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Endolysosome involvement in HIV-1 Tat-induced neuronal amyloid beta production

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Abstract

The increased life expectancy of people living with HIV-1/AIDS is accompanied by increased prevalence of HIV-1 associated neurocognitive disorder (HAND). As well, these individuals are increasingly experiencing Alzheimer's disease (AD)-like neurocognitive problems and neuropathological features such as increased deposition of amyloid beta (A β) protein. Findings that A β production occurs largely in endolysosomes, that HIV-1 transactivator protein (Tat) disrupts endolysosome function - an early pathological feature of AD - and that HIV-1 Tat can increase A β levels prompted us to test the hypothesis that endolysosome dysfunction is associated with HIV-1 Tat-induced increases in neuronal A β generation. Using primary cultured rat hippocampal neurons, we found that treatment with HIV-1 Tat caused such morphological changes as enlargement of endolysosomes as identified with LysoTracker dye and such functional changes as elevated endolysosome pH as measured ratiometrically with LysoSensor dye. The HIV-1 Tat-induced changes in endolysosome function preceded temporally HIV-1 Tat-induced increases in A β generation as measured by ELISA. In addition, we demonstrated that HIV-1 Tat increased endolysosome accumulation of amyloid beta precursor protein and A β as identified by immunostaining with 4G8 antibodies. Furthermore, we demonstrated that treatment of neurons with HIV-1 Tat increased endolysosome accumulation of beta amyloid converting enzyme (BACE-1), the rate limiting enzymatic step for A β production, and enhanced BACE-1 activity. Together, our findings suggest that HIV-1 Tat increases neuronal A β generation and thereby

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Animal protocol was approved by the University of North Dakota Animal Care and Use Committee adherent with the Guide for the Care and Use of Laboratory Animals (NIH publication number 80-23).

All authors have reviewed the contents of the manuscript being submitted, and all authors approve its contents and validate the accuracy of the data.

Disclosure statement

The authors have no current or potential conflicts of interest to report.

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contributes to the development of AD-like pathology in HIV-1 infected individuals by disturbing endolysosome structure and function.

Keywords

HIV-1 Tat; amyloid beta; endolysosome; pH; BACE-1

1. Introduction

Greater than 40 million people worldwide are infected with the human immunodeficiency virus-1 (HIV-1) and pharmacotherapeutic treatment with combined antiretroviral therapeutic drugs has effectively increased the life span of people living with HIV-1/AIDS. Increased as well is the prevalence of HIV-1 associated neurocognitive disorders (HAND), a set of conditions ranging from subtle neuropsychological impairments to profoundly disabling HIV-associated dementia. Indeed, recent epidemiological studies indicate that the prevalence of HAND in the USA is greater than 50% of HIV-1 infected people (Ellis, et al., 2010, Heaton, et al., 2010).

Increasing too is the incidence of Alzheimer's disease (AD)-like neurocognitive problems and neuropathological features of AD such as increased amyloid beta (A β) protein deposition in older HIV-1 infected patients (Achim, et al., 2009, Clifford, et al., 2009, Esiri, et al., 1998, Gelman and Schuenke, 2004, Green, et al., 2005, Nebuloni, et al., 2001, Pulliam, 2009, Xu and Ikezu, 2009). HIV-1 transactivator of transcription protein (Tat) has been shown to increase neuronal A β generation (Aksenov, et al., 2010, Giunta, et al., 2009, Rempel and Pulliam, 2005). Thus, HIV-1 Tat, an HIV-1 viral protein that continues to be implicated as a causative agent in HAND (Agrawal, et al., 2011, Buscemi, et al., 2007, Haughey, et al., 1999, King, et al., 2006b, Nath, et al., 2000, Perez, et al., 2001, Sabatier, et al., 1991, Weeks, et al., 1995), may contribute to the development of AD-like pathology in HIV-1 infected individuals, but by currently ill-defined mechanisms.

HIV-1 Tat is a nonstructural transcriptional regulator essential for the replication of HIV-1. The first exon of HIV-1 Tat encodes for the first 72 amino acids and the second exon encodes for another 14 to 32 amino acids. Others and we have shown that the neuroexcitatory and neurotoxic components of HIV-1 Tat reside within the basic region of exon 1 (Nath, et al., 1996) and that nanomolar concentrations of Tat₁₋₇₂, Tat₁₋₈₆, and Tat₁₋₁₀₁ have similar efficacy and potency in terms of their neurotoxic effects (Aksenov, et al., 2009, Nath, et al., 1996). HIV-1 Tat levels in sera of HIV-1 infected patients have been reported in the nanomolar range, but these levels are almost certainly underestimated given how avidly HIV-1 Tat binds to proteins and cells (Westendorp, et al., 1995, Xiao, et al., 2000). HIV-1 Tat can be transported across the blood-brain barrier (Banks, et al., 2005, Kim, et al., 2003), can be secreted by infected macrophages and microglia, and has been detected in brain of patients with HIV-1 associated dementia (Ellis, et al., 2000, Nath, 2002, Westendorp, et al., 1995). HIV-1 Tat enters neurons rapidly via receptor-mediated endocytosis with the assistance of low-density lipoprotein receptor-related proteins (LRP-1) (Deshmane, et al., 2011, King, et al., 2006a, Liu, et al., 2000, Vendeville, et al., 2004) and is released from endolysosomes into the cytoplasm (Caron, et al., 2004, Liu, et al., 2000, Vives, et al., 1997) most likely through mechanisms involving the high H⁺ gradient maintained by vacuolar H⁺-ATPase (Vendeville, et al., 2004).

Neurons are highly polarized long-lived post-mitotic cells, and they possess an elaborate endolysosome system critical for the maintenance of neuronal function (Nixon and Cataldo, 1995, Nixon and Cataldo, 2006). Increasingly, endolysosome dysfunction, one of the earliest

pathological features of AD that precedes extracellular deposition of A β in brain (Cataldo, et al., 2000), has been implicated in the pathogenesis of AD (Boland, et al., 2008, Cataldo, et al., 2004, Tate and Mathews, 2006) especially the generation of A β . Several lines of evidence indicate that A β is mainly generated in the endolysosome system; A β PP is internalized into endolysosomes (Chyung and Selkoe, 2003, Ferreira, et al., 1993, Perez, et al., 1999, Soriano, et al., 1999) where the rate-limiting enzyme for A β generation, BACE-1, is located (Rajendran, et al., 2008, Shimizu, et al., 2008, Vassar, et al., 1999).

Endolysosome dysfunction has been implicated in the pathogenesis of HAND (Gelman, et al., 2005, Spector and Zhou, 2008, Zhou and Spector, 2008) and we have shown recently that HIV-1 Tat enlarges endolysosomes, elevates endolysosome pH, and disturbs endolysosome function in neurons (Hui, et al., 2012). This prompted us to hypothesize that HIV-1 Tat increases neuronal A β generation by disturbing the structure and function of endolysosomes. As a starting point to test this hypothesis, here we determined the association of HIV-1 Tat-induced endolysosome dysfunction with neuronal A β generation. We found that endolysosome enlargement and elevation of endolysosome pH preceded HIV-1 Tat-induced increases in A β generation. In addition, we demonstrated that HIV-1 Tat increased endolysosome accumulation of amyloid beta precursor protein (A β PP) and A β . Furthermore, we demonstrated that HIV-1 Tat treatment increased endolysosome accumulation of BACE-1 and enhanced BACE-1 activity. Such findings suggest that HIV-1 Tat increases neuronal A β generation and thereby may contribute to the development of AD-like pathology in HIV-1 infected individuals by disturbing endolysosome structure and function.

2. Material and Methods

2.1. Primary cultures of hippocampal neurons

Primary cultures of hippocampal neurons were prepared from embryonic day 18 Sprague-Dawley rats (Harlan Laboratories Inc., WI) using a protocol approved by the University of North Dakota Animal Care and Use Committee adherent with the Guide for the Care and Use of Laboratory Animals (NIH publication number 80-23). Pregnant dams (embryonic day 17) were killed by asphyxiation with CO₂. The fetuses were removed, decapitated, and meninges-free hippocampi were isolated, trypsinized, and plated onto 35-mm poly-D-lysine-coated glass-bottom tissue culture dishes. Neurons were grown in Neurobasal™ medium with L-glutamine, antibiotic/antimycotic and B₂₇ supplement, and were maintained at 37°C and 5% CO₂ for 10–14 days at which time they were used for experimentation. Typically, the purity of the neuronal cultures was greater than 95% as determined by neuronal staining with mouse anti-NeuN and goat anti-MAP2 antibodies (Millipore); astrocytes were identified using a mouse anti-GFAP antibody (Sigma). Neurons were treated either with HIV-1 Tat₁₋₇₂ (a gift from Dr. Avindra Nath) at a concentration of 100 nM, mutant Tat (Tat Δ ₃₁₋₆₁, 100 nM), or phosphate-buffered saline (PBS) as vehicle. The specificity of the actions of Tat₁₋₇₂ was confirmed further using a Tat-specific antibody elution strategy (Buscemi, et al., 2007).

2.2. Quantification of A β levels by ELISA

A β levels were quantified using human/rat A β ₁₋₄₀ and A β ₁₋₄₂ ELISA kits as per the manufacturer's protocol (Wako). Briefly, media from cultured neurons were collected, diluted 1:4 with standard diluent buffer, and quantified using the calorimetric sandwich ELISA method. Each sample was measured in duplicate. Protein concentrations from neurons in each dish were determined by a DC protein assay (Bio-Rad). A β levels were normalized to total protein content in each sample.

2.3. Live cell imaging

The morphology of endolysosomes in living neurons was determined using a LysoTracker dye. After treatment, neurons were loaded with LysoTracker Red DND-99 (50 nM, Invitrogen) for 30 min at 37°C. Fields were chosen at random and at least five images from every experimental condition were acquired by confocal microscopy (Olympus). The sizes of endolysosomes were measured with Image J software and normalized with control cells. Distribution profiles of endolysosome sizes were analyzed with non-linear regression (Gaussian fit) using Prism Graphpad software.

2.4. Measurement of endolysosome pH

Endolysosome pH was measured using a ratio-metric lysosome pH indicator dye (LysoSensor Yellow/Blue DND-160 from Invitrogen); a dual excitation dye that permits pH measurements in acidic organelles independently of dye concentration. Neurons were loaded with 2 μ M LysoSensor for 5 minutes at 37°C. Light emitted at 520 nm in response to excitation at 340 nm and 380 nm was measured every 30 seconds using a filter-based imaging system (Zeiss). The ratios of light excited at 340/380 nm and emitted at 520 nm were converted to pH using a calibration curve established using 10 μ M of the H^+/Na^+ ionophore monensin, and 20 μ M of the H^+/K^+ ionophore nigericin dissolved in 20 mM 2-(N-morpholino) ethane sulfonic acid (MES), 110 mM KCl, and 20 mM NaCl adjusted to pH 3.0 to 7.0 with HCl/NaOH.

2.5. Immunocytochemistry

Neurons were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100, blocked with 5% goat serum, and incubated overnight at 4°C with primary antibodies targeting early endosome antigen-1 (EEA1, 1:500, rabbit polyclonal, Santa Cruz), lysosome associated membrane protein-1 (LAMP1, 1:500, rabbit polyclonal, Santa Cruz), N-terminal A β PP (1:500, mouse monoclonal, Millipore), A β /A β PP (4G8, 1:500, mouse monoclonal, Signet), or BACE-1 (1: 500, mouse monoclonal, Millipore). After washing with PBS, neurons were incubated with corresponding fluorescence-conjugated secondary antibodies including Alexa 488-conjugated goat anti-mouse antibodies (Invitrogen), and Alexa 546-conjugated goat anti-rabbit antibodies (Invitrogen). Neurons were examined using an Olympus confocal microscope. Controls for specificity included staining neurons with primary antibodies without fluorescence-conjugated secondary antibodies (background controls), and staining neurons with only secondary antibodies – these controls eliminated auto-fluorescence in each channel and bleed-through (crossover) between channels. Co-localizations were analyzed with Image J software.

2.6. Immunoblotting

Neurons were lysed with RIPA buffer (Pierce) plus 10 mM NaF, 1 mM Na_3VO_4 and Protease Inhibitor Cocktail (Sigma). After centrifugation (14,000 x g for 10 min at 4 °C), supernatants were collected and protein concentrations were determined with a DC protein assay (Bio-Rad). Proteins (10 μ g) were separated by SDS-PAGE (12% gel) and following transfer to polyvinylidene difluoride membranes (Millipore), membranes were incubated overnight at 4°C with N-terminal A β PP (1: 1000, mouse monoclonal, Millipore) and anti-BACE-1 antibody (1:1000, mouse monoclonal, Millipore). β -actin (1: 10,000, mouse monoclonal, Abcam) was used as a loading control. The immunoblots were developed with enhanced chemiluminescence, and bands were visualized and analyzed by LabWorks 4.5 software on a UVP Bioimaging System (Upland). Quantification of results was performed by densitometry and the results were analyzed as total integrated densitometric volume values (arbitrary units).

2.7. Measurement of BACE-1 enzyme activity

Activity assays of BACE-1 were determined with a BACE-1 activity kit (Calbiochem) according to the protocol provided by the manufacturer. BACE-1 activity was measured using synthetic peptide substrates containing the BACE-1 cleavage site (MCA-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-(Lys-DNP)-OH) at a 50 mM concentration in reaction buffer (50 mM acetic acid pH 4.1, 100 mM NaCl) and equal amount of proteins (10 μ g) from each sample lysate. The fluorescence was measured using a fluorescent microplate reader; excitation wavelength was set at 320 nm and the emission wavelength was set at 383 nm. As a control for specificity, BACE-1 activity was tested in the absence and the presence of the BACE-1 inhibitor, H-Lys-Thr-Glu-Glu-Ile-Ser-Glu-Val-Asn-Stat-Val-Ala-Glu-Phe-OH (Calbiochem). BACE-1 activities were expressed as relative fluorescent units per 10 μ g of protein. Specific activity of BACE-1 was expressed as a ratio of enzyme activity to protein levels as determined by immunoblotting.

2.8. Statistical analysis

All data were expressed as means and SEM values. Statistical significance for multiple comparisons was determined by one-way ANOVA plus a Tukey post hoc test. $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. HIV-1 Tat increased neuronal A β generation

Increasingly, it has been shown that HIV-1 infected individuals on long-term combined anti-retroviral therapeutics have increased depositions of A β in brain as well as increased levels of intraneuronal A β (Achim, et al., 2009, Clifford, et al., 2009, Esiri, et al., 1998, Gelman and Schuenke, 2004, Green, et al., 2005, Nebuloni, et al., 2001, Pulliam, 2009, Xu and Ikezu, 2009). Although the underlying mechanisms remain unclear, HIV-1 Tat has been shown to increase neuronal A β generation (Aksenov, et al., 2010, Giunta, et al., 2009, Rempel and Pulliam, 2005). Here, we determined the extent to which HIV-1 Tat₁₋₇₂ affected A β generation in primary cultures of rat hippocampal neurons and demonstrated that application of HIV-1 Tat₁₋₇₂ (100 nM) for 2 days increased significantly ($p < 0.001$) levels of both A β ₁₋₄₀ and A β ₁₋₄₂ (Figure 1A, B). Treatment with Tat₁₋₇₂ for 1 day did not affect significantly levels of A β ₁₋₄₀ (Figure 1A) and A β ₁₋₄₂ (Figure 1B). The mutant form of HIV-1 Tat (Tat Δ ₃₁₋₆₁, 100 nM) that is not directly neurotoxic (Buscemi, et al., 2007) and that was used as a control did not affect significantly levels of A β ₁₋₄₀ (Figure 1A) and A β ₁₋₄₂ (Figure 1B) even when applied to the neurons for 2 days.

3.2. HIV-1 Tat increased the accumulation of A β PP and A β in endolysosomes

A β results from proteolytic cleavage of its precursor protein A β PP catalyzed by β - and γ -secretase enzymes mainly in endolysosomes (Almeida, et al., 2006, Cataldo, et al., 2004, Takahashi, et al., 2002, Van Broeck, et al., 2008). Furthermore, increased intraneuronal A β production, especially in endolysosomes, occurs in HIV-1 infected individuals (Achim, et al., 2009). Thus, we determined next the extent to which HIV-1 Tat affected specifically in endolysosomes the accumulation of A β PP and A β in primary cultured neurons using double immunofluorescent staining techniques. We found some extent expression of A β PP (Figure 2A) and A β (Figure 3A) in control neurons and that there was increased expression levels of A β PP (Figure 2B, C) and A β (Figure 3B) in neurons that were treated with HIV-1 Tat₁₋₇₂ (100 nM) for 2 days. Furthermore, treatment with HIV-1 Tat₁₋₇₂ (100 nM) for 2 days changed the distribution of and increased expression levels of EEA1-labeled endosomes (Figure 2A and 3A) and of LAMP1-labeled endolysosomes (Figure 2B and 3B). When HIV-1 Tat₁₋₇₂ (100 nM) was applied to neurons for 2 days and colocalization studies were performed, we found that A β PP increasingly colocalized with EEA1-labeled endosomes

(Figure 2A) and LAMP1-labeled endolysosomes (Figure 2B), and that A β increasingly ($p < 0.001$) colocalized with EEA1-labeled endosomes (Figure 3A) and LAMP1-labeled endolysosomes (Figure 3B).

3.3. HIV-1 Tat enlarged the size of endolysosomes and elevated endolysosome pH

In neurons and other cells, HIV-1 Tat enters cells via receptor-mediated endocytosis (Deshmane, et al., 2011, King, et al., 2006a, Liu, et al., 2000, Vendeville, et al., 2004) and HIV-1 Tat accumulates first in endolysosomes. Because alterations in the structure and function of endolysosomes have been implicated in the pathogenesis of both AD and HAND, we determined next the extent to which HIV-1 Tat affected the structure and function of endolysosomes. In living neurons, we identified endolysosomes with LysoTracker, and found that treatment with HIV-1 Tat for 1 and 2 days increased significantly ($p < 0.05$) the size of endolysosomes (Figure 4A). In contrast, treatment of neurons with mutant Tat $_{\Delta 31-61}$ for 1 or 2 days did not affect significantly endolysosome size (Figure 4A). In addition, we demonstrated that the distribution of sizes was significantly shifted to larger profiles upon HIV-1 Tat $_{1-72}$ treatment for two days; the mean size of endolysosomes was 0.49 ± 0.15 ($n = 40$) for control neurons, 0.52 ± 0.14 ($n = 36$) for neurons treated with mutant Tat $_{\Delta 31-61}$, and 0.74 ± 0.37 ($n = 67$) for neurons treated with HIV-1 Tat $_{1-72}$ (Figure 4B). The observations by others and us that HIV-1 Tat accumulates in endolysosomes and our findings described above that HIV-1 Tat alters endolysosome morphology led us to determine next the extent to which HIV-1 Tat affected endolysosome function. Because the intra-endolysosome pH is critical for endolysosome function, we determined the extent to which HIV-1 Tat affected endolysosome pH using lysoSensor dye. We found that treatment of the cultured hippocampal neurons with HIV-1 Tat for 1 or 2 days elevated significantly ($p < 0.001$) endolysosome pH (Figure 5).

3.4. HIV-1 Tat increased endolysosome accumulation of BACE-1 and enhanced BACE-1 activity

The rate-limiting step in A β production is catalyzed by the beta-site A β PP-cleaving enzyme 1 (BACE-1) that is present in endolysosomes and its activity is pH dependent (Rajendran, et al., 2008, Shimizu, et al., 2008, Vassar, et al., 1999). Given our observations that HIV-1 Tat increased both the size and the pH of endolysosomes we determined next the extent to which HIV-1 Tat increased A β production by affecting the levels of expression, enzyme activity, and intracellular distribution of BACE-1 in primary cultured neurons. We found that HIV-1 Tat treatment increased significantly ($p < 0.05$) protein levels of BACE-1 (Figure 6A) and enhanced significantly ($p < 0.001$) the specific activity of BACE-1, which was expressed as a ratio of BACE-1 activity to protein levels as determined by immunoblotting (Figure 6B). When intracellular distribution patterns of BACE-1 were examined in control neurons we found enhanced accumulation of BACE-1 in endosomes as identified with EEA1 antibodies (Figure 6C), but little accumulation of BACE-1 in endolysosomes as identified with LAMP1 antibodies (Figure 6D). However, HIV-1 Tat treatment for 2 days increased markedly the accumulation of BACE-1 in both endosomes (Figure 6C) and endolysosomes (Figure 6D), and increased significantly ($p < 0.001$) the co-localization of BACE-1 with endosomes (Figure 6C) and endolysosomes (Figure 6D).

4. Discussion

Increased life span of HIV-1 infected individuals has been accompanied by an increased prevalence of HAND (Ellis, et al., 2010, Heaton, et al., 2010) and an increased incidence of AD-like pathology including increased deposition of neuronal A β (Achim, et al., 2009). Recently, HIV-1 Tat a non-structural HIV-1 protein that continues to be implicated in the pathogenesis of HAND (Merino, et al., 2011, Nath, et al., 1996, Nuovo, et al., 1994) was

reported to increase neuronal A β generation (Aksenov, et al., 2010, Giunta, et al., 2009, Rempel and Pulliam, 2005) by as yet unclear underlying mechanisms. Here we report that HIV-1 Tat increased neuronal A β generation and that this was accompanied by altered endolysosome structure and function, increased endolysosome accumulations of A β PP, BACE-1 and A β , and enhanced BACE-1 expression levels and specific activity. Our findings suggest that HIV-1 Tat-induced endolysosome dysfunction underlies the development of AD-like pathology in HIV-1 infected individuals.

Neurons are long-lived post mitotic cells possessing elaborate endolysosomes that when dysfunctional can cause neurodegeneration and contribute to the development of a variety of neurodegenerative diseases including AD and HAND. HIV-1 Tat enters neurons via receptor-mediated endocytosis mainly with the assistance of LRP-1 (Deshmane, et al., 2011, Liu, et al., 2000) and once inside neurons HIV-1 Tat accumulates within endolysosomes. Thus, HIV-1 Tat could by virtue of its accumulation in endolysosomes affect structural and functional features of neuronal endolysosomes. Indeed, we found that HIV-1 Tat₁₋₇₂ at a concentration of 100 nM, but not mutant Tat _{Δ 31-61}, enlarged markedly the size of neuronal endolysosomes and elevated endolysosome pH.

Although the underlying mechanisms are unknown, the arginine rich domain of HIV-1 Tat between amino acid residues 49 and 57 could be responsible for the HIV-1 Tat-induced elevation of endolysosome pH. Indeed, we have found that a series of other arginine rich peptides including penetratin, an amino acid domain from the Antennapedia protein (sequence 43–58) of *Drosophila*, a flock house virus coat peptide (sequence 35–49), and oligoarginines (R9) all have the ability to elevate endolysosome pH (unpublished observations). Others have shown that arginine rich peptides have the ability to escape endolysosomes using high proton gradient found in endolysosomes (Drin, et al., 2003, Fischer, et al., 2004, Henriques, et al., 2006, Magzoub, et al., 2005, Potocky, et al., 2003) and here we postulate that an as yet unidentified proton-dependent peptide transporter in endolysosomes might be responsible. Such a peptide transporter could transport arginine rich peptides such as HIV-1 Tat using the arginine rich domain as a signal, and during the transporting process protons would leak out and endolysosome pH would be elevated. Low pH is critical for the degradation of internalized materials, the trafficking and fusion of endolysosomes, and the formation of autophagosomes (Marshansky and Futai, 2008, Ravikumar, et al., 2010, Williamson, et al., 2010). Therefore, the elevation of endolysosome pH that we observed could result in alterations in the digestive capability of endolysosomes as evidenced by decreased specific activity of three different endolysosome enzymes, increased accumulation of internalized material, and alterations in structural and functional features of endolysosomes consistent with increased vulnerability of neurons to injury and degeneration (Wong and Cuervo, 2010).

One of the earliest pathological features of AD is altered morphological and functional feature of endolysosomes (Boland, et al., 2008, Tate and Mathews, 2006). Indeed, endosome enlargement has been noted in brains of AD patients and in non-demented patients with early signs of AD, in Down's syndrome individuals, and in patients bearing the ApoE4 allele (Arriagada, et al., 2007, Cataldo, et al., 2004). As well, endosome enlargement was found to precede extracellular deposition of A β in brain (Cataldo, et al., 2000). Furthermore, pathological changes in endolysosomes may contribute to A β production, a pathological hallmark of AD. Specifically it has been found that A β PP and its cleavage products are present in clathrin-coated vesicles that are part of the endocytic pathway (Ferreira, et al., 1993)(Harris, Milton et al. 2010), that A β production is decreased in cultured cells that were stably transfected with an A β PP construct where the C-terminal endocytic targeting signal was removed (Perez, et al., 1999, Soriano, et al., 1999), that A β production is decreased in cells transfected with dominant negative dynamin to prevent endocytosis (Chyung and

Selkoe, 2003), that BACE-1 a key enzyme for amyloidogenesis is localized in endosomes and its activity is pH dependent (Rajendran, et al., 2008, Shimizu, et al., 2008, Vassar, et al., 1999), and that A β is accumulated in endolysosomes of neurons from AD brain (Cataldo, et al., 2004).

Disturbed endolysosomes have been noted in brain of HIV-1 infected individuals (Gelman, et al., 2005, Spector and Zhou, 2008, Zhou and Spector, 2008) and a recent finding that increased A β accumulation in neuronal endolysosomes in HIV-1 infected individual (Achim, et al., 2009) suggests that endolysosome dysfunction contributes to increased A β generation in HIV patients. Consistent with this notion, we demonstrated that HIV-1 Tat disturbed endolysosome structure and function, promoted neuronal A β production, and increased endolysosome accumulation of both A β PP and A β . Although detailed molecular underlying mechanisms were not explicitly explored, we propose two mechanisms by which HIV-1 Tat increases neuronal A β generation. First, HIV-1 Tat may promote A β PP internalization. As mentioned earlier, Tat enters neuronal endolysosomes via receptor-mediated endocytosis with the assistance of LRP-1 (Deshmane, et al., 2011, Liu, et al., 2000), and it is known that LRP-1 interacts directly with A β PP (Klug, et al., 2011, Waldron, et al., 2008, Waldron, et al., 2006). Thus, the binding of HIV-1 Tat with LRP-1 and subsequent receptor-mediated endocytosis could promote A β PP internalization. Our observation that HIV-1 Tat increases endolysosome accumulation of A β PP seems to support this premise. Second, BACE-1, the rate-limiting enzyme in the production of A β , is present in endosomes and its activity is pH-dependent with an optimal pH around 5.0 (Rajendran, et al., 2008, Shimizu, et al., 2008, Vassar, et al., 1999). Thus, the observed elevation of endolysosome pH could be responsible for enhanced BACE-1 enzyme activity and increased A β production. In addition, BACE-1 has been shown to be degraded in lysosomes under more acidic conditions (pH < 4) (Koh, et al., 2005). Thus, the observed elevation of endolysosome pH could lead to decreased degradation of BACE-1 and increased accumulation of BACE-1 in endolysosomes, which also results in increased A β production. In addition, our observations that HIV-1 Tat-induced endolysosome dysfunction occurs prior to increased A β production suggest that HIV-1 Tat disrupts endolysosome function and subsequently affects A β production (Jin, et al., 2004). Collectively, elevated endolysosome pH could contribute to HIV-1 Tat-induced increases in neuronal A β generation.

Taken together, these findings suggest strongly that the altered structure and function of endolysosomes plays an important role in HIV-1 Tat-induced neuronal A β generation and contributes directly to the development of AD-like pathology in HIV-1 infected individuals. Our findings also suggest that blocking HIV-1 Tat endocytosis and improving endolysosome function are potential therapeutic targets against HAND.

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Figure 1. HIV-1 Tat increased neuronal Aβ generation

(A) HIV-1 Tat₁₋₇₂ treatment (100 nM) for 2 days increased significantly levels of Aβ₁₋₄₀, when compared with control groups (n=5; ***p<0.001). Neither HIV-1 Tat₁₋₇₂ (100 nM) treatment for 1 day nor mutant Tat (Tat_{Δ31-61}, 100 nM) treatment for 2 days affected significantly levels of Aβ₁₋₄₀. (B) HIV-1 Tat₁₋₇₂ treatment (100 nM) for 2 days increased significantly levels of Aβ₁₋₄₂, when compared with control groups (n=5; ***p<0.001). Neither Tat₁₋₇₂ (100 nM) treatment for 1 day nor mutant Tat (Tat_{Δ31-61}, 100 nM) treatment for 2 days affected significantly levels of Aβ₁₋₄₂.

Figure 2. HIV-1 Tat increased endolysosome accumulation of AβPP

(A) Although there is some extent co-localization of AβPP with endosomes (EEA1) in control neurons, HIV-1 Tat₁₋₇₂ treatment (100 nM) for 2 days increased significantly the co-localization of AβPP with endosomes (EEA1) ($n = 11$, *** $p < 0.001$). Bar = 10 μ m. (B) There is little co-localization of AβPP with endolysosomes (LAMP1) in control neurons, but HIV-1 Tat₁₋₇₂ treatment (100 nM) for 2 days increased markedly the co-localization of AβPP with endolysosomes (LAMP1) ($n = 9$, *** $p < 0.001$). Bar = 10 μ m. (C) HIV-1 Tat₁₋₇₂ treatment (100 nM) for 2 days increased significantly the protein levels of AβPP ($n = 6$, * $p < 0.05$).

Figure 3. HIV-1 Tat increased endolysosome accumulation of Aβ

(A) Although there is some extent co-localization of Aβ/AβPP (4G8) with endosomes (EEA1) in control neurons, HIV-1 Tat₁₋₇₂ treatment (100 nM) for 2 days increased markedly the co-localization of Aβ/AβPP (4G8) with endosomes (EEA1) (n = 10, **p<0.01). Bar = 10 μm. (B) There is little co-localization of Aβ/AβPP (4G8) with endolysosomes (LAMP1) in control neurons, but HIV-1 Tat₁₋₇₂ treatment (100 nM) for 2 days increased markedly the co-localization of Aβ/AβPP (4G8) with endolysosomes (LAMP1) (n=12, ***p<0.001). Bar = 10 μm.

Figure 4. HIV-1 Tat disturbed the structure of neuronal endolysosomes

(A) HIV-1 Tat₁₋₇₂ treatment (100 nM) for 1 day and 2 days increased significantly the size of endolysosomes (lysoTracker) in primary cultured hippocampal neurons (**p*<0.05). Mutant Tat Δ_{31-61} treatment (100 nM) for 2 days did not affect the size of endolysosomes. Bar = 10 μ m. (B) The sizes of endolysosomes from control neurons (n = 40) and neurons treated with mutant Tat Δ_{31-61} (n = 37) or HIV-1 Tat₁₋₇₂ (n = 67) for 2 days were plotted and analyzed with non-linear regression.

Figure 5. HIV-1 Tat elevated endolysosomes pH

Endolysosome pH was measured ratio-metrically with a LysoSensor dye. HIV-1 Tat₁₋₇₂ treatment (100 nM) for 1 day and 2 days increased significantly neuronal endolysosome pH (n=8; *** p<0.001).

Figure 6. HIV-1 Tat increased endolysosome accumulation of BACE-1 and enhanced BACE-1 activity

(A) HIV-1 Tat₁₋₇₂ treatment (100 nM) for 2 days increased significantly protein levels of BACE-1 (n=6, *p<0.05). (B) HIV-1 Tat₁₋₇₂ treatment (100 nM) for 2 days increased significantly specific activity of BACE-1 (n=8; ***p<0.001). BACE-1 activity was expressed as relative fluorescent units per 10 µg of protein. Specific activities of BACE-1 were expressed as a ratio of BACE-1 activity to protein levels as determined by immunoblotting. (C) There is some extent co-localization of BACE-1 with endosomes (EEA1) in control neurons, and HIV-1 Tat₁₋₇₂ treatment (100 nM) for 2 days increased markedly the co-localization of BACE-1 with endosomes (EEA1) (n = 12, ***p<0.001). Bar = 10 µm. (D) There is little co-localization of BACE-1 with endolysosomes (LAMP1) in control neurons, but HIV-1 Tat₁₋₇₂ treatment (100 nM) for 2 days increased markedly the

co-localization of BACE-1 with endolysosomes (LAMP1) (n = 10, ***p<0.001). Bar = 10 μ m.