HIV-1gp120 Induces Neuronal Apoptosis through Enhancement of 4-Aminopyridine-Sensitive Outward K+ Currents

Lina Chen1,3, Jianuo Liu1, Changshui Xu1, James Keblesh1, Weijin Zang3, Huangui Xiong1,2*

1Neurophysiology Laboratory, Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, Nebraska, United States of America, 2Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, Nebraska, United States of America, 3Department of Pharmacology, College of Medicine, Xi’an Jiaotong University, Xi’an, People’s Republic of China

Abstract

Human immunodeficiency virus type 1 (HIV-1)-associated dementia (HAD) usually occurs late in the course of HIV-1 infection and the mechanisms underlying HAD pathogenesis are not well understood. Accumulating evidence indicates that neuronal voltage-gated potassium (Kv) channels play an important role in memory processes and acquired neuronal channelopathies in HAD. To examine whether Kv channels are involved in HIV-1-associated neuronal injury, we studied the effects of HIV-1 envelope glycoprotein 120 (gp120) on outward K+ currents in rat cortical neuronal cultures using whole-cell patch techniques. Exposure of cortical neurons to gp120 produced a dose-dependent enhancement of A-type transient outward K+ currents (IA). The gp120-induced increase of IA was attenuated by T140, a specific antagonist for chemokine receptor CXCR4, suggesting gp120 enhancement of neuronal IA via CXCR4. Pretreatment of neuronal cultures with a protein kinase C (PKC) inhibitor, GF109203X, inhibited the gp120-induced increase of IA. Biological significance of gp120 enhancement of IA was demonstrated by experimental results showing that gp120-induced neuronal apoptosis, as detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and caspase-3 staining, was attenuated by either an IA blocker 4-aminopyridine or a specific CXCR4 antagonist T140. Taken together, these results suggest that gp120 may induce caspase-3-dependent neuronal apoptosis by enhancing IA via CXCR4-PKC signaling.

Introduction

Human immunodeficiency virus type 1 (HIV-1)-infected individuals often suffer from neurological complications such as memory loss, mental slowing, and gait disturbance [1]. The severity of such impairments can vary, ranging from asymptomatic neurocognitive impairment, to mild neurocognitive disorder and to HIV-associated dementia (HAD), which are now collectively referred to as HIV-associated neurodegenerative disorders (HAND) [2]. The mechanisms of neuronal injury in HAND, including reduction of synaptic contacts [3], dendritic pruning [4], and selective neuronal loss [5] and apoptosis [6], remain incompletely understood. Current consensus holds that secreted soluble factors such as cytokines, chemokines, excitatory amino acids, nitric oxide, arachidonic acid and metabolites, and viral proteins, diffuse within the central nervous system (CNS) to directly or indirectly damage neurons [7,8]. In particular, the role of HIV-1 envelope glycoprotein 120 (gp120) in HAND neuropathology has drawn considerable research attention.

In HIV-1-infected brain, gp120 may be shed off from virions or secreted as a soluble substance by HIV-1-infected mononuclear phagocytes. To model its effects in the CNS, gp120 was introduced into neuronal cultures and found to induce neuronal apoptosis [9,10] even at very low concentrations [11]. This in vitro gp120-mediated apoptosis was then confirmed with ex vivo organotypic hippocampal slice preparations [12], transgenic over-expression of glial gp120 [13,14], and direct stereotactic intracranial injection [15,16]. A number of researchers have established that gp120-induced apoptosis can be prevented by blocking or down-regulating the CXCR4 receptors [17,18,19], indicating gp120 can induce apoptosis through the CXCR4. While research into the apoptotic pathways downstream of gp120 binding continue, new insights into the process of apoptosis are also being made, in particular with regard to the crucial role of voltage-gated potassium (Kv) channels.

Across various cell types, the process of apoptosis is characterized by cell volume decreases, caspase activation, and DNA fragmentation, with accumulating evidence demonstrating K+ homeostasis involvement in each stage [20,21]. Since the original experiment by Shan Ping Yu, et al. [22] demonstrating K+ ionophore insertion was sufficient to initiate and sustain apoptosis in neurons, this sequence has been observed with various neuronal insults and accompanied by increased K+ channel current [23]. Furthermore, K+ channel blockade and high K+ medium have been found to prevent apoptosis in cultured cortical neurons [22,24,25], cerebellar granule neurons [26,27], and rat hippo-
were seeded at a density of 5 × 10^5/dish (or well) in poly-D-lysine-coated 35 mm culture dishes or 6 well plates and maintained in serum-free supplement, 1% penicillin/streptomycin, 0.2% fetal bovine serum (FBS) and 0.25 mM L-glutamine (Invitrogen) for 7–10 days. The recorded cells were held at −60 mV during voltage clamping. The electrodes were advanced towards cells by a Burleigh micromanipulator (PC-5000, EXFO, Canada). After establishment of the whole-cell patch configuration, the cells were allowed to stabilize for 3–5 min before tests.

Electrophysiology
Whole-cell patch recordings were performed in rat cortical neuronal cultures in 35 mm tissue culture dishes on the stage of an inverted Nikon microscope (TE 300) using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Patch electrodes, made from borosilicate glass micropipettes (WPI Inc. Sarasota, FL) with a P-97 micropipette puller (Sutter Instruments, Novato, CA), had tip resistance of 5.0–8.0 MΩ. The electrodes were advanced towards cells by a Burleigh micromanipulator (PC-5000, EXFO, Canada). After establishment of the whole-cell patch configuration, the cells were allowed to stabilize for 3–5 min before tests.

The neuronal cells were identified by their triangular-shaped morphology and their firing of action potentials in response to a depolarizing current injection. Chemical reagents were applied through incubation (2 h) in 95% CO2 and 5% O2 at 37°C. Data were analyzed by Clampfit 10.2 (Molecular Devices). For each set of experiments, the instantaneous outward currents generated by voltage steps from −60 mV to +60 mV were measured and analyzed.

**Materials and Methods**

**Animal**

Pregnant Sprague-Dawley rats were purchased from Jackson Laboratory (Bar Harbor, Maine) and maintained under ethical guidelines for care of laboratory animals at the University of Nebraska Medical Center. All animal-use procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of University of Nebraska Medical Center (IACUC # 00-062-07).

**Primary cortical neuronal culture**

Purified cortical neurons were prepared from rat embryos described previously [29]. Briefly, female Sprague-Dawley rats with 18–19 days of gestation were anesthetized and embryonic pups were surgically removed. Cerebral cortices were harvested and digested using 0.25% trypsin and 200 U deoxyribonuclease I (Sigma-Aldrich, St. Louis, MO) in Hank’s Buffered Salt Solution (HBSS) (In Vitrogen, Carlsbad, CA) at 37°C for 15 min. After washing in HBSS, tissue mixtures were centrifuged, decanted and sequentially passed through a 100 μm- and 40 μm-mesh. The cells were seeded at a density of 5 × 10^5/dish (or well) in poly-D-lysine-coated 35 mm culture dishes or 6 well plates and maintained in neurobasal medium (Invitrogen) supplemented with 2% B-27 serum-free supplement, 1% penicillin/streptomycin, 0.2% fetal bovine serum (FBS) and 0.25 mM L-glutamine (Invitrogen) for 7–10 days. The purity of neural cells was determined by staining with microtubule-associated protein-2 (MAP-2, a mature neuronal marker) antibody (Millipore, Temecula, CA) and more than 90% of MAP-2 positive cells were obtained.

**Immunocytochemistry**

Neurons growing on poly-D-lysine-coated coverslips were treated with gp120 in the presence or absence of T140, 4-AP, or as indicated. After 24 h treatments, neurons were fixed in 4% paraformaldehyde and blocked in phosphate buffered saline (PBS) containing 10% normal goat serum and 0.1% Triton X-100 for 30 min at room temperature. Neurons were incubated with primary antibody anti-caspase 3 (1:200 Cell Signaling Technology, Beverly, CA) or mouse monoclonal anti-NeuN (1:100; Millipore) diluted in blocking solution for 2 h, followed by application of AlexaFluor 488 (1:500) and AlexaFluor 594 (1:500) secondary antibody (Invitrogen) in PBS+10% HIGS for 1 h. The fluorescent images were captured using Olympus DP70 camera and DP Controller Ver. 2.1.1 software. The values of fluorescent intensity were acquired using ImageJ software (National Institutes of Health) and then normalized with cell numbers.

**TUNEL assays**

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed to evaluate apoptotic neurons by using in situ cell death detection kit, AP® (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s instructions. Briefly, rat cortical neurons grown on poly-D-lysine-coated coverslips at a density of 1 × 10^5/well in 24 well plates were pretreated with CXCR4 blocker, T140 (50 nM, kindly provided by Dr. Nobutaka Fujii, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto 606-8511, Japan) or K+ channel blocker 4-AP (5 μM) 30 min before addition of 500 pM gp120 (ImmunoDiagnostics, Inc., Woburn, MA). After treatments of 24 h, the neurons were fixed with 4% paraformaldehyde and permeabilized 0.1% Triton X-100 (Sigma) in 0.1% sodium citrate solution containing 10% normal goat serum and 0.1% Triton X-100 for 30 min at room temperature. Neurons were incubated with TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and fluorescein-labeled nucleotides at 37°C for 60 min. After final wash, coverslips were mounted in ProLong Gold antifade reagent (Molecular Probes, Eugene, OR) with 4’,6-diamidino-2-phenylindol (DAPI) and visualized by fluorescent microscope using a ×20 objective. The TUNEL-positive cells were analyzed by NIH ImageJ software and the percentage of TUNEL-positive cells (green) was normalized to total DAPI-positive cells from 12 microscopic fields.
Statistics

All data were expressed as mean ± SEM and graphed using Origin 8.0 software (OriginLab, Northampton, MA) unless otherwise indicated. Statistical analyses were performed by one-way ANOVA analysis or by Student’s t tests. A minimum p value of 0.05 was estimated as the significance level for all tests.

Results

Expression of outward K⁺ currents in cultured rat cortical neurons

Outward K⁺ currents generated by voltage steps (Fig. 1A) were recorded in rat cortical neurons after 7–10 days in culture. The average peak current amplitude, generated by a voltage step from −60 mV to +60 mV, was 3541.9 ± 369.5 pA (Fig. 1B, n = 8). Addition of TEA (20 mM) to the bath solution reduced the peak current to 2940.6 ± 383.5 pA (Fig. 1C, n = 8), producing a reduction of ~17% on peak current. The peak current was further reduced to 905.3 ± 110.8 pA (Fig. 1D, n = 8) by addition of 4-AP (5 mM) to the bath solution, resulting in a reduction of ~69% on the remaining peak current. The 4-AP-sensitive outward K⁺ currents were isolated by subtraction of the currents recorded in the presence of both TEA and 4-AP from those recorded in the presence TEA alone (Fig. 1E). Thus, the reduction on peak current produced by 4-AP was ~50% when measured at the voltage step of +60 mV.

Enhancement of neuronal Iₐ by gp120

It is well-known that gp120 induces neuronal apoptosis [8] and that enhancement of outward K⁺ currents is believed to be an essential pathway in programmed cell death [22,24,25]. To examine whether gp120 induces neuronal apoptosis via increasing Iₐ, we first tested the effects of gp120 on Iₐ in primary rat cortical neuronal cultures. The 4-AP-sensitive Iₐ and the 4-AP-insensitive delayed rectifier like outward K⁺ currents (Iₒ) were recorded by addition of TEA (20 mM) and 4-AP (3 mM) to the extracellular solution, respectively. Incubation of rat cortical neurons with gp120 (200 pM) for 2 h produced an enhancement of Iₐ with an average instantaneous current density of 89.2 ± 3.4 pA/pF (n = 120) recorded in control neurons (without gp120 incubation), the difference was statistically significant (Fig. 2, p < 0.05). In contrast, the Iₒ density recorded in neurons with and without incubation with gp120 were 77.4 ± 4.0 pA/pF (n = 33) and 76.8 ± 4.6 pA/pF (n = 30), respectively. The difference was not statistically significant (Fig. 2, p > 0.05). These results indicate that gp120 differentially enhances Iₐ in cultured rat cortical neurons. The enhancement of Iₐ induced by gp120 was dose-dependent, with average current densities of 89.2 ± 3.4 pA/pF (n = 115), 102.6 ± 4.3 pA/pF (n = 63), 115.3 ± 5.0 pA/pF (n = 57) when neuronal cells were incubated with gp120 at concentrations of 200, 400 and 800 pM, respectively. The differences are statistically significant (p < 0.01), demonstrating an enhancement of Iₐ by gp120 (Fig. 3).

gp120 increases Iₐ via CXCR4

C-X-C chemokine receptor type 4 (CXCR4), a co-receptor for HIV-1 infection, is expressed in the brain in a variety of cell types including neurons [8,31,32]. Studies have shown that gp120 induces neuronal injury via CXCR4 [17,33]. To assess whether gp120-induced enhancement of Iₐ is mediated through activation of neuronal CXCR4, we studied the effects of T140, a specific CXCR4 receptor antagonist [34,35], on its blockade of gp120-induced enhancement of Iₐ. As shown in Fig. 4, incubation of cortical neurons with gp120 (200 pM) increased the Iₒ density by 33.4%, from 78.7 ± 2.6 pA/pF (n = 120) to 99.2 ± 3.4 pA/pF (n = 115). Addition of T140 (50 nM) to the incubation medium blocked gp120 enhancement of the Iₒ with an average current density of 77.4 ± 2.5 pA/pF (n = 70). In comparison with the gp120-induced enhancement, the difference is statistically significant (p < 0.05), suggesting gp120 increase of neuronal Iₒ via CXCR4. Application of T140 alone had no apparent effect on neuronal Iₒ (Fig. 4).

Involvement of PKC signaling in gp120-mediated increase of Iₐ

As an extracellular toxic molecule, gp120 represents one of the many exogenous signals that must be integrated by neurons. The neurons accomplish this through evolutionarily conserved signaling cascades, often comprised of reversibly modifiable kinases such as the protein kinase C (PKC) family of isozymes [36]. PKC can be activated by G-protein coupled receptors such as CXCR4 through production of its direct activator diacylglycerol (DAG) and can have either pro- or anti-apoptotic effects depending on the
particular stimuli, cell type, and isozyme activated [37,38,39]. Recently, PKC activation was shown to be involved in increasing I_A and neuronal apoptosis in rat cerebellar granule neurons [27]. In order to determine whether gp120 increases I_A through activation of PKC, we used phorbol myristate acetate (PMA) to mimic DAG activation of PKC and GF109203X as a PKC inhibitor. Figure 5 shows brief incubation of rat cortical neurons with PMA produced an increase of I_A similar to those produced by gp120, with an average current density of 94.4 ± 5.2 pA/pF (n = 49). The difference is statistically significant (p < 0.01) when compared to the average current density of 78.7 ± 2.6 pA/pF (n = 128) recorded in control neurons, indicating an increase neuronal I_A via PKC. The involvement of PKC signaling in gp120-mediated enhancement of neuronal I_A was demonstrated by experimental results showing that co-incubation of cortical neurons with gp120 and PKC inhibitor GF109203X significantly (p < 0.01) attenuated gp120-associated increase of I_A. The average current densities recorded in the absence and presence of GF109203X were 89.2 ± 3.4 pA/pF (n = 115) and 75.2 ± 3.7 pA/pF (n = 57), respectively.

Inhibition of gp120-induced apoptosis by 4-AP and T140

Activation of Kₗ channel has been considered an essential pathway in programmed cell death [21]. To investigate whether the gp120-mediated enhancement of I_A contributes to gp120-induced neuronal injury, we examined the protective effects of 4-AP on gp120-induced neuronal apoptosis in rat cortical neuronal cultures. Cell nuclei were labeled with DAPI staining (blue) and apoptotic cells were determined by TUNEL staining (green) of fragmented DNA (Fig. 6A). Visualized by TUNEL staining, incubation of neuronal cultures with gp120 for 24 h resulted in neuronal apoptosis in a dose-dependent manner as shown in Fig. 6B. At the concentrations of 200 pM and 500 pM, the percentages of apoptotic neurons induced by gp120 were 23.4 ± 3.6% and 29.3 ± 2.8% (n = 4, each in triplicate, the same in the followings of this section), respectively, compared to

Figure 2. Enhancement of I_A by gp120. A shows representative current traces recorded in control (Ctrl, left) and gp120-treated (gp120, right) rat cortical neurons in the presence of TEA (20 mM, upper) and 4-AP (5 mM, lower). The voltage protocol utilized to generate outward K⁺ currents were the same as shown in Fig. 1A. Note that gp120 enhanced I_A (upper) with no apparent effect on delayed rectifier like I_K (lower). B is a summarized bar graph illustrating gp120 enhancement of the I_A, but not the I_K. Each value represents the mean ± SEM. * p < 0.05; gp120 (n = 115) vs Ctrl (n = 128) for I_A.

doi:10.1371/journal.pone.0025994.g002

Figure 3. gp120 increased neuronal I_A in a dose-dependent manner. Panel A illustrates the I–V plots of I_A current densities in the absent and present of gp120 at different concentrations indicated. * p < 0.05, ** p < 0.01, gp120 200 pM (n = 115), 400 pM (n = 63), 800 pM (n = 57) vs Ctrl (n = 128), respectively. Panel B is a bar graph showing the average percentage of gp120-induced increase of neuronal I_A when the instantaneous peak currents generated in response to +60 mV voltage step were measured. ** p < 0.01 as indicated.

doi:10.1371/journal.pone.0025994.g003
6.1±1.0% (n = 4) of apoptotic cells observed in control, the difference was statistically significant (Fig. 6), demonstrating gp120 induces neuronal apoptosis in primary rat neuronal cultures. The gp120-induced neuronal apoptosis was partially blocked either by 4-AP or the CXCR4 antagonist T140 (Fig. 6). In one subset of cortical neuronal cultures co-incubated with 4-AP (5.0 mM) and gp120 (200 pM or 500 pM), the average percentages of apoptotic neurons were 9.3±0.9% and 13.8±2.5%, respectively. In comparison with the average percentage of apoptotic neurons observed in neuronal cultures incubated with gp120 alone, the differences were statistically significant (Fig. 6, p<0.01, n = 4), indicating that involvement of I_A in gp120-induced neuronal apoptosis was partially blocked.

Figure 4. gp120 increases neuronal I_A via CXCR4. A, Representative I_A current traces recorded in the presence of 20 mM TEA from a control neuron (Ctrl) and neurons incubated with 200 pM gp120 (gp120), gp120+T140 (gp120+T140) and 50 nM T140 (T140), respectively. B, Bar graph showing the average instantaneous peak current amplitude (% of control) generated by a voltage step from −60 mV to 60 mV. Note gp120 produced a significant increase of peak I_A and this increase was blocked by T140, a CXCR4 receptor antagonist, indicating gp120 increase of neuronal I_A via CXCR4. * p<0.05, gp210 (n = 115) vs Ctrl (n = 128); # p<0.05, gp120 (n = 115) vs gp120+T140 (n = 70).

doi:10.1371/journal.pone.0025994.g004

Figure 5. Involvement of PKC in gp120-mediated enhancement of neuronal I_A. A, I_A currents recorded in the presence of 20 mM TEA from a control neuron and neurons incubated with gp120 (200 pM), gp120+GF109203X (5 μM, a PKC inhibitor), and GF109203X (5 μM), respectively. B I–V curves illustrating I_A current densities generated by voltage steps in control neurons (n = 128) and neurons treated with gp120 (n = 115), gp120+GF109203X (n = 57) and GF109203X alone (n = 55). * p<0.05, ** p<0.01, gp120 vs Ctrl; # p<0.01, gp120+GF109203X vs gp120. C is a bar graph plotting the average peak I_A currents (% of ctrl) measured at +60 mV. Note that gp120 enhanced neuronal I_A and this enhancement was blocked by a specific PKC inhibitor GF109203X. Incubation of cortical neurons with PMA, a PKC activator (100 nM, n = 49), for 30 min also produced a significant enhancement of neuronal I_A, suggesting that PKC pathway is involved in gp120-mediated enhancement of neuronal I_A. * * p<0.01 and ## p<0.01 for comparisons as indicated.

doi:10.1371/journal.pone.0025994.g005
apoptosis. In another subset of cortical neuronal cultures co-incubated with T140 (50 nM) and gp120 (200 pM or 500 pM), the average percentage of apoptotic neurons was significantly (p<0.01, n = 4) decreased to 7.0±1.2% and 12.4±3.1% (Fig. 6), respectively, further supporting our aforementioned findings that gp120 enhances neuronal IA via CXCR4.

Blockade of IA inhibits gp120-induced caspase-3 activation

It has been shown that gp120 induces neuronal injury via activation of caspase-3 [13,19,40]. Our TUNEL results revealed that gp120 enhancement of IA underlies gp120-induced neuronal apoptosis as blocking IA decreased gp120-associated neuronal apoptosis. We further investigated if caspase-3 is the downstream pathway of gp120 enhancement of IA in rat cortical neurons. Caspase-3 was detected with anti-caspase 3 staining (green) and neurons were marked with anti-NeuN staining (red). Neuronal cultures incubated with gp120 for 24 h exhibited a significant increase of apoptotic neurons and that application of T140 or 4-AP significantly attenuated the gp120-induced increase of neuronal apoptosis. 12 randomly selected visual fields were counted in each group **p<0.01, ***p<0.001. a; 200 pM; b; 500 pM. doi:10.1371/journal.pone.0025994.g006

Several pieces of additional data collected point towards a possible mechanism. First, the CXCR4 inhibitor T140 was able to block both gp120-mediated increases in IA and apoptosis. Given the accumulation of research demonstrating a role for CXCR4 signaling in gp120 apoptosis [17,41], the effects on apoptosis are perhaps unsurprising. To our knowledge, however, this is the first work demonstrating outward K+ currents can be increased through CXCR4 signaling. Combined with ample research having established a critical role for K+ channel in initiating and sustaining apoptosis [22,25], these results may perhaps indicate a toxic mechanism of gp120 that has thus far been unappreciated. It should be noted that while many of the experiments regarding apoptotic K+ current have focused on sustained delayed rectifier currents, there have been other reports of 4-AP-sensitive outward K+ currents contributing to apoptosis [26,42,43]. In any case, it is the chronic efflux and intracellular depletion of K+ that is necessary for apoptosis, which in pathological conditions could occur via IA. Our finding that gp120 increases IA and that 4-AP blocks gp120-induced apoptosis stands as evidence of this possibility. Since our evidence indicates this occurs via the G-protein coupled CXCR4 receptors, we next attempted to identify an intracellular signaling cascade, such as PKC, that could be responsible for this pathological increase in IA.

In general, PKC activation occurs through G-protein coupled receptors, which when signaled activate phospholipase C (PLC) to hydrolyze phosphatidylinositol-4,5-bisphosphate (PIP2) to DAG [38,44]. PKC isoforms can be categorized according to the combination of phosphatidylserine (PS), DAG, and Ca2+ required for activation as conventional (PS, DAG, and Ca2+), novel (PS and DAG), or atypical (PS) [44]. In this experiment, we used the phorbol ester PMA to mimic PKC activation of conventional and novel isoforms. We found this activation of PKC to mimic gp120 increases in IA. However, precision targeting of PKC isoforms has revealed that, in general, conventional isoforms (such as PKCα) have anti-apoptotic roles while novel isoforms (such as PKCδ) have pro-apoptotic roles [37,39]. The relative expression of different PKC isoforms also varies with tissue, subcellular localization, and even between neuronal compartments [38], complicating the situation. Therefore, while PKC activation has been shown elsewhere to decrease K+ channel current [45,46,47] and in some cases has neuroprotective effects [48,49], PKC activators have been shown to induce apoptosis in many cell types.
including neurons [27,54] and chemical inhibition or mutation of apoptotic PKC isozymes in many cases attenuates this apoptosis [50,54,55,56]. Under our experimental conditions, the apoptotic effects of brief exposure to PMA were confirmed by assay of reactive oxygen species (data not shown). Furthermore, PKC inhibition using GF109203X was protective against gp120-mediated apoptosis. While not definitive, these experiments lay the groundwork for understanding the intracellular signals involved in apoptotic gp120 increases in IA and indicate a possible role of apoptotic PKC pathways.

With our other mechanistic studies, the emerging picture indicates a possible scenario whereby gp120 affects apoptosis through G-protein-coupled CXCR4 receptor signaling and activates apoptotic PKC–caspase-3 pathways. Overall, the finding that gp120 mediates enhancement of IA could have relevance at several levels within the context of HAND. The first and most obvious biological significance is the contribution of enhancement of IA to gp120-induced apoptosis. Current consensus holds that HIV-infected and activated macrophages migrate across a weakened blood-brain barrier and secrete soluble viral proteins in addition to cellular factors that lead to direct or indirect neuronal damage [8]. Of these viral proteins, substantial evidence has implicated gp120 in the neurotoxic brain pathology underlying HAND [10,13,15]. Gp120-induced neuronal damage has been linked to NMDA receptor excitotoxicity and CXCR4 receptor [8]; however, until now, the involvement of K+ homeostasis in the apoptotic process [20,21,23] in association with HIV-1-associated neurodegeneration has largely been unappreciated. Bringing together these two avenues of research may help complete the mechanism of gp120-induced neurotoxicity and HAND neuropathology.

Secondly, cognitive decline in HAND may result as much from neuronal dysfunction as from neuronal loss, an idea supported by experimental results showing alterations in cell layer volume [57] and dendritic morphology [4] correlate with HAD [58]. Indeed, extensive cell death is not always present when symptoms manifest [59] and antiretroviral therapy (ART) treatment has been known to lead to cognitive improvement [60,61,62,63], suggesting the underlying pathology of HAD may be in part reversible. This is consistent with a channelopathy hypothesis originally described by Dr. Ben Gelman [64], which led our laboratory to investigate K+ channel involvement in HAND [65]. K+ channels are well known to regulate membrane potential and thereby the repolarization, discharge frequency, and waveforms of action potentials (AP). Due to a negative equilibrium potential relative to the AP threshold, outward K+ currents are essentially inhibitory. Not surprisingly, decreased outward K+ currents have been found to correspond with improved memory and long-term potentiation (LTP), while increased outward K+ currents correspond to learning and memory deficiencies [65]. Continuing research involving outward K+ currents has given broad support to this general concept. Whether dendritic, somatic, axonal, or terminal, K+ 1.1 (with β subunit), K+ 1.4, or K+ 4, appropriate A-type current has been shown to be crucial for LTP induction, learning and memory, axonal signal propagation, and terminal transmitter release [65]. Therefore, increased outward K+ currents could be expected to have a deleterious effect on neuronal function with subsequent induction of apoptosis. In previous experiments, we found injection of HIV-infected macrophages into severe combined immune deficient (SCID) mice basal ganglia inhibits LTP and impairs radial arm water maze performance, measures of learning and memory that were restored with systematic administration of the K+ channel blocker 4-AP [66]. The present research more

Figure 7. Activation of caspase-3 is involved in gp120 enhancement of neuronal IA and resultant neuronal apoptosis. A, Photomicrograph of neuronal cultures treated with gp120 in the absence or presence of T140 or 4-AP. Caspase-3 was labeled with anti-caspase-3 antibody (green) and nuclei were labeled with NeuN (red). Note that gp120 increased the caspase-3 positive cells and this increase was attenuated by either T140 or 4-AP. Twelve different areas were measured in each group, and the experiments were done in triplicates (magnification ×40). B, A bar graph illustrates the average fluorescence density of caspase-3 detected in different experimental conditions. The fluorescence intensity in the control (Ctrl) group was normalized as 100%. *** p<0.001. doi:10.1371/journal.pone.0025994.g007

Figure 8. A schematic diagram illustrating the potential pathways for gp120 enhancement of neuronal IA and resultant neuronal apoptosis. doi:10.1371/journal.pone.0025994.g008
specifically implicates gp120-induced increases in IA as the underlying mechanism of neuronal dysfunction and eventual cell death in HAND.

While gp120 is supposed to increase IA by direct action on neurons, it should be noted that 5-10% of the cells present in culture are glia. Gp120 can also interact with glia and promote the release of immunological factors [67,68] that could also potentially alter outward K+ currents [65]. Previous experiments in our laboratory have demonstrated that conditioned media recovered from immune-activated macrophages increase neuronal outward K+ currents and induce neuronal apoptosis [69] suggesting the gp120 increases of neuronal outward K+ currents may be in some part attributable to indirect activation of bystanders such as glial cells and resultant production of soluble substances. This issue is significant in the sense of understanding the precise mechanism of gp120-induced increases in IA and apoptosis. However, the key finding here remains that gp120 does indeed induce enhancement of neuronal outward K+ currents and apoptosis, which can be attenuated by K+ channel blocker 4-AP. While many questions still exist, knowledge of K+ channel dysfunction induced by gp120 and other soluble factors can serve as a starting point for developing adjunctive therapies to target the disrupted neuronal function in HAND.

In summary, the experimental data provide in vivo evidence that the HIV-1gp120 increases IA via CXCR4+PKC pathway leading to neuronal apoptosis. The enhancement of IA resulted in neuronal apoptosis by activation of caspase-3 (Fig. 8) and the gp120-induced neuronal apoptosis was significantly attenuated by 4-AP, a K+ channel antagonist. These results suggest that K+ channels are involved in HIV-1-associated neuropathogenesis.

Acknowledgments

The authors thank Ms. Robin Taylor and Mr. Bryan Katabiass for reading the manuscript. The authors extend a special thanks to Ms. Julie Ditter, Ms. Robin Taylor and Ms. John Belling for their excellent administrative support.

Author Contributions

Conceived and designed the experiments: XJ JL CX. Performed the experiments: LC JL CX. Analyzed the data: LC JL WZ HX. Wrote the paper: JK HX.

References

1. McArthur JC, Brew BJ, Nath A (2005) Neurological complications of HIV infection. Lancet Neurol 4: 543–553.
2. Antinori A, Arendt G, Becker JT, Brew BJ, Byrd DA, et al. (2007) Updated research nosology for HIV-associated neurocognitive disorders. Neurology 69: 1789–1799.
3. Fuhr DP, Heaton RK, Marrotte TD, Ellis RJ, McCutchan JA, et al. (1999) Cortical synaptic density is reduced in mild to moderate human immunodeficiency virus neurocognitive disorder. HNRC Group. HIV Neurobehavioral Research Center. Brain Pathol 9: 209–217.
4. Muzio E, Heaton RK, Marrotte TD, Ellis RJ, Waly CA, et al. (1997) Dendritic injury is a pathological substrate for human immunodeficiency virus-related cognitive disorders. HNRC Group. The HIV Neurobehavioral Research Center. Ann Neurol 42: 963–972.
5. Masliah E, Ge N, Achim C, Hansen L, Waly C (1992) Selective neuronal vulnerability in HIV encephalitis. J Neuropath Exp Neurol 51: 593–599.
6. Petito CK, Roberts B (1995) Evidence of apoptotic cell death in HIV encephalitis. American Journal of Pathology 146: 1121–1130.
7. Gonzalez-Scarano F, Martin-Garcia J (2003) The neuropathogenesis of AIDS. Nat Rev Immunol 5: 69–81.
8. Kaul M, Garden GA, Lipton SA (2001) Pathways to neuronal injury and apoptosis in HIV-associated dementia. Nature 410: 988–994.
9. Singh IN, Goody RJ, Dean C, Ahmad NM, Lutz SE, et al. (2004) Apoptotic pathway in HIV-associated dementia. J Neurosci Res 75: 75–82.
10. McLaughlin B, Pal S, Tran MP, Parsons AA, Barone GC, et al. (2001) p38 activation is required upstream of proinflammatory current enhancement and caspase cleavage in thid oxidant-induced neuronal apoptosis. J Neurosci 21: 3303–3311.
11. Redman PT, Jefferson BS, Ziegler CR, Mortensen OV, Torres GE, et al. (2006) A vital role for voltage-dependent potassium channels in dopamine transporter-mediated 6-hydroxydopamine neurotoxicity. Neuroscience 143: 1–6.
12. Hu GL, Liu Z, Zeng XM, Liu ZQ, Chen XH, et al. (2006) 4-aminoypyridine, a Kv channel antagonist, prevents apoptosis of rat cerebellar granule neurons. Neuropharmacology 51: 757–746.
13. Hu GL, Zeng XM, Zhou MH, Shi YT, Cao H, et al. (2008) Kv1.1 is associated with neuronal apoptosis and modulated by protein kinase C in the rat cerebellar granule cell. J Neurochem 106: 1125–1137.
14. Shen QJ, Zhao YM, Cao DX, Wang XL (2009) Contribution of Kv channel subunits to glutamate-induced apoptosis in cultured rat hippocampal neurons. J Neurosci Res 87: 3133–3140.
15. Flavin MP, Coughlin K, Ho LT (1997) Soluble macrophage factors trigger apoptosis in cultured hippocampal neurons. Neuroscience 80: 437–448.
16. Klee R, Ficker E, Heinemann U (1995) Comparison of voltage-dependent potassium currents in rat pyramidal neurons acutely isolated from hippocampal regions CA1 and CA3. J Neurophysiol 74: 1982–1995.
17. Laurie DJ, Putzke J, Zieglgansberger W, Seeburg PH, Tolle TR (1995) The distribution of splice variants of the NMDAR1 subunit mRNA in adult rat brain. Brain Res Mol Brain Res 32: 94–108.
18. Hesselgesser J, Horuk R (1996) Chemokines and chemokine receptor expression in the central nervous system. J Neurovirol 5: 13–26.
19. Ohagen A, Ghosh S, He J, Huang K, Chen Y, et al. (1999) Apoptosis induced by infection of primary brain cultures with diverse human immunodeficiency virus type 1 isolates: evidence for a role of the envelope. J Virol 73: 897–906.
20. Tanamahura H, Hiramatsu K, Mizumoto M, Ueda S, Kusano S, et al. (2003) Enhancement of the T140-mediated pharmacophores leads to the development of more potent and bio-stable CXCR4 antagonists. Org Biomol Chem 1: 3663–3669.
21. Tanamahura H, Fujisawa H, Hiramatsu K, Mizumoto M, Nakashima H, et al. (2004) Identification of a CXCR4 antagonist, a T140 analog, as an anti-rheumatoid arthritis agent. FEBS Lett 569: 99–104.
22. Andewson P (1997) Kinase cascades regulating entry into apoptosis. Microbiol Mol Biol Rev 61: 33–46.
23. Dempsey EC, Newton AC, Mochly-Rosen D, Fields AP, Reyland ME, et al. (2000) Protein kinase C isoforms and the regulation of diverse cell responses. An J Physiol Lung Cell Mol Physiol 279: L429–438.
38. Liu WS, Heckman CA (1998) The seventh wave of PKC regulation. Cell Signal 10: 529–542.

39. Reyland ME (2007) Protein kinase Cdelta and apoptosis. Biochem Soc Trans 35: 1001–1004.

40. Bachis A, Major EO, Mocchetti I (2003) Brain-derived neurotrophic factor inhibits human immunodeficiency virus-1/gp120-mediated cerebellar granule cell death by preventing gp120 internalization. J Neurosci 23: 5715–5722.

41. Catani MV, Corasaniti MT, Navarra M, Nistico G, Finazzi-Agro A, et al. (2000) gp120 induces cell death in human neuroblastoma cells through the CXCR4 and CCR5 chemokine receptors. J Neurochem 74: 2373–2379.

42. Beaumais F, Michel L, Dubertret L (1995) Human eosinophils in culture undergo a striking and rapid shrinkage during apoptosis. Role of K+ channels. J Leukoc Biol 57: 831–835.

43. Elkhatera D, Platoshyn O, Krick S, Yu Y, McDaniel SS, et al. (2001) Bcl-2 modulates of dendritic K+ channels in hippocampus involves a mitogen-activated protein kinase pathway. J Neurosci 21: 4660–4668.

44. Schrader LA, Bornbaum SG, Nadim BM, Ren Y, Bui D, et al. (2001) ERK/ MAPK regulates the Kv4.2 potassium channel by direct phosphorylation of the pore-forming subunit. Am J Physiol Cell Physiol 290: C552–561.

45. Newton AC (2001) Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. Chem Rev 101: 2553–2564.

46. von Bir Mais F, Da Silva WC, Bevilacqua LR, Medina JH, Izquierdo I, et al. (2007) On the participation of hippocampal PKC in acquisition, consolidation and reconsolidation of spatial memory. Neuroscience 147: 57–45.

47. Yuan LL, Adams JP, Swank M, Sestack JD, Johnston D (2002) Protein kinase modulation of dendritic K+ channels in hippocampus involves a mitogen-activated protein kinase pathway. J Neurosci 22: 4680–4686.

48. Fattore M, Fattore M, Fattore M, Fattore M, Fattore M, et al. (2000) PKCdelta. J Biol Chem 275: 7574–7582.

49. Ito Y, Mishra NC, Yoshida K, Kharbanda S, Saxena S, et al. (2001) Mitochondrial targeting of JNK/SAPK in the phorbol ester response of myeloid leukemia cells. Cell Death Differ 8: 794–800.

50. Takanaka Y, Gavrilides MV, Misuuchi Y, Fujii T, Kazanietz MG (2003) Protein kinase C promotes apoptosis in LNCaP prostate cancer cells through activation of p38 MAPK and inhibition of the Akt survival pathway. J Biol Chem 278: 33753–33762.

51. Zhu D, Jiang X, Wu X, Tian F, Mearos K, et al. (2004) Inhibition of protein kinase C promotes neuronal survival in low potassium through an Akt-dependent pathway. Neurotox Res 6: 281–289.

52. Felipo V, Minana MD, Grojis R, et al. (2003) Inhibitors of protein kinase C prevent the toxicity of glutamate in primary neuronal cultures. Brain Res 604: 192–196.

53. Humphries MJ, Linessed KH, Schneider JC, Nakayama I, Anderson SM, et al. (2006) Suppression of apoptosis in the protein kinase Cdelta null mouse in vivo. J Biol Chem 281: 9728–9737.

54. Sa MJ, Madeira MD, Rueda C, Volk B, Mota-Miranda A, et al. (2000) AIDS does not alter the total number of neurons in the hippocampal formation but induces cell atrophy: a stereological study. Acta Neuropathol (Berl) 99: 643–653.

55. Gray F, Adle-Biasset E, Chretien F, Lorin de la Grandmaison G, Force G, et al. (2003) Neuropathology and neurodegeneration in human immunodeficiency virus infection. Pathogenesis of HIV-induced lesions of the brain, correlations with HIV-associated disorders and modifications according to treatments. Clin Neuropathol 20: 146–155.

56. Seifeddin D, Dwykaerts C, Vaesen R, Belztt F, Brunet P, et al. (1993) HIV-1-associated cognitive/motor complex: absence of neuronal loss in the cerebral neocortex. Neurology 43: 1492–1499.

57. Ferrando S, van Geijlsp D, McElhiney M, Gogggin K, Sewell M, et al. (1996) Highly active antiretroviral treatment in HIV infection: benefits for neuropsychological function. AIDS 12: 805–7.

58. McCutchan JA, Wu JW, Robertson K, Koletar SL, Ellis RJ, et al. (2007) HIV suppression by HAART preserves cognitive function in advanced, immune-reconstituted AIDS patients. AIDS 21: 1109–1117.

59. Parsons TD, Braaten AJ, Hall CD, Robertson KR (2006) Better quality of life with neuropsychological improvement on HAART. Health Qual Life Outcomes 4: 11.

60. Sacktor N, Nakasui S, Skolasky R, Robertson K, Wong M, et al. (2006) Antiretroviral therapy improves cognitive impairment in HIV+ individuals in sub-Saharan Africa. Neurology 67: 311–314.

61. Gelman SS, Soukup VM, Schuenke KW, Keberly MJ, Holzer C, et al. (2004) Acquired neuronal channelopathies in HIV-associated dementia. J Neuroimmunol 157: 111–119.

62. Keblesh J, Hu D, Xiong H (2009) Voltage-gated potassium channels in human immunodeficiency virus type-1 (HIV-1)-associated neurocognitive disorders. J Neuroimmunol 204: 60–70.

63. Keblesh JP, Hou H, Gendelmann HE, Xiong H (2009) 4-Aminopyridine improves spatial memory in a murine model of HIV-1 encephalitis. J Neuroimmune Pharmacol 4: 60–70.

64. Gendelmann HE, Lipton SA, Tardieu M, Bukrinsky MI, Nottet HS (1994) The gp120 Induces Neuronal Apoptosis by Increase IA