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The HIV Protein gp120 Alters Mitochondrial Dynamics in Neurons

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Abstract

Neurotoxicity of human immunodeficiency virus-1 (HIV) includes synaptic simplification and neuronal apoptosis. However, the mechanisms of HIV-associated neurotoxicity remain unclear, thus precluding an effective treatment of the neurological complications. The present study was undertaken to characterize novel mechanisms of HIV neurotoxicity that may explain how HIV subjects develop neuronal degeneration. Several neurodegenerative disorders are characterized by mitochondrial dysfunction; therefore, we hypothesized that HIV promotes mitochondrial damage. We first analyzed brains from HIV encephalitis (HIVE) by electron microscopy. Several sections of HIVE subjects contained enlarged and damaged mitochondria compared to brains from HIV subjects with no neurological complications. Similar pathologies were observed in mice overexpressing the HIV protein gp120, suggesting that this viral protein may be responsible for mitochondrial pathology found in HIVE. To gain more information about the cellular mechanisms of gp120 neurotoxicity, we exposed rat cortical neurons to gp120 and we determined cellular oxygen consumption rate, mitochondrial distribution, and trafficking. Our data show that gp120 evokes impairment in mitochondrial function and distribution. These data suggest that one of the mechanisms of HIV neurotoxicity includes altered mitochondrial dynamics in neurons.

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Compliance with Ethical Standards

Conflicts of interest The authors declare no conflicts of interest.

Keywords

Gp120ADA; Fis-1; HIVE; Mitochondrial respiration; Oxygen consumption; Tom 20

Introduction

The entry of human immunodeficiency virus-1 (HIV) into the central nervous system (CNS) leads to neurotoxic events culminating in axonal injury (Ellis et al. 2007; Spudich and Gonzalez-Scarano 2012) and loss of cognitive and motor function (McArthur et al. 2005), termed HIV-associated neurological disorders (HAND). Approximately 17–20 % of HAND patients suffer from HIV encephalitis (HIVE) (Everall et al. 2009; Gelman 2015), a neuro-inflammatory condition characterized by the presence of activated and infected microglia, multinucleated giant cells, astrogliosis, and myelin loss (Davies et al. 1997; Everall et al. 2005; Wiley and Achim 1994). The current antiretroviral therapy prolongs the life span of HIV positive subjects and reduces HIVE but does not necessarily stop HAND. Thus, adjunct therapies are needed. The key to developing therapies is more knowledge of the molecular and cellular mechanisms whereby HIV is neurotoxic in virally suppressed patients.

HIV, through its viral envelope protein gp120, activates neurotoxic pathways that lead to atrophy of neuronal processes (Toggas et al. 1994), neuronal dysfunction, and cell loss (Kaul 2008). Such pathways include dysregulated calcium homeostasis (Haughey and Mattson 2002), activation of oxidative stress (Mattson et al. 2005), induction of the proapoptotic transcription factor p53 (Garden et al. 2004; Khan et al. 2005), and mitochondrial fission/fusion (Fields et al. in press). These pathway may also be affected seen in neurons with impaired mitochondrial dynamics. It has been suggested that oxidative stress and neuronal apoptosis in HIV subjects is due to altered mitochondrial metabolism (Bennett et al. 2014; Opii et al. 2007). Mitochondria play a role in neuronal survival through a variety of mechanisms. They regulate Ca^{2+} homeostasis, thus affecting neurotransmission and synaptic plasticity (Levy et al. 2003), as well as control the production of reactive oxygen species (ROS), which is crucial for neuronal survival (Gleichmann and Mattson 2011). Moreover, mitochondria control high energy intermediates, such as ATP. Neuronal function depends on ATP because neurons have a high energy demand and need ATP at distant regions such as axonal and dendritic synapses (Berthet et al. 2014; Dickey and Strack 2011; Merrill et al. 2011). In fact, disruption of energy because of mitochondrial dysfunction has been linked to numerous neurodegenerative diseases (Burte et al. 2015).

To maintain energy homeostasis, neurons require an efficient system to transport and distribute mitochondria to axons and dendrites. The mitochondrial transport to distal axons and dendrites depends upon the polarity and organization of neuronal microtubules (MTs) [reviewed in (Sheng 2014)], which are the major avenue for intracellular transport of many organelles and synaptic vesicles. A reduced axonal transport of mitochondria, because of an impaired MT transport, has been shown to lead to axonal and dendritic degeneration (Chang et al. 2006; Shirendeb et al. 2012). The goal of this study was to provide evidence that HIV, through gp120, may drive the neuropathological mechanisms of axonal degeneration by altering mitochondrial movement and/or dynamics. We show that the brains of HIVE

subjects, as well as gp120 transgenic mice, which reproduce most of the neuropathological scenario seen in HIVE, exhibit enlarged mitochondria as well as altered mitochondrial protein levels in their neurons. We then used gp120 in vitro to confirm that gp120 alters mitochondrial movements and consequently their dynamics in neurons.

Materials and Methods

Human Cohort

Postmortem brains were obtained from the HIV Neurobehavioral Research Center (HNRC) and California Neuro-Acquired Immunodeficiency Syndrome Tissue Network at the University of California, San Diego. A total of 13 HIV positive cases were used, which were classified neuropathologically by presentation of HIVE (Table 1) (Valcour 2013). The diagnosis of HIVE was based on the presence of microglial nodules, astrogliosis, HIV p24-positive cells, and myelin pallor. Cases had neuromedical and neuropsychological examinations within a median of 12 months before death. Most cases died as a result of acute bronchopneumonia or septicemia and autopsy was performed within 24 h of death. Autopsy findings were consistent with AIDS and the associated pathology was most frequently due to systemic cytomegalovirus, Kaposi sarcoma, and liver disease. Subjects were excluded if they had a history of CNS opportunistic infections or non-HIV-related developmental, neurologic, psychiatric, or metabolic conditions that might affect CNS functioning (e.g., loss of consciousness exceeding 30 min, psychosis, and substance dependence).

Gp120 Transgenic Mice

12-month-old mice (either sex) expressing high levels of gp120 under the control of the glial fibrillary acidic protein promoter and wild-type (wt) littermates were used. These transgenic (tg) mice have been previously characterized (Toggas et al. 1994). Mice were euthanized within 1 week after the neurocognitive (water maze) testing (Toggas et al. 1994), either by intracardial exsanguination (paraformaldehyde) for histochemical analysis or by decapitation for biochemical analysis. All studies were carried out following the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and approved by Animal Care and Use Committee. Efforts were made to minimize animal suffering and to reduce the number of animals used.

Analysis of Mitochondrial Size and Cristae

Sections from human frontal cortex or gp120 tg mouse brains were fixed, embedded, and sectioned with the ultramicrotome. To analyze the relative changes in size of mitochondria, a total of 25 cells were analyzed. Cells were randomly acquired from three grids. Grids were analyzed with a Zeiss OM 10 electron microscope as previously described (Rockenstein et al. 2001). Electron micrographs were obtained at a magnification of $\times 25,000$. All the analyses of images were conducted on blind-coded samples. After the results were obtained, the code was broken, and data were analyzed with the StatView program (SAS Institute, Inc., Cary, NC). For Cristae analysis, the electron micrographs of the mitochondria were digitally scanned and analyzed with Image J. An average of 100 mitochondria were analyzed. A threshold was set and the total area of the mitochondria as well as the area

occupied by the cristae was obtained, and results were expressed as % area of the mitochondria occupied by cristae.

Immunohistochemistry

Immunohistochemistry of tissue sections was carried out as previously described (Masliah et al. 2003). In brief, free-floating 40- μ m-thick vibratome sections of human or mouse brains were washed with Tris buffered saline (TBS, pH 7.4), pre-treated in 3 % H₂O₂, blocked with 10 % serum (Vector Laboratories), 3 % bovine serum albumin (Sigma), and 0.2 % gelatin in TBS-Tween (TBS-T). Sections were incubated at 4 °C overnight with anti-Tom20 or anti-Tom40 antibodies (1:500; Santa Cruz Biotechnology, Dallas, TX). Sections were then incubated with a correspondent secondary antibody (1:75, Vector), followed by Avidin D-horseradish peroxidase (HRP, ABC Elite, Vector) and reacted with diaminobenzidine (DAB, 0.2 mg/ml) in 50 mM Tris (pH 7.4) with 0.001 % H₂O₂ for 1 h at room temperature (RT). Control experiments consisted of incubation with pre-immune rabbit serum. Sections were imaged with a digital Olympus microscope and assessment of levels of Tom20 and Tom40 immunoreactivity was performed utilizing the Image-Pro Plus program (Media Cybernetics, Silver Spring, MD). For each case, a total of three sections (10 images per section) were analyzed in order to estimate the average number of immunostained cells per unit area (mm²) and the average intensity of the immunostaining (corrected optical density). All experiments were conducted blind-coded; code was broken after analysis was performed.

Western Blot

Frontal cortex tissues from human and mouse brains were homogenized and fractionated using a buffer that facilitates separation of the membrane and cytosolic fractions (1.0 mmol/L HEPES, 5.0 mM benzamidine, 2.0 mM 2-mercaptoethanol, 3.0 mM EDTA, 0.5 mM magnesium sulfate, and 0.05 % sodium azide; final pH 8.8). In brief, as previously described, (Hashimoto et al. 2002) tissues from human and mouse brain samples (100 mg) were homogenized in 0.7 ml of fractionation buffer containing phosphatase and protease inhibitor cocktails (Calbiochem, San Diego, CA). Samples were precleared by centrifugation at 5000 $\times$ g for 5 min at RT. Supernatants were retained and were centrifuged at 436,000 $\times$ g for 1 h at 4 °C (Beckman Coulter, Brea, CA). This supernatant was collected, as representing the cytosolic fraction, and the pellets were resuspended in 0.2 ml of buffer and homogenized for the membrane fraction.

After determination of the protein content of all samples by BCA Protein assay (Thermo Fisher Scientific, Rockford, IL), homogenates were loaded (20 μ g total protein/lane), separated on 4–12 % Bis–Tris gels, electrophoresed in 5 % HEPES running buffer, and blotted onto Immobilon-P 0.45 μ m membrane using NuPage transfer buffer. The membranes were blocked in either 5 % non-fat milk, 1 % BSA in phosphate buffered saline (PBS) + 0.05 % Tween-20 (PBST) or in 5 % BSA in PBST for 1 h. Membranes were incubated overnight at 4 °C with primary antibodies for Tom20 (1:1000) or Tom40 (1:1000). Following visualization, blots were stripped and probed with a mouse monoclonal antibody against β -actin (1:2000, Millipore, Billerica, MA) as a loading control. All blots were then washed in PBS, 0.05 % Tween-20 and then incubated with corresponding secondary antibodies (1:5000 American Qualex) and visualized with enhanced chemiluminescence

reagent (ECL, Perkin-Elmer). Images were obtained and semi-quantitative analysis was performed with the VersaDoc gel imaging system and Quantity One software (Bio-Rad).

Primary Rat Cortical Neurons

Cortical neuronal cultures were prepared from the cortex of embryonic (E17–18) Sprague–Dawley rats (Charles River, MA) following an established protocol (Avdoshina et al. 2010). These studies were done in strict accordance with the Laboratory Animal Welfare Act, with National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, and after approval from the Animal Care and Use Committee of Georgetown University. Cells were seeded onto poly-L-lysine pre-coated plates in Neurobasal Medium containing 2 % B27 supplement, 25 nM glutamate, 0.5 mM L-glutamine, and 1 % antibiotic–antimycotic solution (Invitrogen, Carlsbad, CA). Cultures were grown at 37 °C in 5 % CO₂/95 % air for 7 days on glass coverslips. Cultures contained ~5 % of non-neuronal cells. All gp120s were purchased from Immunodiagnostics Inc, Woburn, MA. After treatment, neurons were fixed in 4 % paraformaldehyde/phosphate buffer with 4 % sucrose for 20 min at RT. Fixed cells were blocked and permeabilized in 5 % non-fat milk in TBS-T (150 mM NaCl, 20 mM Tris-base, pH 7.5, 0.1 % Triton X100) for 1 h at RT. Cells were incubated overnight at 4 °C with mouse monoclonal microtubule-associated protein 2 (MAP2) antibody (1:5000; Sigma-Aldrich, MO) alone or in combination with rabbit polyclonal anti-Tom20 (1:2000; Santa Cruz, CA). Coverslips were washed with PBST and correspondent fluorescence-conjugated secondary antibodies (1:2000; Invitrogen, CA) were applied for 1 h at RT. Coverslips were washed with TBS-T and mounted with Fluoro-Gel with TES buffer (Electron Microscopy Science, PA). Cells were imaged using an FV300 laser confocal scanning system attached to an Olympus IX-70 (Tokyo, Japan) upright microscope. Image scale was calibrated and length of MAP2 positive processes was measured in 10 neurons per field using ImageJ.

Stochastic Optical Reconstruction Microscopy (STORM)

STORM was performed using a Nikon A1 confocal microscope with CFI SR Apochemat TIRF ×100 oil objective and Andor Technology iXon3 897 EMCCD camera. Samples were treated as previously described (Huang et al. 2008). In brief, primary rat cortical neurons were maintained on glass coverslips (1×10^5 /ml) for 7 days, then fixed with 3 % PFA and 0.1 % glutaraldehyde for 10 min, and reduced with 0.1 % NaBH₄ for 7 min at RT. Cells were washed three times with 0.1 % sodium cacodylate buffer, and blocking buffer (5 % non-fat milk with 0.2 % Triton) was applied for 20 min at RT. Rabbit anti-Tom20 (1:200; Santa Cruz, CA) and rat anti-tubulin (1:5000; Abcam, MA) were used overnight to label mitochondria and cytoskeleton, respectively. Correspondent fluorescence-conjugated secondary antibody solution (1:2000; Invitrogen, CA) was applied for 1 h at RT. Samples were post-fixed with the same initial fixation solution mentioned above, and coverslips were mounted using imaging buffer with cysteamine (MEA), as described by Nikon, and used immediately. Secondary fluorophores were bleached using 647 and 561 nm lasers until blinking was evident (1–3 min), followed by image recording. Mitochondrial shape and size was measured manually using Nikon AR analysis software (NIS-elements Advanced Research software, Nikon, Japan).

Mitochondrial Function by Seahorse

Mitochondrial stress test (MST) reagents were purchased from Sigma-Aldrich and diluted in DMSO (Sigma-Aldrich). MST was measured by monitoring cellular oxygen consumption rate (OCR) with an XF24e Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA). Primary rat neuronal cells were plated (5×10^5 cells/well) in poly-D-lysine-coated, V7 24-well plates (Seahorse Bioscience) and left to adhere overnight. One hour before the experiment, NBM media was removed and cells were incubated in non-buffered DMEM (Sigma-Aldrich), containing 25 mM glucose (Sigma-Aldrich), 0.23 mM sodium pyruvate, and 0.5 mM L-glutamine (both from Thermo Fisher Scientific) for 1 h prior to assessing basal OCR. Cells were then placed in the XF24e Analyzer where OCR was measured first at basal conditions, and then measured following three subsequent injections of oligomycin, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), and a mixture of Rotenone and Antimycin A (Rot + AA). The cellular bioenergetic parameters determined were ATP-linked respiration, proton leak, maximal, and reserve capacity. ATP-linked respiration was derived from the difference between OCR at baseline and respiration following oligomycin addition. The difference in OCR between Rot + AA and oligomycin represents the amount of oxygen consumed that is due to proton leak. Maximal OCR was determined by subtracting the OCR after Rot + AA addition from the OCR induced by FCCP. Lastly, the spare capacity was calculated by the difference between maximal (FCCP) and basal respiration. After MST, OCR measurements were normalized to pmol O_2 /min/ μ g protein, by lysing cells in their individual wells and calculating protein level using the Bradford method.

Time-Lapse Imaging

Rat cortical neurons were cultured onto glass-bottom dishes for 10 days, then used for time-lapse imaging with a Zeiss Z1 Axioscope equipped for fluorescence, live cell imaging, and integrated stage environmental chamber (37 °C and 5 % CO_2). Cells were placed onto the incubated stage for 15 min prior to pretreatment with gp120, or Mitotracker deep red 633 (Life Tech #M-22426). A definite focus was utilized with a $\times 40$ oil objective to ensure the target cell stayed in focus. Treatments were gently applied to the glass-bottom dishes either just prior, or during, time-lapse imaging. Images were captured every 10 min or 20 s for up to 3 h.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism software (GraphPad Software, Inc.). Results are depicted as a mean $\pm$ standard error of mean. For a comparison of more than two groups, an ANOVA test, followed by a proper post hoc test for multiple comparisons, was applied. *p* values of <0.05 indicate statistical significance.

Results

Neuronal Mitochondria are Damaged and Elongated in HIVE Subjects

Mitochondrial health can be revealed by the size of individual mitochondria as well as the integrity of mitochondrial cristae (Costa et al. 2010). Hence, we sought to analyze mitochondrial morphology in HIV + and HIVE subjects (Table 1) utilizing transmission

electron microscopy (TEM). Mitochondrial morphology was analyzed by assessing the size of mitochondria as well as the integrity of cristae (inner membrane). Well-formed cristae were observed in neurons from HIV + brains, suggesting that mitochondrial morphology is preserved (Fig. 1a). Conversely, neuronal mitochondria from HIVE subjects were filled with discontinuous and damaged cristae (Fig. 1a). Analysis of mitochondrial size revealed an ~80 % increase in their diameter in HIVE brains compared to those from HIV+ subjects (Fig. 1b), suggesting increased mitochondrial size.

Changes in mitochondrial size can be the result of abnormal mitochondrial dynamics (fusion and fission) (Chen and Chan 2009). There are several proteins that play a role in mitochondrial dynamics, these include the Tom complex, that is responsible for the formation of mitochondrial transport channels and recognition of mitochondrial preproteins (Hanson et al. 1996; Rapaport 2005), the inner membrane fusion protein OPA1, and the fission regulator Fis-1. Western blot analysis of brain lysates revealed that the levels of Tom20 and 40 were increased in HIVE (Fig. 1c). OPA1 immunoreactivity as well as the levels of the chaperone protein mtHSP70, which plays a role in mitochondrial aggregation, did not differ between HIV and HIVE (Fig. 1c, d). Furthermore, the levels of Fis-1 were significantly decreased in HIVE (Fig. 1c, d). Because lower Fis-1 levels can lead to mitochondrial elongation (Yoon et al. 2006), these results are in line with our TEM data showing enlarged mitochondria in HIVE.

Neuronal Mitochondria are Damaged and Elongated in Brains of gp120 tg Mice

Gp120 tg mice develop neurodegeneration accompanied by gliosis, similar to HIVE (Toggas et al. 1994). Therefore, we used these mice to establish whether gp120 plays a role in the altered morphology of mitochondria observed in HIVE. Neurons in gp120 tg mice contained enlarged and damaged mitochondria with electron dense regions and malformed cristae compared to wt mice (Fig. 2a, b). Moreover, as in HIVE subjects, the levels of Tom 20 and 40 were increased (Fig. 2c, d). Likewise, Fis-1 immunoreactivity was decreased in gp120 tg mice versus wt (Fig. 2c, d). Thus, gp120 tg mice recapitulate a scenario similar to what was found in the HIVE cohort.

Gp120 Induces Mitochondrial Damage

Altered morphology of mitochondria alone may not be sufficient to prove damage. To examine whether gp120 promotes faulty mitochondrial function, we exposed rat cortical neurons to gp120ADA and then performed a mitochondrial stress test (MST) using XF24e extracellular flux analyzer, as described in Materials and Methods. We have used gp120ADA because it is the envelope protein from the predominant strain of HIV in the brain (Gorry et al. 2001). The MST allows for the calculation of oxygen consumption rate (OCR), maximal and spare respiratory capacity, and ATP production when using the ATP synthase inhibitor oligomycin, the mitochondrial oxidative phosphorylation uncoupler FCCP, and a mixture of inhibitors for electron transport chain complexes I and III, rotenone + antimycin A (Rot + AA), respectively. Figure 3 shows the effect of gp120 (5 nM) on OCR. A significant decrease in the basal OCR was observed with gp120 when compared to boiled gp120 (Fig. 3a). We then tested mitochondrial function using mitochondrial inhibitors as described above. Oligomycin caused a substantial decrease in OCR in control cells,

indicating a decrease in ATP-linked respiration. Gp120 potentiated the effect of oligomycin in a time-dependent manner suggesting that gp120 might affect mitochondrial bioenergetics capacity. To determine whether gp120 affects the maximal respiratory capacity, FCCP was injected into the media. While in control cells, FCCP raised OCR to a high maximal rate, the uncoupler failed to obtain the same effect in gp120-treated neurons (Fig. 3b), suggesting that the mitochondrial respiratory capacity had been “exhausted.” Lastly, injection of Rot + AA significantly inhibited respiration in both control and gp120-treated neurons, with a significantly greater effect on control neurons (Fig. 3c). Overall, our data suggest that gp120 reduces mitochondrial bioactivity.

Gp120 Reduces Mitochondria in Neuronal Processes

The mechanism whereby gp120 causes mitochondrial damage is not easily defined. Damaged mitochondria can result from defective mitochondrial transport and accumulation. To examine whether gp120 changes the distribution of mitochondria, we exposed rat cortical neurons to gp120 for various time points and then we visualized mitochondria by Tom20 immunoreactivity in MAP2 positive processes. The time-course experiments were necessary in order to establish whether gp120 changes mitochondrial distribution prior to synaptic pruning, which occurs within 6 h. In control neurons, Tom20 immunoreactivity was scattered in both MAP2 positive neuronal processes and soma and did not appear punctated (Fig. 4, white arrows). After gp120 treatment, we observed a time-dependent reduction of Tom20 immunoreactivity in the processes. Moreover, Tom20 immunoreactivity in MAP2 positive processes appeared punctated (Fig. 4, white arrows), suggesting loss of mitochondria in processes or change in their distribution.

Gp120 Increases Mitochondria Size

Alteration of mitochondrial distribution could lead to altered mitochondrial morphology. To establish whether gp120 changes in mitochondrial distribution lead to increased mitochondrial size, we determined the effects of gp120 on mitochondrial area and length using stochastic optical reconstruction microscopy (STORM). Mitochondria in control neurons exhibited a round shape (Fig. 5a, left panel) typical of healthy mitochondria. Neurons exposed to gp120ADA for 24 h displayed mitochondria with morphological abnormalities (Fig. 5a, right panel) manifested by their longer length (Fig. 5b) and larger size (Fig. 5c). These abnormalities appear to be consistent with mitochondrial damage (Song et al. 2011) and are reminiscent of what we were able to observe in HIV cases.

Gp120 Blocks Mitochondrial Movement

To examine whether gp120 alters mitochondrial movements, we analyzed mitochondrial trafficking in rat cortical neurons. Mitochondrial trafficking was determined by live images using MitoTracker Deep Red 633, a fluorescent dye that stains mitochondria in living cells. In control neurons, a number of mitochondria were seen to undergo a bidirectional transport in both axons and dendrites (video, panel A), with an average speed of $5.04 \pm 4.4 \mu\text{m}/\text{min}$. Some mitochondria remained stationary or paused after movements. Upon exposure to gp120ADA (video, panel B), most of mitochondrial bidirectional transport came to a stop within a few seconds (average speed $0.24 \pm 1.7 \mu\text{m}/\text{min}$) and remains stationary for the entire recording period (60 min), suggesting that gp120 decreases mitochondrial trafficking.

This phenomenon was followed by swollen regions along axons (video, panel B, upper portion) which are characteristic of cytoskeleton breakdown following oxidative stress (Wang et al. 2012).

Discussion

The goal of this study was to explore new cellular mechanisms of HIV neurotoxicity that could explain the selective degeneration of neuronal processes. Because abnormal regulation of mitochondrial homeostasis is linked to a number of neurodegenerative diseases (Wallace 2005), we focused on the hypothesis that HIV, through gp120, impairs mitochondrial bioenergetics. We first observed that in brains of HIVE, mitochondria exhibit damaged cristae and are enlarged and elongated. These morphological alterations were not seen in HIV + subjects without encephalitis, who demonstrate no cognitive impairments. Moreover, we observed that gp120 tg mice, which display synaptic simplification similar to HIVE (Toggas et al. 1994), or neurons exposed to gp120 in vitro, present the same morphological and biochemical changes in mitochondria size that we have detected in HIVE samples. In addition, we found punctated or fragmented mitochondria in gp120-treated neural processes, which are seen during the early phase of apoptosis (Lee et al. 2004). Lastly, we show that exposure of neurons to gp120 in vitro decreases mitochondrial respiratory capacity denoting dysfunctional mitochondria. Overall, our data suggest that HIV, through gp120, impairs mitochondrial function.

Mitochondria are undergoing dynamic process to change their number, distribution, and morphology. This process is maintained by a balance between fission and fusion (Westermann 2010). Although we did not investigate whether gp120 alters these two phenomena in this study, a recent report has shown that gp120 promotes mitochondrial fusion through increased mitofusin 1 and decreased dynamin-like 1, proteins that control mitochondrial fusion and fission, respectively (Fields et al. in press). In support of this finding, our data show that Fis-1, a mitochondrial outer membrane protein that regulates mitochondrial fission (van der Bliek et al. 2013) is decreased in the brain of both HIVE and gp120 tg mice. Fis-1 down regulation can lead to progressive elongated mitochondria (Stojanovski et al. 2004) accompanied by early senescence (Yoon et al. 2006). Thus, it is plausible to suggest that the decrease in Fis-1 could play a role in HIV/gp120-mediated changes in mitochondrial morphology and function.

In many diseases, mitochondrial dysfunction occurs when oxidative modification of the respiratory chain complexes occurs. This event amplifies and promotes further oxidative damage. Our results also show that gp120 impairs neuronal mitochondrial respiration, measured by maximal, and spared oxygen consumption, which could lead to bioenergetic dysfunction and production of ROS. Previous studies have suggested that ROS and other toxins could cause neuronal cell death in HIV subjects (Li et al. 2005). The production of ROS is frequent in neurological disorders and aging (Mattson et al. 2008). This correlation supports the notion that HIV subjects may develop a pattern of brain degradation similar to aging (Thomas et al. 2013) because of an impaired mitochondrial function that induces an early senescent. However, more studies are needed to support this hypothesis.

Survival of neurons involves anterograde and retrograde axonal transport of proteins and organelles, including mitochondria. Mitochondrial biogenesis occurs in the cell body and then the mitochondria are transported to different cellular locations such as axons and dendrites, where ATP is in high demand. Larger size mitochondria may be transported along axons less efficiently (Sheng and Cai 2012). Furthermore, damaged mitochondria tend to accumulate within the soma rather than synaptic terminals (Li et al. 2004). These considerations suggest that in HIV, mitochondria are more stationary within the soma and cannot deliver a continuous supply of ATP to axonal or dendritic terminals, which may undergo retrograde degeneration. Live imaging data confirmed that neurons exposed to gp120 exhibit a profound impairment of mitochondrial trafficking to a point when mitochondria stop their bidirectional moving. Defective mitochondrial transport has been suggested to cause synaptic injury in some neurodegenerative diseases (Burte et al. 2015; Itoh et al. 2013). Thus, impaired mitochondrial trafficking by gp120 could be one of the key pathogenic mechanisms to explain how HIV produces synaptic simplification and neuronal degeneration. In addition, our results show that gp120 produces an imbalance in mitochondrial distribution between the soma and processes. This event occurs concomitantly to an impaired oxygen production, which is a characteristic of damaged mitochondria. Mitochondria can also return to the soma when their integrity is damaged. Our data cannot distinguish whether damaged mitochondria are those accumulating in neuronal processes or in the soma or both. Based on HIV and gp120 tg mice samples, in which damaged mitochondria appear to be seen in the soma, it is plausible to suggest that gp120 damages both somal and axonal mitochondria. More studies are needed to confirm this suggestion.

The shape, integrity, and size of mitochondria are controlled by their trafficking along MTs. In this study, we show that gp120 significantly blocks mitochondria trafficking, which could lead to altered mitochondria distribution. There are several cellular mechanisms that could explain this toxic effect of gp120. For instance, the hallmark of Huntington's disease, polyQ repeats mutant huntingtin, has been shown to interact with mitochondrial fission GTPase and decrease mitochondrial transport while increasing mitochondrial fragmentation (Song et al. 2011). Thus, gp120 may share some similarities with other toxic proteins. On the other hand, we and others have previously shown that gp120 is endocytosed into neurons (Bachis et al. 2003; Berth et al. 2015; Teodorof et al. 2014), associates with MTs, and is retrogradely transported to cause the loss of distal neurons (Bachis et al. 2006). Preliminary data have showed that gp120 can be localized inside human neurons and that a relative short domain of gp120 binds to the carboxy terminal tail of beta tubulin, a component of neuronal MTs (Mocchetti et al. 2014). Thus, the alteration in mitochondrial dynamics that was observed by Fields et al., and in this study could be initiated by gp120 binding to MTs. Nevertheless, we cannot rule out that gp120 may affect mitochondrial trafficking by mechanisms unrelated to MTs. More experiments are needed to prove this hypothesis.

In conclusion, alterations in mitochondrial distribution is believed to initiate neurodegeneration because an arrest in mitochondrial transport negatively influences energy distribution within synapses (Pekkurnaz et al. 2014). Indeed, various neurological diseases are now considered a consequence of abnormal or altered mitochondrial transport. HIV infection of the CNS causes distinct mitochondrial alterations, and a similar scenario is induced by gp120. Remarkably, these effects occur even in the absence of the virus

suggesting that this protein is sufficient to initiate an irreversible neurodegenerative process that may overlap with other endogenous neurotoxins or other pathophysiological insults. Our discovery provides new significant data that will help in the design of adjunct therapies against HAND.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Neuronal mitochondria are damaged and mitochondrial marker expression is altered in brains of HIVE subjects. **a** Cortical sections from HIV + and HIVE subjects (Table 1) were analyzed by TEM to visualize mitochondria. *Black arrows* and enlargements denote mitochondrial cristae (bar = 1 μm). **b** Quantification of mitochondrial diameter and cristae (see “Materials and Methods” section). **c** Representative Western blot analysis of brain lysates for Tom40, Tom20, mtHSP70, OPA1, and Fis-1 immunoreactivity. Numbers on the left indicate the relative molecular weight for each protein measured. **d** Densitometry analyses were performed on Tom40, Tom20, mtHSP70, OPA1, and Fis-1 band intensity and normalized to actin. * $p < 0.05$ by unpaired t test

Fig. 2.

Gp120 tg mice exhibit damaged neuronal mitochondria. **a** Examples of mitochondria visualized by TEM in cortical sections from 12-month-old wt and gp120 tg mice. *Black arrows* and *insets* indicate mitochondrial cristae (bar = 1 μm). **b** Quantification of average mitochondrial diameter and cristae in wt and gp120 tg brains. **c** Brain lysates were analyzed by immunoblot for Tom40, Tom20, mtHSP70, OPA1, and Fis-1 immunoreactivity. **d** Densitometry analyses (normalized to actin) of blots shown in **c**. * $p < 0.05$ by unpaired t test

Fig. 3.

gp120 reduces mitochondrial function. **a** OCR was measured in rat cortical neurons exposed for the indicated time points to either gp120 (5 nM), oligomycin (1 μM), FCCP (500 nM) or Rot + AA (1 μM, each), as described in “Materials and Methods” section. Changes in maximal respiration (**b**) and spare respiratory (**c**) capacity was determined as described in “Materials and Methods” section. Data are the mean ± SEM of 6 samples from two independent experiments. * $p < 0.001$ versus control; *ND* not detectable

Fig. 4.

Tom20 immunoreactivity is punctated in gp120-treated neurons. Rat cortical neurons were exposed to boiled gp120 (control) or to gp120ADA (5 nM) for the indicated time points. Cells were then fixed and co-stained for MAP2 (*red*) and Tom20 (*green*), and analyzed by confocal microscopy. *Arrows* denote punctated mitochondria. *Yellow* (*red + green*) denotes overlapping of markers. Mag $\times 60$ (Color figure online)

Fig. 5. gp120 changes mitochondrial size. **a** Cortical neurons were exposed to boiled gp120 (control), or gp120ADA for 24 h. Cells were then fixed and co-stained for MAP2 and Tom20, and analyzed by STORM to quantify area (**b**) and length (**c**) of mitochondria. Data are expressed as % of control (*red line*) from three independent experiments. Data are *** $p < 0.0001$ versus control (ANOVA and Tukeys' test). Please note that control neurons contains rounded mitochondria (*white circles*), whereas gp120-treated neurons exhibit elongated mitochondria (*white ellipsis*) (Color figure online)

Table 1

Characteristic of human study samples

Group	Number	Gender (M/F)	Age	Plasma VL log	CSF VL log	CD4
HIV+	7	6/1	47.8 ± 11.2	3.3 ± 1.4	1.9 ± 0.4	122.8 ± 137.3
HIVE	6	7/1	42.3 ± 8.5	4.4 ± 1.3	4.2 ± 1.4	66.1 ± 75.1

The diagnosis of HIVE was based on the presence of microglial nodules, astrogliosis, HIV p24-positive cells, and myelin pallor. Cases had neuromedical and neuropsychological examinations within a median of 12 months before death