose-regulated protein (GRP78)	P20029	5.6 ↑	ER stress
3704	SEC23-interacting protein (S23IP)	Q6NZC7	3.1 ↑	ER re-organization
5609	Apoptosis-resistant E3 ubiquitin protein ligase 1 (AREL1)	Q8CHG5	5.9 ↑	UPS degradation
3606	U7 snRNA-associated Sm-like protein LSm11 (LSM11)	Q8BUV6	3.6 ↑	mRNA processing
6611	Dihydropyrimidinase-related protein 2 (DPYL2)	O08553	10.0 ↓	Neuronal development and polarity, as well as in axon growth and guidance
7204	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1 (GBB1)	P62874	45 ↓	transmembrane signaling systems.
8108	Myosin-10 (MYH10)	Q61879	12.5 ↓	cytoskeleton re-organization
6605	Occludin (OCLN)	Q61146	3.4 ↑	cell-cell junction
8003	Translationally-controlled tumor protein (TCTP)	P63028	3.0 ↑	calcium binding and microtubule stabilization

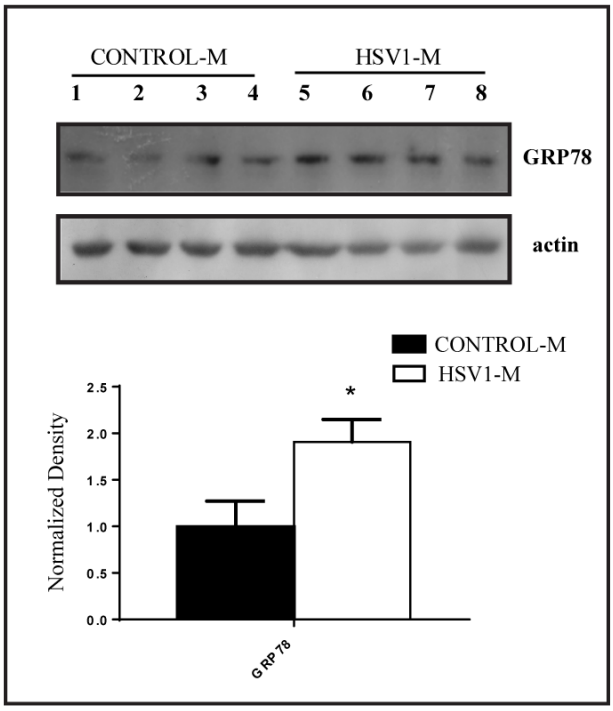

Figure 10. Multiple HSV-1 reactivations induce GRP78 increase. Levels of GRP78 were investigated by the aid of specific antibodies in brain homogenates from CONTROL-M and HSV1-M sacrificed following 7 TSs. Actin expression level was used as sample loading control. Densitometric analysis of immunoreactive signals normalized to actin are shown in the graphs: values represent the normalized fold changes in protein levels from HSV-M with respect to CONTROL-M (mean $\pm$ SEM); * $p < 0.05$ vs CONTROL-M.

5. DISCUSSION

In the present study we demonstrated that repeated HSV-1 reactivations in wild type mouse induce the appearance and progressive accumulation of neurodegenerative and oxidative damage hallmarks typical of AD brain.

Our recurrent HSV-1 reactivation model was set up using female BALB/c mice, since they are reported as suitable for HSV-1 infection (Kastrukoff et al 2012), and sublethal dose of the neurotropic HSV-1 F strain to avoid virus-induced encephalitis (HSE) and death. In particular, we carried out HSV-1 inoculation in young mice by snout abrasion since labial infection is the common way used by the virus to infect humans. Notably, we chose female BALB/c mice also because many studies reported evidence on gender differences in the AD progression. Indeed, not only the female 3xTgAD (a transgenic mouse with AD phenotype) mice have augmented senile plaque load and a more extensive Aβ accumulation respect to males (Clinton et al 2007, Carroll et al 2010), but also female wild type mice showed a higher γ-secretase activity and specificity for Ab42 production (Placanica et al 2009).

Usually, HSV-1 reactivation in *in vivo* models is induced by different stimuli such as cornea UV-irradiation (Shimeld et al 1999), administration of immunosuppressive agents (methylprednisolone or cyclophosphamide), intraperitoneal injection of anti-nerve growth factor antibodies (Birmanns et al 1993) or thermal stress (Huang et al 2011, Sawtell et al 1992). The latter method, was reported to induce virus spreading and inflammation in animal brain (Thompson et al 2010). Thus, we induced HSV-1 reactivations in mice through thermal stress, also mimicking what usually occurs in humans, where virus reactivations are usually triggered by fever and artificial hyperthermia (Sawtell 1992, Boak et al 1934).

Our results showed that repeated HSV-1 reactivations induced an accumulation of hyperphosphorylated tau and APP amyloidogenic products in mouse cortex and hippocampus. In particular, we found accumulation of CTF fragments (WB analysis in Fig. 2) and Aβ (IF analysis and ThS staining in Fig. 3), resulted from an abnormal amyloidogenic APP processing. These data are in line to what previously reported by our and other groups for *in vitro* (De Chiara et al 2010, Piacentini et al 2011, Alvarez et al 2012) and *in vivo* (Martin et al 2014) HSV-1 infection. However, these studies are limited to Aβ production following a short period after primary HSV-1 infection *in vitro* and *in*

vivo models and no data are so far available regarding the long-term effects of HSV-1 infection and reactivations on Aβ hyperproduction. In particular, it is unknown whether, once produced after virus reactivation, this peptide is degraded by cellular enzymes or accumulates in neurons producing its detrimental effects. Our data clearly show that repeated HSV-1 reactivations cause an increasing Aβ accumulation, indicating that this virus-induced effect is not restored over time, but is worsened by the number of virus reactivations. This may be due to an impairment in Aβ clearance. Further studies are required to clarify this issue.

A similar effect was found for the other AD hallmark, that is hyperphosphorylated tau. In particular, through WB analysis with AT100 antibody, that specifically recognizes both PHF and tau phosphorylated at Ser212Thr214 (pSer212Thr214-tau), we showed a significant increase in pSer212Thr214-tau in cortices and hippocampi from 13-month-old HSV1-M after 7 TSs compared with matched CONTROL-M. This double phosphorylation is a specific marker for AD. In fact, pSer212Thr214-tau is reported as less able to bind microtubule and more prone to create self-assembly aggregates (Gong and Iqbal, 2008). Interestingly, we found an increase in a higher molecular weight band recognized by AT100 antibody in 13-month-old HSV1-M undergone 7 TSs, suggesting that hyperphosphorylated tau had started to aggregate in PHFs.

Among the kinases involved in tau phosphorylation, GSK-3β, a pleiotropic kinase that plays a key role in AD (Takashima et al 2006; Leroy et al 2007), was reported from our group to be up-regulated following HSV-1 infection (Piacentini et al 2015, Civitelli 2015). Thus, we decided to study tau phosphorylation at sites reported to be targets of GSK-3β. In particular, we focused on phosphorylation at Thr205 and Ser396, found increased in *in vitro* and *in vivo* AD experimental models (Paudel et al 1993, Krstic et al 2012) as well as in AD patients (Mondragón-Rodríguez et al 2014). We showed that tau phosphorylation at Thr205 is higher in brain tissues following HSV-1 reactivations, especially in older HSV1-M (Fig.4). On the contrary, in this study we did not find any significant increment in phosphoSer396 tau, although other authors showed that HSV-1 induce Ser396 phosphorylation in neuronal cultures (Zambrano et al 2008) and in in vivo model of HSV-1 infection (Martin et al 2014). These controversial results may be due to the different experimental models used and different time of the analyses (several months vs hours or up to 60 days p.i.), as well as the different methodologic approaches (WB vs IF).

The brain is particularly susceptible to the detrimental activity of oxidative stress. This is because the brain is an organ that needs a large amount of oxygen (20% of the body consumption) and contains a large amount of polyunsaturated peroxidizable fatty acids along with high levels of iron that act as a prooxidant. In addition, lipid peroxidation leads to the production of toxic compounds such MDA and HNE which in turn may cause neuronal apoptosis (McCracken et al 2000). Thus, since oxidative stress has been implicated in many neurodegenerative disorders, including AD (Chen et al 2012, Hensley et al 1995, Butterfield et al 2011, Kim et al 2015) and oxidative stress conditions were found following HSV-1-infections in different cell types (Santana et al 2013, Schachtele et al 2010, Nucci et al 2000), we verified whether recurrent HSV-1-infections may trigger oxidative damages and in turn neurodegeneration. We revealed that recurrent HSV-1 reactivation in mouse cortex cause increased oxidative modification in proteins and lipids (HNE, 3-NT and CP), resembling what occurs in the progression of AD (Ansari et al 2010, Sultana et al 2010). In particular, studies from Butterfield's group demonstrated that many proteins in brain from MCI and AD subjects are more oxidatively modified compared with controls, and that these irreversible modifications interfere with their function (Sultana et al 2009). Indeed, oxidative alterations in proteins involved in specific pathways, including energy metabolism, axonal integrity, chaperone machinery and antioxidant systems, correlate with the pathology of MCI and AD (Sultana et al 2010). Hence, our data support the hypothesis that oxidative stress conditions induced by HSV-1 reactivation can concur to the virus-induce neurodegeneration.

To further investigate this hypothesis, according to results from in vitro experiments (Fig. 8) and on the basis of reported data showing that HNE adduct results in alteration of protein function (Guéraud et al 2010), we performed a redox-proteomic approach to identify specific targets of lipid peroxidation in the cortices from HSV1-M sacrificed after 7TSs at 13-monthof age. Interestingly, all the 13 identified proteins (Table 1) that we found modulated by HSV-1 recurrent infection are involved in important cellular processes, such as energy metabolism, (DHPR, PGAM1, IDH3A, ATPA) protein folding (GRP78, S23IP), degradation process (AREL1, LSM11) and cell structure (DPYL2, GBB1, MYH10, OCLN, TCTP). These pathways have been linked to AD onset and progression (Cenini et al 2014).

Among the identified HNE-protein adducts, we focus our attention on GRP78 (BiP) that is a key protein in UPR response, a pathway involved in ER stress. In particular, GRP78 is a member of the heat shock protein 70 (Hsp70) family of proteins, which function as

molecular chaperones by binding transiently to proteins into ER and facilitating their folding, assembly, and transport (Yang et al 1998). Interestingly, some papers showed that GRP78 is involved in Aβ clearance and in tau phosphorylation (Kakimura et al 2011, 2012, Endres and Reinhardt 2013, Chu et al 2014). Indeed, GRP78 was demonstrated to interact with APP, reducing Aβ40 and Aβ42 secretion. This transient interaction may impair access of APP to beta e gamma-secretases within the ER/Golgi or may influence APP metabolism by facilitating its correct folding (Yang et al 1998). In addition, GRP78 was found to bind GSK-3β enhancing its phosphorylation activity, thus causing a consequent increase in tau phosphorylation (Liu et al 2012). Di Domenico and colleagues demonstrated that oxidative damage, among which those mediate by lipid peroxidation, may result in GRP78 inability to bind misfolded proteins, providing a plausible mechanistic link between deficits in molecular chaperones, accumulation of misfolded proteins, risk of cognitive decline and neurodegeneration (2013). In this line, our results, showing an increase in GRP78 bearing HNE modifications in those HSV-1-M that displayed the main accumulation of neurodegenerative hallmarks, suggest that the oxidative deactivation of this protein may play a role in the virus-induced neurodegenerative effects. On the other hand, we also found an increase in GRP78 expression in the same animal brains (Fig. 10). This may be due to ER stress induced by both virus replication cycles and virus induced accumulation of misfolded proteins. Consistently, Hoozemans and colleagues evidenced increased levels of GRP78 in AD brains, confirming that it is a ER stress marker in AD pathogenesis (Hoozemans et al 2005).

Overall, our data from redox proteomic analysis suggest that recurrent HSV-1 infections induce in mouse brain a condition of generalized stress where UPR response, regulation of the cytoskeleton network, protein or nucleic acids degradation pathways are potentially impaired. Actually, we found in HSV1-M cortices some proteins with downregulated levels of HNE-adducts, suggesting that recurrent HSV-1 infection may preserve their function. This may reflect a viral strategy to survive in host cells and/or cell defence against virus replication and damaging effects. Further studies are required to clarify these points.

In conclusion, our data demonstrate that damages produced by recurrent HSV-1 infections into the brain result in the progressive accumulation of neurodegenerative hallmarks, suggesting that recurrent HSV-1 reaching the brain induce an AD-like phenotype. Among them, a crucial role seems played by the constant oxidative stress alterations induced by repeated HSV-1 reactivations.

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