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ORIGINAL ARTICLE

HIV increases the release of dickkopf-1 protein from human astrocytes by a Cx43 hemichannel-dependent mechanism

Juan Andres Orellana,* Juan Carlos Sáez,†,‡ Michael Vander Lann Bennett,§ Joan Weinberger Berman,¶,** Susan Morgello†† and Eliseo Alberto Eugenin*,§§

*Public Health Research Institute (PHRI), Rutgers New Jersey Medical School, Rutgers The State University of New Jersey, Newark, New Jersey, USA

†Departamento de Fisiología, Pontificia Universidad Católica de Chile, Santiago, Chile

‡Centro Interdisciplinario de Neurociencias de Valparaíso, Valparaíso, Chile

§Department of Neuroscience, Albert Einstein College of Medicine, Bronx, New York, USA

¶Department of Pathology, Albert Einstein College of Medicine, Bronx, New York, USA

**Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York, USA

††Department of Neurology, Mount Sinai Medical Center, New York, New York, USA

§§Department of Microbiology and Molecular Genetics, Rutgers New Jersey Medical School, Rutgers The State University of New Jersey, Newark, New Jersey, USA

Abstract

Human immunodeficiency virus-1 (HIV) is a public health issue and a major complication of the disease is NeuroAIDS. *In vivo*, microglia/macrophages are the main cells infected. However, a low but significant number of HIV-infected astrocytes has also been detected, but their role in the pathogenesis of NeuroAIDS is not well understood. Our previous data indicate that gap junction channels amplify toxicity from few HIV-infected into uninfected astrocytes. Now, we demonstrated that HIV infection of astrocytes results in the opening of connexin43 hemichannels (HCs). HIV-induced opening of connexin43 HCs resulted in dysregulated secretion of dickkopf-1 protein (DKK1, a soluble wnt pathway inhibitor). Treatment of mixed cultures of neurons and astrocytes with DKK1, in the absence of HIV infection, resulted in the collapse of neuronal processes. HIV infection of mixed cultures of human neurons and astrocytes also resulted in the collapse of neuronal processes through a DKK1-dependent mechanism. In addition, dysregulated DKK1 expression in astrocytes was observed in human brain tissue sections of individuals with HIV encephalitis as compared to tissue sections from uninfected individuals. Thus, we demonstrated that HIV infection of astrocytes induces dysregulation of DKK1 by a HC-dependent mechanism that contributes to the brain pathogenesis observed in HIV-infected individuals.

Keywords: dementia, gap junctions, HIV, neuroAIDS.

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Despite effective combination of antiretroviral treatment, normal $CD4^+$ T-cell counts, and low to undetectable viral load, human immunodeficiency virus type 1 (HIV) results in neurological complications in 40–60% of individuals (Boisse

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Address correspondence and reprint requests to Eliseo A. Eugenin, Public Health Research Institute (PHRI) and the Department of Microbiology and Molecular Genetics, Rutgers New Jersey Medical School, Rutgers The State University of New Jersey, 225 Warren Street, Newark, NJ 07103, USA. E-mail: eliseo.eugenin@rutgers.edu

Abbreviations used: Cx43 HC, connexin43 hemichannels; DKK-1, Dickkopf-1; GFAP, glial fibrillary acid protein; GJ, gap junction; HIV, human immunodeficiency virus; PBS, phosphate-buffered saline; Panx, pannexins.

et al. 2008; Letendre 2011). These HIV-associated neurocognitive disorders include HIV-associated dementia and the less severe mild neurocognitive disorder. Importantly, even in the current combination antiretroviral treatment era, a significant number of HIV-infected cells may remain in the CNS, including microglia/macrophages (Cosenza *et al.* 2002) and astrocytes (Conant *et al.* 1994; Tornatore *et al.* 1994; An *et al.* 1999; Eugenin and Berman 2007, Eugenin *et al.* 2011). Although the evidence indicates that only a small fraction of astrocytes is infected with HIV, they are the most abundant cell type in the brain, and constitute a significant viral reservoir.

Normally astrocytes participate in multiple functions within the CNS (Giaume 2010), but their role in NeuroAIDS disorders is still not well understood and only recently has been revisited (Hazleton *et al.* 2010). Previous studies by our group showed that despite the small fraction of astrocytes that become infected with HIV and the minimal to undetectable viral replication, significant bystander apoptosis, blood–brain barrier disruption, and cellular dysfunction are observed by a mechanism involving gap junction (GJ) channels (Eugenin and Berman 2007; Eugenin *et al.* 2011), but the role of hemichannels (HC) has not been examined.

GJs are aggregates of channels connecting the cytoplasmic compartments of the coupled cells and providing direct cytoplasmic continuity between the cells allowing electrical and metabolic coordination (Saez *et al.* 2003). A GJ channel is formed by the docking of two hemichannels (one contributed by each of the joined cells), and each hemichannel is composed of six protein subunits termed connexins (Cx). Connexin are a highly conserved protein family encoded by 21 genes in humans and 20 in mice with orthologs in other vertebrate species as well as ascidians. In addition, a glycoprotein family of three members in humans and rodents, unrelated to Cxs but with a similar membrane topology, termed pannexins (Panx), has been shown to form hemichannels in the surface of vertebrate cells (Bennett *et al.* 2012). Recent evidence indicates that hemichannels composed of Cx or Panx in non-junctional membranes can open to the extracellular space under appropriate conditions and allow diffusional exchange between the cytoplasmic compartment and extracellular milieu (Saez *et al.* 2010).

Here, we show that HIV infection of human astrocytes increases the opening of Cx43, but not Panx1 hemichannels, which results in increased expression and secretion of Dickkopf-1 (DKK1), a soluble inhibitor of Wnt signaling. Addition of DKK1, in the absence of HIV infection, to mixed cultures of human neurons and astrocytes results in collapse of neuronal processes. Up-regulation of DDK1 expression is also observed in astrocytes present in brain section from HIVE subjects. Our results provide the basis of a novel mechanism for the spread of damage mediated by astroglial connexin43 hemichannels (Cx43 HC) in response to HIV infection.

Materials and methods

Reagents and antibodies

Dulbecco's modified Eagle's medium, fetal bovine serum, penicillin/streptomycin (P/S), and trypsin-EDTA were purchased from Invitrogen (Carlsbad, CA, USA). The HIV isolates, HIV_{ADA}, $HIV_{JR\text{-}CSF}$, and HIV_{Bal}, were from the National Institutes of Health AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health (Germantown, MD, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise designated. Antibodies to Cx43 and glial fibrillary acid protein (GFAP) were obtained from Cell Signaling (Boston, MA, USA). Gap26, ^{10}panx1, and scrambled peptides were obtained from Peprotech (Rocky Hill, NJ, USA). DKK1 blocking and immune staining antibodies were obtained from R & D systems (Minneapolis, MN, USA). $Cx43^{E2}$, a previously characterized Cx43 HC antibody to the second extracellular loop was kindly provided by Dr Jean Jiang, Department of Biochemistry, University of San Antonio, USA (Orellana *et al.* 2013).

Cell culture

Cortical tissue from human fetuses was obtained as part of an ongoing research protocol approved by the Albert Einstein College of Medicine and Rutgers University (protocol02-21-12-01A1 and Pro2012001303). The preparation of astrocyte and neuronal cultures was performed as described previously (Eugenin and Berman 2003; Eugenin *et al.* 2003, 2007; Eugenin *et al.* 2011). U87, an astrocytoma cell line transfected with CD4 and CCR5, was used as a model of HIV-infected astrocytes. Upon infection these cells maintain Cx43 expression, gap junctional communication, and HC on the surface of the cells, like primary astrocytes. In addition, these cells are highly susceptible to HIV infection (~ 80–90%) in contrast to the lower prevalence (5–8%) in primary astrocytes. Thus, we infected U87CD4CCR5 cells with HIV for 2 days and mRNA microarray was performed using a Human whole genome onearray™ system (n = 3, Phalanx, Hsinchu, Taiwan, www.phalanxbiotech.com).

HIV infection of primary cultures of human astrocytes

Confluent cultures of human fetal astrocytes were infected by incubation with viral stocks (20–50 ng p24/mL/1 × 10^6 cells) of HIV_{ADA}, $HIV_{JR\text{-}CSF}$, or HIV_{Bal}, using a previously described protocol (Ohagen *et al.* 1999; Eugenin *et al.* 2003). Briefly, astrocyte cultures were exposed to the virus for 24 h. The medium was removed and astrocytes were washed extensively to eliminate the unbound virus before addition of fresh medium. Immunofluorescence analyses for GFAP and HIV-p24 indicated that cells infected with HIV were astrocytes, and that cultures showed no contamination with CD68 positive microglial cells (data not shown). Our published data indicated that infection with HIV_{ADA}, $HIV_{JR\text{-}CSF}$, or HIV_{Bal} always resulted in ~ 5% infectivity despite the amount of virus used (Eugenin and Berman 2007; Eugenin *et al.* 2011).

Dye uptake and time-lapse fluorescence imaging

For "snapshot" experiments, control and treated cells were exposed to 5 μM ethidium (Etd) bromide and TOPRO for 10 min at 37°C in culture medium. Then they were washed five times for 2 min each with Hank's balanced salt solution (in mM: 137 NaCl, 5.4 KCl, 0.34 Na_2HPO_4, 0.44 KH_2PO_4, 1.2 $CaCl_2$ at pH 7.4), and

fixed at 28°C with 2% paraformaldehyde for 30 min, mounted in antifade reagent conjugated with 4′,6-diamidino-2-phenylindole (DAPI) and examined in a confocal laser-scanning microscope. Stacks of consecutive confocal images taken with a 63X objective at 500-nm intervals were acquired sequentially with two lasers (argon 488 nm and helium/neon 543 nm), and Z projections were reconstructed using Leica confocal software. For time-lapse fluorescence imaging, cells were plated on dishes in Locke's solution (containing (in mM) 154 NaCl, 5.4 KCl, 2.3 $CaCl_2$, 5 HEPES, and pH 7.4) with 5 μ*M* Etd *and TOPRO*. Phase-contrast and fluorescence microscopy with time-lapse imaging were used to record cell appearance and fluorescence intensity changes in each condition as described previously. No differences in dye uptake were found before the addition of the virus. Images of Etd uptake were analyzed with the Image J program (NIH software). For data representation, the average of four independent background fluorescence intensity measurements (F_B, expressed as arbitrary units, AU) was subtracted from the fluorescence intensity in each cell (F_1). Results of this calculation (F_1–F_B) [or F–F_0] for 20 cells were averaged and plotted vs. time. Slopes of dye uptake were calculated using Microsoft Excel software and mean values of slopes were compared using GraphPad Prism software (La Jolla, CA, USA).

Immunofluorescence and apoptosis assays

Cultures of human fetal astrocytes and mixed cultures of neurons and astrocytes were grown on coverslips and fixed and permeabilized in cold 70% ethanol for 20 min at −20°C. Cells were incubated in terminal deoxynucleotidyl transferase dUTP nick end labeling reaction mixture (Roche, Mannheim, Germany) at 37°C for 1 h, washed three times in phosphate-buffered saline (PBS), and incubated in blocking solution for 30 min at 28°C. Cells were incubated in diluted primary antibody (anti-GFAP, antineuron-specific beta III tubulin, and DDK1; 1 : 800, 1 : 2000, and 1 : 300, respectively) overnight at 4°C. Cells were washed several times with PBS at 28°C and incubated with the appropriate secondary antibodies conjugated to fluorescein isothiocyanate or Cy3 for 1 h at 28°C, followed by another wash in PBS for 1 h. Coverslips were then mounted using antifade reagent with DAPI, and cells were examined by confocal microscopy as described above. Antibody specificity was confirmed by replacing the primary antibody with a non-specific myeloma protein of the same isotype or non-immune serum.

Human tissue sections

Autopsy brain tissue was collected as part of the IRB approved protocols of the Manhattan HIV Brain Bank (U01MH083501). We analyzed tissues from five uninfected donors as controls and from five donors with HIV encephalitis. All human brains were subjected to postmortem ischemic events, owing to time of tissue processing. Sections of 10-μm thickness were processed for immunofluorescence and confocal microscopy as described above.

ELISA for DKK1 and HIV-p24

DKK1 and HIV-p24 levels were determined by ELISA according to the manufacture's protocols (R & D Systems and Perkin Elmer, Boston, MA, USA, respectively).

Statistical analysis

Paired *t* tests were used to calculate significance. A value of $p < 0.05$ was considered significant.

Results

HIV induces opening of Cx43 hemichannels in primary cultures of human astrocytes

To examine whether HC opening is modulated by HIV, human primary astrocytes were infected with HIV using three different viral strains. Astrocytes were exposed to HIV_{ADA}, HIV_{Bal}, or $HIV_{JR\text{-}CSF}$ (20–50 ng/mL) for 24 h and then evaluated for hemichannel activity by Etd uptake (charge +1) at different times after infection. Etd only crosses the plasma membrane in healthy cells by passing through relatively non-specific large channels, such as connexin and pannexin hemichannels, and becomes fluorescent upon binding to intracellular nucleotides (Orellana *et al.* 2011). Time-lapse fluorescence imaging revealed that similar to rodent astrocytes (Orellana *et al.* 2010), human astrocytes cultured under control conditions exhibited a basal Etd uptake (Fig. 1a and b). After HIV_{ADA} infection, the uptake of Etd (Fig. 1a) and TOPRO (Fig. 1a) gradually increased, approaching a plateau at 5–7 days post-exposure, to ~ 650% of the basal rate (Fig. 1b). Similar results were obtained after stimulation with HIV_{Bal} or $HIV_{JR\text{-}CSF}$ (data not shown). These findings were corroborated using "snapshot" experiments of TOPRO (charge +2) uptake (Fig. 1a), another dye employed to measure HC activity (Sandilos *et al.* 2012). Despite the fact that only 5–8% of the cells became productively infected with HIV (Eugenin & Berman 2007, Eugenin *et al.* 2011), all astrocytes exhibited increased Etd and TOPRO uptake after HIV stimulation (Fig. 1a). No differences were found in dye uptake induced by the three HIV strains tested (data not shown). In addition, we observed an increase in GFAP staining in HIV exposed cultures (Fig. 1a). Increased GFAP has been observed *in vitro* and *in vivo* as a marker of astroglial activation in HIV-infected individuals (Petito *et al.* 2001; Anderson *et al.* 2003; Vanzani *et al.* 2006).

The possible role of Cx43 HC in the HIV-induced Etd uptake was examined using specific blockers of Cx43 HC, a $Cx43^{E2}$ antibody (Orellana *et al.* 2011) and the blocking mimetic peptide Gap26 with an amino acid sequence identical to a segment of the second extracellular loop of Cx43 (Evans *et al.* 2006). $Cx43^{E2}$ (1 : 500) and Gap26 (200 μM) reduced Etd uptake rate to control values; La^{3+}, a general blocker of connexin HC, caused a similar reduction in Etd uptake (Fig. 2). Pre-treatment with the pre-immune antibodies (1 : 500, data not shown) and a scrambled peptide of Gap26 did not affect the rate of HIV-induced Etd uptake (Fig. 2), supporting the specificity of $Cx43^{E2}$ and Gap26. In contrast ^{10}panx1 (200 μM) and probenecid (200 μM), both Panx1 HC blockers (Pelegrin and Surprenant 2006; Silverman *et al.*

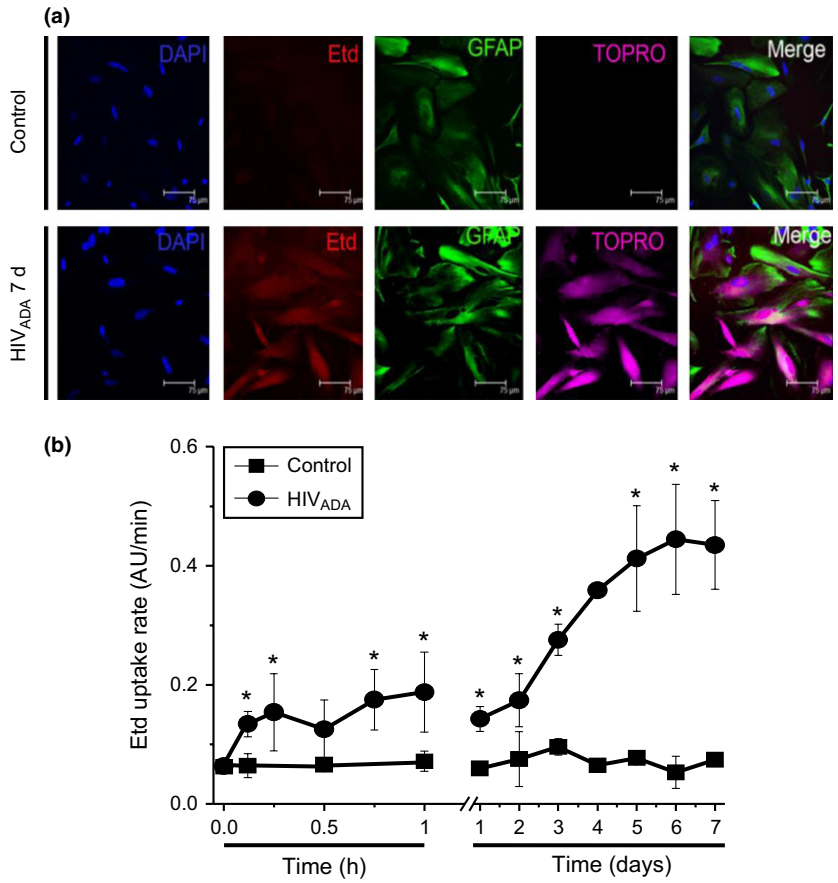

Fig. 1 Human immunodeficiency virus (HIV) increases Etd uptake in human primary astrocytes. (a) Representative snapshot of the time lapse described in (b) of dye uptake in control conditions or 168 h after HIV_{ADA} infection (HIV_{ADA} 7 days). Astrocytes were identified by glial fibrillary acid protein (GFAP) staining. Bar: 75 μm. (b) Quantification of the time course of Etd uptake rate, obtained from human primary astrocyte cultures under control conditions (lines with squares) or after HIV_{ADA} infection (lines with circles). Each value corresponds to the mean ± SD of the Etd intracellular intensity present in at least 20 cells per time point, $^*p < 0.005$ vs. control conditions, $n = 4$.

2008), failed to reduce the rate of HIV-induced Etd uptake, ruling out the possible involvement of Panx1 HC in this phenomenon. Taken together, these data indicate that HIV infection induces Cx43 HC opening in primary human astrocytes.

Chemokine receptors involved in HIV entry do not induce hemichannel opening in cultured human astrocytes

Recent findings of our laboratory indicate that different HIV strains and chemokines that bind receptors associated with HIV entry (CCR5 or CXCR4) increase the opening of Panx1 HC in human $CD4^+$ T lymphocytes (Orellana *et al.* 2013). Thus, to examine whether chemokines that bind CCR5 or CXCR4 participate in the opening of Cx43 HC in human astrocytes, a time course of Etd uptake, as described in Fig. 1b, was performed. RANTES/CCL5 (100 ng/mL), MIP-1α/CCL3 (100 ng/mL), and MIP-1β/CCL4 (100 ng/mL), all chemokine ligands for CCR5, failed into increase the rates of Etd uptake (Fig. 3a–c). Increasing concentrations (to 300 or 500 ng/mL, $n = 5$) also did not affect the rates of Etd uptake (data not shown). Moreover, stimulation with stromal cell derived factor-1 (SDF-1/CXCL12, 100 ng/mL), a physiological ligand for CXCR4, did not affect the rate of Etd uptake, until 7 days (168 h) post treatment; however, after 7 days no further increase in Etd uptake was detected (Fig. 3d). No chemokine treatment reproduced the HIV-induced time course of Etd uptake (compare to Fig. 1), ruling out mediation by CCR5 or CXCR4 and differentiating the mechanism from Panx-1 HC opening in $CD4^+$ T lymphocytes in response to HIV infection (Orellana *et al.* 2013).

To further identify the mechanism of Cx43 HC opening, we characterized whether HC opening was caused by infection with (VSV)-HIV_{BAL}, a pseudotyped HIV with a vesicular stomatitis viral envelope, that does not require viral membrane interaction to enter the cells (Superti *et al.* 1987). Infection with this pseudotyped virus did not increase the rate Etd uptake as compared to HIV treatment, suggesting that

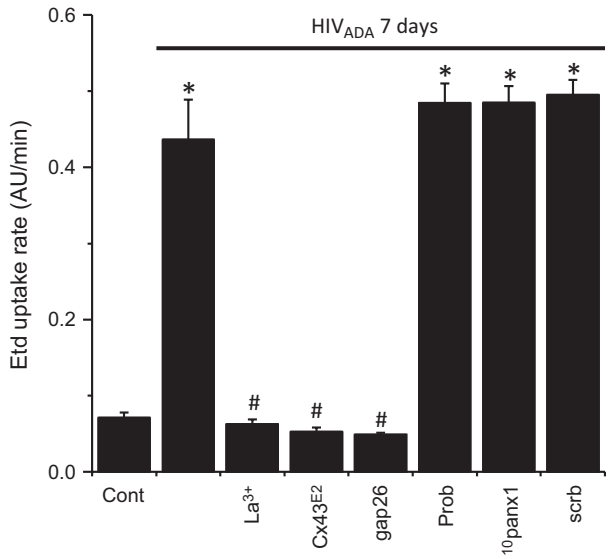

Fig. 2 HIV infection induces opening of astroglial Cx43 HC. Etd uptake rate for human primary astrocytes exposed to HIV$_{ADA}$ for 7 days is shown. Control cells have minimal Etd uptake (Cont). HIV infection resulted in significant increase in Etd uptake (*$p < 0.005$). Addition of Cx43 HC blockers, lanthanum ions (La^{3+}, 200 μM), Cx43^{E2} antibody (1 : 500 dilution), or peptide Gap26 (200 μM) abolished the increase of Etd uptake induced by HIV infection. In contrast, blockers of pannexins (Panx)1 HCs, such as Probenecid (Prob, 500 μM), and ^{10}Panx1 (200 μM) did not alter the Etd uptake induced by the virus. The scrambled peptide for Gap26 and ^{10}Panx1 (scrb, 200 μM) did not affect Etd uptake induced by the virus. Each value corresponds to the mean ± SD, (* represents significance of $p < 0.005$ as compared with control conditions (cont), #represents significant difference as compared to HIV infection alone, $p < 0.005$, $n = 4$).

virus–plasma membrane interaction is required for the HIV-induced Cx43 HC activity (data not shown). Previous reports indicated that the mannose receptor is involved in HIV entry into astrocytes (Liu *et al.* 2004). However, ligands for this receptor such as albumin, dextran, or insulin did not increase Etd uptake in primary astrocytes (data not shown). Altogether these results indicate that the opening of Cx43 HC does not depend on chemokine–chemokine receptor or mannose receptors interaction, but results from HIV interaction with the plasma membrane and thus differs from the mechanism recently described for HIV-induced opening of Panx1 HC in CD4$^+$ T lymphocytes (Orellana *et al.* 2013).

Opening of Cx43 hemichannels modulates DKK1 secretion induced by HIV infection of astrocytes

Our previous studies indicated that HIV infection of astrocytes results in bystander apoptosis of neighboring uninfected astrocytes by a GJ-dependent mechanism (Eugenin & Berman 2007; Eugenin *et al.* 2011, Eugenin *et al.* 2012), but these studies did not examined the role of HC. Despite the low rate of infection and the low number of infected astrocytes, significant changes in the secretion of inflammatory factors and neurotransmitters (glutamate) occurred (Eugenin & Berman 2007; Eugenin *et al.* 2011, Eugenin *et al.* 2012). Blockade of Cx43 HC did not alter the number of HIV-infected astrocytes (7.3 ± 2.1% vs. 6.9 ± 3.5% in the presence of La^{+3}, Cx43^{E2} or gap 26). In addition, HIV viral replication and bystander apoptosis (HIV-infected astrocytes affect neighboring uninfected cells) were not altered by the HC blockers. Thus, to identify additional HIV factors altering neuronal and glial functions, cDNA microarray analysis was performed using HIV-infected astrocytes. We used the astrocytoma cell line, U87, transfected with CD4 and CCR5 (80–90% of the cells become infected, instead of primary cells where only 5–8% of the cells become infected) for the microarray analysis. Three independent cDNA microarrays consistently indicated that DKK1 mRNA was the main up-regulated mRNA (7.2 ± 2.8 folds, $p = 6.1 \times 10^{-5}$, $n = 3$) in response to HIV infection.

DKK1 is a secreted protein member of the dickkopf family and is involved in embryonic development through the inhibition of the Wnt signaling pathway (Zorn 2001). DKK-1 interacts with LRP5/6 and the coreceptor Kremen 1/2. This triggers LRP5/6 endocytosis, thereby preventing formation of the LRP5/6–Wnt–Frizzled complex and reducing cellular differentiation induced by the Wnt signaling pathway (Kawano and Kypta 2003). Dysregulation of DKK1 secretion have been associated with cancer and Alzheimer's disease (Rosi *et al.* 2010; Purro *et al.* 2012). Coordination of the Wnt pathway is mediated by GJ channels (van der Heyden *et al.* 1998; Ai *et al.* 2000; Xu *et al.* 2001; Du *et al.* 2008). However, the role of HC or GJ channels in HIV infection and Wnt signaling remains unknown.

Recent studies of HIV infection linked DKK1 expression and β-catenin pathways with changes in viral replication (Li *et al.* 2011). However, the mechanisms by which DKK1 is dysregulated during HIV infection in glial cells remain to be elucidated. For all studies illustrated HIV$_{ADA}$ was used, however, similar data were obtained for HIV$_{JR\text{-}CSF}$ and HIV$_{BAL}$ (not shown). During the time period analyzed (0–7 days post-HIV stimulation), cultures of human astrocytes not treated with virus release low and stable levels of DKK1 into the medium as determined by ELISA (Fig. 4A). Blockade of Cx43 HC by using Cx43^{E2} and Gap 26, or inhibition of Panx1 HC with ^{10}Panx1 did not alter secretion of DKK1 in these conditions (Fig. 4a). Thus, in the absence of virus Cx HC do not play a role in secretion of DKK1.

In contrast to untreated cultures, extracellular levels of DKK1 rose as the duration of HIV infection increased (Fig. 4b). Notably, the Cx HC blockers, Cx43^{E2} or Gap26, increased the HIV-induced DKK1 secretion at 1–4 days post-HIV infection, but later on (5–7 days post-HIV) DKK1 release returned to the level at or below that observed after 1 day (Fig. 4b). ^{10}Panx-1 did not alter secretion of DKK1 during the time period analyzed (Fig. 4b), indicating that

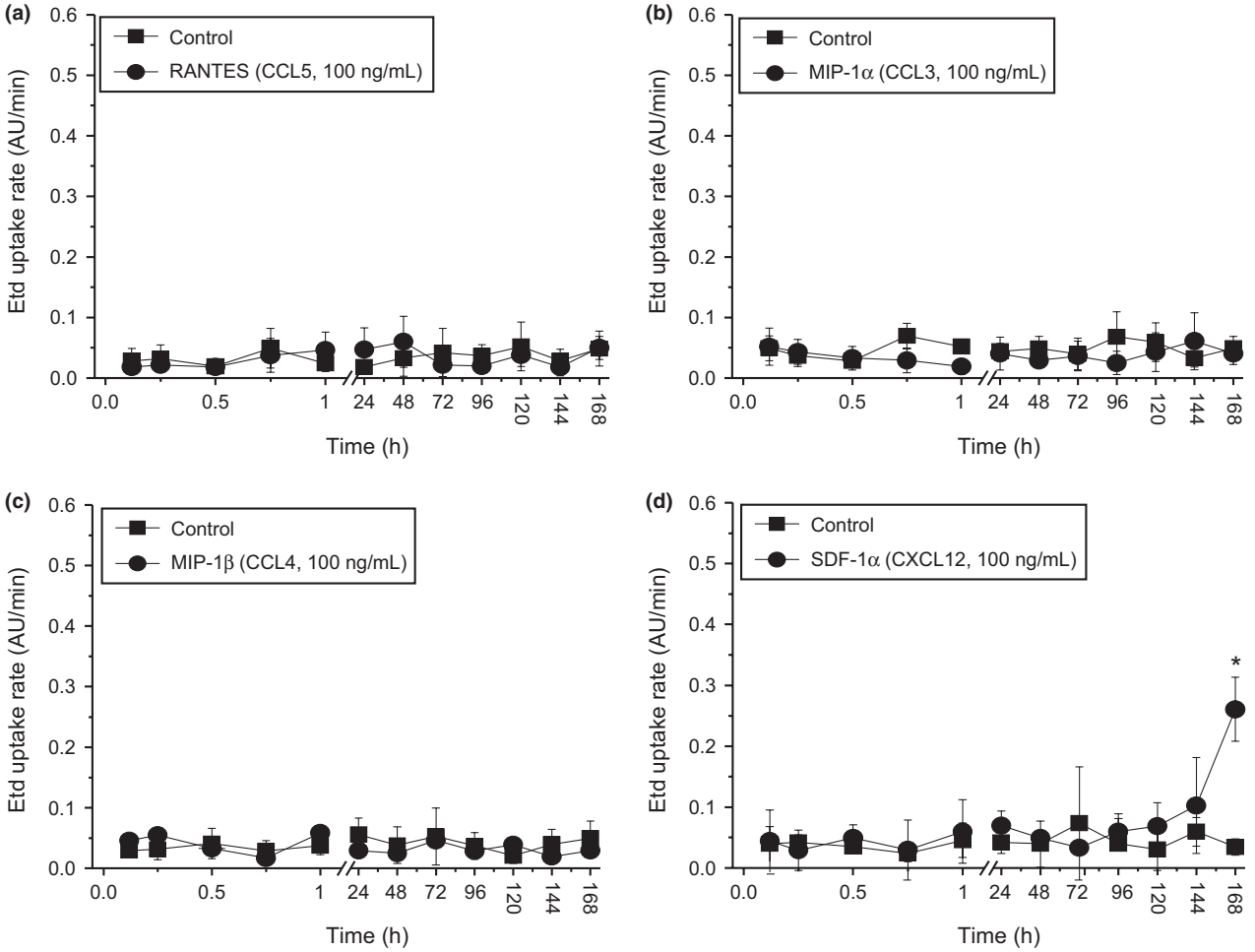

Fig. 3 Chemokines involved in human immunodeficiency virus (HIV) infection do not open HCs on human primary astrocytes. Etd uptake measurements were perform in human primary cultures of astrocytes under control conditions (lines with squares) or after chemokine treatments (lines with circles): RANTES (CCL5, a), MIP-1α (CCL3, b), MIP-1β (CCL4, c) and SDF-1α (CXCL12, d). No differences were obtained using higher concentrations (300 and 500 ng/mL) of these chemokines involved in HIV infection (*$p < 0.003$, $n = 4$), except by SDF-1α at 7 days post treatment only.

Panx1 HC do not participate in the HIV-induced DKK1 secretion in human astrocytes. Our data indicate that opening of Cx43 HC in response to HIV infection (Fig. 4b, 2–4 days) maintains DKK1 secretion at low level because blocking opening of the channel enhanced DKK1 secretion. After 4 days Cx43 HC opening participates in the increased secretion of DKK1 (5–7 days) because blocking Cx43 HC reduced DKK1 secretion (Fig. 4b, 5–7 days). In all these experiments, no viral replication was detected; thus, changes in secretion of DKK1 were not associated with enhanced viral replication as determined by HIV-p24 ELISA (data not shown).

DKK1 is up-regulated in human brain from subjects with HIV encephalitis

To examine where DKK1 is normally expressed in the CNS and to identify the cell type involved, uninfected and HIV encephalitic brain sections were analyzed by immunofluorescence and subsequent confocal microscopy. Sections obtained from adult uninfected individuals showed minimal DKK1 reactivity and did not colocalize with GFAP positive cells, suggesting that under control conditions, astrocytes do not express DKK1 (Fig. 5, uninfected). Confocal analysis of brain sections obtained from patients with HIV encephalitis showed increased DKK1 staining compared with normal controls in reactive astrocytes as well as in other cells (Fig. 5, HIVE). The inset in the HIVE merge is shown enlarged in the next row images, demonstrating that these astrocytes express a significant amount of DKK1 (Fig. 5, HIVE, third row). Negative controls using IgGs and control sera did not show positive staining (Fig. 5, Negative). DKK1 expression was associated with the presence of other cell types, in addition to astrocytes, such as endothelial cells (data not shown). In summary, DKK1 expression is up-regulated in

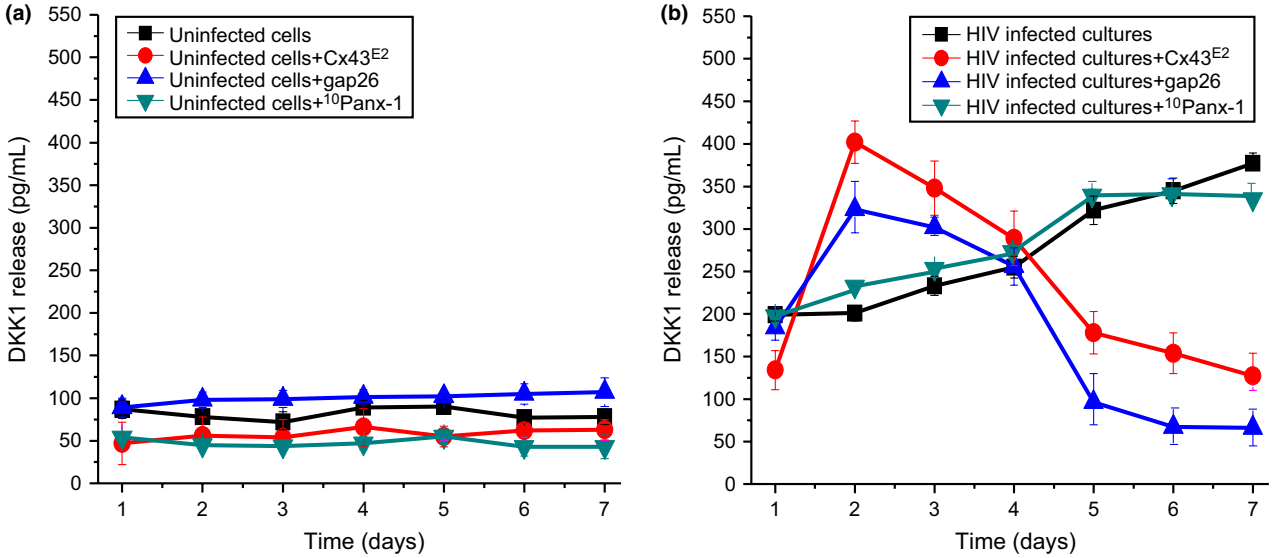

Fig. 4 The opening of Cx43 HCs enhanced secretion of Dickkopf-1 (DKK1) in response to human immunodeficiency virus (HIV) infection of astrocytes. (a) In control condition, minimal secretion of DKK1 was detected during the time course analyzed (1–7 days). Blockers of Cx43 HCs did not alter the secretion of DKK1 during the time course examined in uninfected cells. The blockers used were $Cx43^{E2}$ (1 : 500), gap 26 (200 μM), and ^{10}panx-1 (200 μM). All highly effective blockers of these channels as described in figure 2. (b) HIV infection of human primary astrocytes increased DKK1 release in a time-dependent manner (black line with squares) as compared with uninfected conditions [see (a), black line with squares]. Blocking HCs with $Cx43^{E2}$ (red line with circles) or gap26 (blue line with upright triangles) after HIV infection results in increased secretion of DKK1 as compared to HIV infection alone during the first 3 days post infection. After 5 days post-infection blocking HCs reduces secretion of DKK1. Blocking pannexins (Panx)1 HC with ^{10}Panx-1 peptide also did not block secretion of DKK1 in response to HIV infection (purple line with triangles) ($n = 6$, all numbers are significant as compared with uninfected cells, except days 5–7 of HIV infected cultures+gap26).

astrocytes and other brain cells in HIVE, supporting the idea that HIV infection increases the secretion of DKK1 in the CNS during HIV invasion of the brain.

DKK1 participates in neuronal damage, but not in HIV infection/reactivation in human astrocytes

To determine the effect of DKK1 on neuronal function, in the absence and presence of HIV infection, we stimulated mixed primary cultures of neurons and astrocytes with increasing concentrations of DKK1, mimicking the curve secretion induced by HIV infection (Fig. 4b). In addition, we HIV-infected cultures of astrocytes in the presence and absence of neutralizing antibodies to DKK1 or blocking gap26, a blocking Cx43 HC peptide, as well as controls (non-immune IgG or scrambled peptide). Neuronal cultures were treated with recombinant DKK1 (300 or 500 pg/mL), and then levels of apoptosis, neuronal process length, HIV infectivity, and viral replication were determined. DKK1 treatment of uninfected mixed cultures of neurons and astrocytes did not alter neuronal apoptosis or HIV infectivity after 0.5, 1, 2, 7, and 14 days as assayed by terminal deoxynucleotidyl transferase dUTP nick end labeling, ELISA, or immunofluorescence for p24 (data not shown). However, DKK1 (300 or 500 pg/mL) treatment, in the absence of HIV infection, resulted in significant collapse of neuronal processes in mixed cultures of neurons and astrocytes (Fig. 6, white bars), as determined by staining and quantification of the length of the neuronal processes using the imaging program NIS Elements Advance Research. A similar morphology of collapsed neuronal processes has been observed in the brain of HIV-infected individuals, but the mechanism of synaptic compromise remains unknown (Adamson *et al.* 1996; Epstein and Gelbard 1999; Kolson 2002; Kim *et al.* 2008). In addition, DKK1 treatment of mixed cultures of neurons and astrocytes did not open connexin HC (data not shown), suggesting that the opening of HC induced by the virus regulates expression and subsequent secretion of DKK1.

HIV infection of these mixed cultures of neurons and astrocytes also resulted in reduction in neuronal processes in a similar manner to that observed after DKK1 treatment (Fig. 6, yellow bars). The pre-inculation of the cultures with neutralizing antibodies to DKK1 (Fig. 6, green bars) or Cx HC blocking peptide, gap26 (Fig. 6, pink bars), reduced the neuronal damage associated with HIV infection, suggesting that DKK1 as well as opening of Cx43 HC are essential for the neuronal processes collapse. Addition of IgG or scrambled peptide was not protective against HIV-induced neuronal processes collapse (Fig. 6, last bar). Thus, we propose that DKK1 is one of the mediators of synaptic compromise in the context of HIV infection. Therefore, DKK1 and its

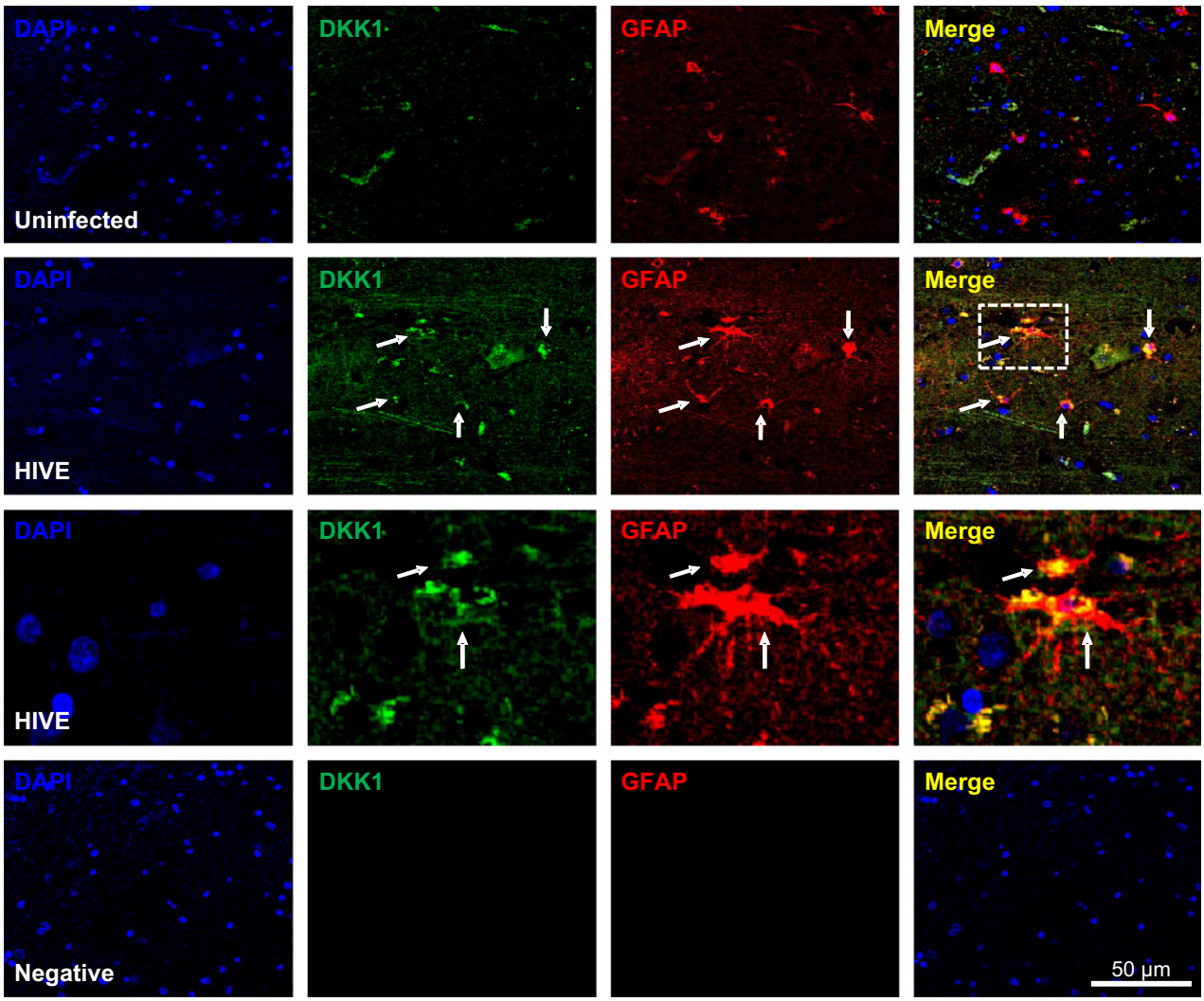

Fig. 5 Dickkopf-1 (DKK1) expression is increased in human brain tissue sections obtained from individuals with human immunodeficiency virus (HIV) encephalitis. Brain sections from five uninfected individuals and from five with HIV encephalitis (HIVE) were evaluated by immunohistochemistry and confocal microscopy. Astrocyte expression of DKK1 (FITC-green) was evaluated using glial fibrillary acid protein (GFAP, an astrocyte marker, red staining). In uninfected tissue sections, minimal detection of DKK1 was observed and minimal colocalization with GFAP-positive cells was detected (uninfected row). In contrast, in HIVE tissue sections, DKK1 staining was increased and colocalization with GFAP-positive cells was increased (HIVE row). The inset in the HIVE row, Merge, was magnified to demonstrate colocalization of DKK1 with GFAP-positive cells (HIVE third row). Negative control for DKK1 or GFAP antibodies did not show staining (negative row). DAPI staining was used in counter-staining. Arrows represent colocalization of GFAP and DKK1. Thus, DKK1 was consistently elevated in all encephalitic tissue examined (n = 5 cases by condition).

secretion in response to HIV infection of the CNS may be one of the elements that contribute to synaptic collapse and to the pathogenesis of NeuroAIDS.

Discussion

Here, we demonstrate that HIV stimulation of human primary astrocytes results in increased secretion of DKK1, *in vitro* and *in vivo*. Moreover, our results show that opening of astroglial Cx43 HC participates in the up-regulation and mechanism of secretion of DKK1 in response to HIV infection. This altered secretion of DKK1 for HIV infection of astrocytes may contribute to the neuronal compromise often observed in HIV-infected subjects.

Astrocytes accomplish critical functions within the CNS, but their role in several CNS diseases, especially in HIV infection, has been largely ignored. The fraction of astrocytes that are infected by HIV *in vivo* and *in vitro* is low, and viral production is low to undetectable. Previous studies performed in our laboratory indicate that HIV infects only 5–8%

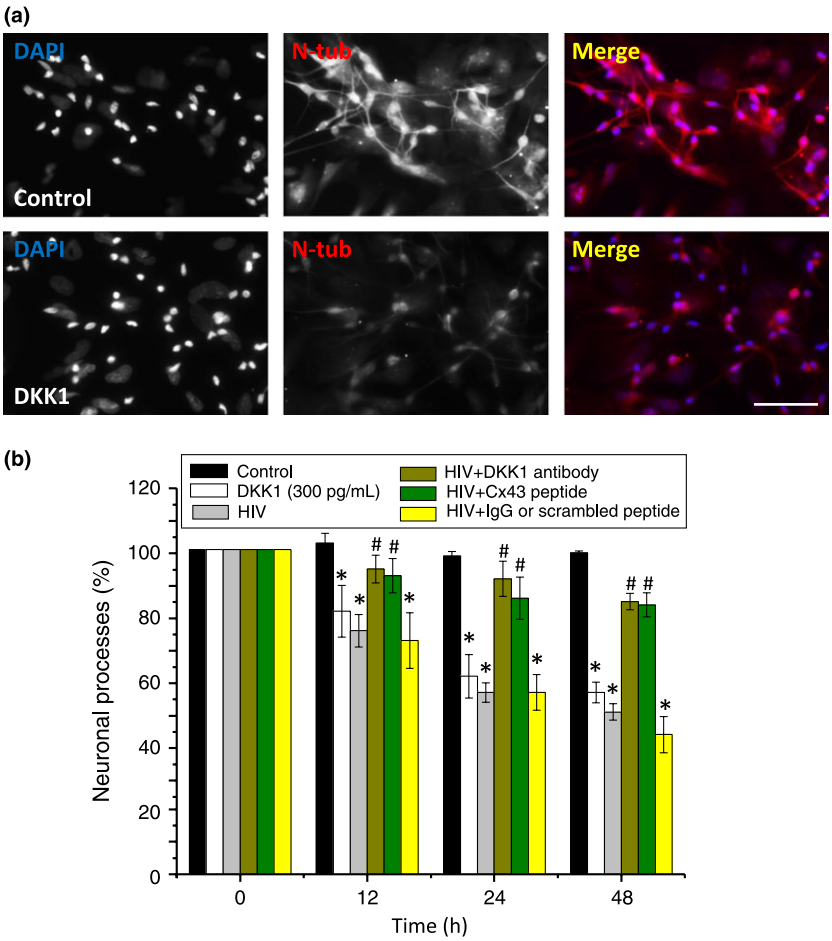

Fig. 6 Dickkopf-1 (DKK1) treatment of human immunodeficiency virus (HIV) infection of mixed cultures of neurons and astrocytes result in collapse of neuronal processes. To examine the potential consequences of DKK1 enhanced secretion induced by HIV, we determined the number and length of neuronal processes in response to DKK1 treatment in the absence or presence of HIV infection. (a) Examples of neuronal processes in control conditions (control) and after 24 h of DKK1 (DKK1, Bar: 300 μm) treatment as analyzed by confocal microscope and imaging software. Neuronal cultures were stained for neuronal specific beta III tubulin (N-tub, red staining) and DAPI to identify nuclei (DAPI). Treatment of neuronal cultures with DKK1 (300 ng/mL, the same concentration released in response to HIV infection) reduced the length and numbers of neuronal processes. Bar: 300 μm. (b) Quantification of the length of the neuronal processes in control and DKK1 treated conditions using an imaging program indicates that DKK1 decreased significantly in 40% the number neuronal processes (white bars) as compared with control conditions (black bars), without altering the total number of neurons during the time course analyzed (data not shown). Similar results were found using 500 pg/mL of DKK1 (data not shown). HIV infection of these cultures resulted in similar decay in neuronal processes as compared to DKK1 treatment (yellow bars). Pre-incubation of cultures with a neutralizing antibody to DKK1 or gap26 (Cx43 peptide), abolished the damage induced by HIV infection, indicating that DKK1 and opening of Cx43 HC are essential to trigger neuronal damage. The use of IgG control or scrambled peptide did not alter the time course of neuronal damage observed by HIV infection. $^{*}p < 0.007$ as compared with control untreated/uninfected conditions, $^{\#}p < 0.005$ as compared with HIV infection alone, $n = 5$.

of primary human astrocytes, and viral replication is minimal to undetectable (Eugenin & Berman 2007; Eugenin *et al.* 2011, Eugenin *et al.* 2012). Despite the low rates of infection and viral production, HIV toxicity appears to spread to neighboring uninfected cells by a mechanism mediated by GJs (Eugenin & Berman 2007; Eugenin *et al.* 2011, Eugenin *et al.* 2012). Thus, the few infected astrocytes generate unknown intracellular toxic mediators that diffuse through GJs into uninfected cells (astrocytes and endothelial cells) resulting in apoptosis (Eugenin & Berman 2007, Eugenin *et al.* 2011). However, our early work did not characterize the contribution of HC to HIV CNS damage. The opening of HC results in release of several metabolites into the extracellular space including ATP, glutamate, NADH, and

prostaglandins (Orellana *et al.* 2012), which contribute to cellular and immune activation (Eugenin *et al.* 2012; Orellana *et al.* 2012). In the context of HIV infection, we and others have described that extracellular ATP as well as particular ATP receptors, P_2X1, P_2X7, P_2Y1, and P_2Y2, are required for efficient infection and viral replication in immune cells (Seror *et al.* 2011; Hazleton *et al.* 2012). However, it was unknown whether HIV modulates the opening of HC in astrocytes, and whether the HC had a role in the pathogenesis of NeuroAIDS disorders. In the current work, we demonstrated that HIV exposure results in the opening of Cx43 HC which participates in the mechanism of DKK1 expression and secretion. Our results indicate that DKK1 secretion is modulated by the opening of Cx43 but not Panx1 HC. Indeed, blockers of Cx43 HC revealed that these channels can play a dual role in DKK1 secretion.

DKK1 is a soluble factor that inhibits the Wnt pathway by binding to LRP5/6 and kremen and causing internalization of the Wnt receptor, LRP5/6 (Kawano and Kypta 2003). DKK1 dysregulation contributes to neuronal death in several CNS diseases, such as Alzheimer's disease (Zorn 2001; Rosi *et al.* 2010; Matrisciano *et al.* 2011; Purro *et al.* 2012), but no data are available on infectious diseases and DKK1 expression in astrocytes. The Wnt pathway participates in HIV replication in astrocytes (Li *et al.* 2011; Al-Harthi 2012), and here we described that application of DKK1 to primary cultures of human neurons and astrocytes did not alter apoptosis, viral infectivity, or replication. Importantly, confocal analysis of human brain sections indicates that DKK1 is up-regulated *in vivo* in NeuroAIDS as compared to uninfected subjects. These results are in agreement with our *in vitro* data, suggesting that HIV infection of the CNS results in expression and up-regulation of DKK1, especially in astrocytes and potentially other GFAP negative cells. Most of the astrocytes expressing DKK1 were hyperreactive, a common feature of glial cells in HIV-infected individuals (Petito *et al.* 2001; Anderson *et al.* 2003; Vanzani *et al.* 2006). Experiments in rodents indicate that neurons in distress secrete DKK1 (Busceti *et al.* 2007; Mastroiacovo *et al.* 2009; Rosi *et al.* 2010; Matrisciano *et al.* 2011; Munji *et al.* 2011; Purro *et al.* 2012). In these reports, it is clear that DKK1 expression is mostly in neurons. However, our data *in vitro* and *in vivo* indicate that human astrocytes also express and secrete high amounts of DKK1 resulting in neuronal synaptic compromise. Here, we show that DKK1 expression and secretion in response to HIV resulted in significant decrease in number and length of neuronal processes. Neuronal processes are important players in the formation and stability of synapses (Knobel *et al.* 1999; Moreno-Lopez and Gonzalez-Forero 2006; Chen *et al.* 2012). Decrease in neuronal processes results in synaptic impairment, loss and subsequently cognitive disease. Importantly, mixed cultures of neurons and astrocytes treated with agonists of NMDA receptors induce a fast secretion of DKK1 in association with a decrease in nuclear β-catenin, which is indicative of inhibition of the Wnt pathway (Cappuccio *et al.* 2005). Previous studies indicate that HIV infection of astrocytes dysregulates glutamate metabolism (Eugenin & Berman 2007), and most of the HIV toxicity observed in neurons is mediated by activation of NMDA receptors (Eugenin *et al.* 2003, 2007; King *et al.* 2006). Thus, alterations in glutamate metabolism and DKK1 secretion may be involved in neuronal/synaptic compromise in response to HIV infection.

Recently, we demonstrated that HIV infection results in opening of Panx1 HC in $CD4^+$ T lymphocytes, depending on the viral isolate used and the associated chemokine receptors involved in HIV entry (Orellana *et al.* 2013). It is widely known that binding of the HIV envelope protein gp120 to the host CD4, CCR5, and/or CXCR4 receptors in $CD4^+$ T lymphocytes depends on the viral isolate used (Choe *et al.* 1996; Dragic *et al.* 1996; Wu *et al.* 1996, 1997). However, HIV infection of astrocytes is CD4 independent (Schweighardt *et al.* 2001; Liu *et al.* 2004). Although only 5–8% of astrocytes become infected, all astrocytes exhibit increased Cx43 HC activity in response to HIV infection. Infection of astrocytes with VSV-HIV which does not require virus receptor interaction [CD4 and CCR5 and/or CXCR4, (Superti *et al.* 1987)] does not change HC activity in human astrocytes, again suggesting that neither chemokine nor CD4 receptors are required for the opening of these channels. In agreement, chemokine stimulation of astrocytes did not cause opening of Cx43 HC. Another receptor in astrocyte involved in HIV entry is the mannose receptor (CD206, MRs) (Liu *et al.* 2004); however, ligands for this receptor did not open Cx43 HC and in addition, only 50% of the cells express this receptor, but 100% of the cells respond to HIV exposure. Thus, viral replication or infection is not required for HC opening, but interaction of the envelope with the membrane is essential. Importantly, the mechanism of HIV-induced HC opening in human astrocytes is different from that in human $CD4^+$ T lymphocytes. Thus, further experiments will be required to identify the mechanism(s) underlying HIV-induced opening of Cx43 HC.

Our studies demonstrated that HIV infection of astrocytes induces opening of Cx43 HC, and we propose that the relatively non-specific increase in permeability participates in the CNS dysfunction observed in HIV-infected individuals. These data provide a novel mechanism of damage in NeuroAIDS and indicate potential new targets for therapeutic interventions, such as DKK1 and glycogen synthase kinase-3 beta inhibitors, to reduce the ongoing CNS effects of HIV.

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Conflict of interest

None.

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