Int J Physiol Pathophysiol Pharmacol 2023;15(3):75-87
www.ijppp.org /ISSN:1944-8171/IJPPP0150824

Original Article

HIV-1 envelope protein gp120 modulation of glutamate effects on cortical neuronal synapses: implications for HIV-1-associated neuropathogenesis

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Received April 20, 2023; Accepted May 17, 2023; Epub June 15, 2023; Published June 30, 2023

Abstract: Despite the introduction of combined antiretroviral therapy (cART) HIV-1 virus persists in the brain in a latent or restricted manner and viral proteins, such as gp120, continue to play a significant disease-inciting role. Gp120 is known to interact with *N*-methyl-*D*-aspartate (NMDA) receptors (NMDARs) resulting in neuronal injury. Glutamate is the main excitatory neurotransmitter in the brain and plays an important role in cognitive function and dysregulation of excitatory synaptic transmission impairs neurocognition. It is our hypothesis that gp120 may alter synaptic function via modulating glutamate function from a physiological molecule to a pathophysiological substance. To test this hypothesis, we studied the modulatory effects of gp120 and glutamate on NMDAR-mediated spontaneous excitatory postsynaptic current ($sEPSC_{NMDAR}$) and dynamic dendritic spine changes in rat cortical neuronal cultures. Our results revealed that gp120 and glutamate each, at low concentrations, had no significant effects on $sEPSC_{NMDAR}$ and dendritic spines, but increased $sEPSC_{NMDAR}$ frequency, decreased numbers of dendritic spines when tested in combination. The observed effects were blocked by either a CXCR4 blocker or an NMDAR antagonist, indicating the involvements of chemokine receptor CXCR4 and NMDARs in gp120 modulation of glutamate effects. These results may imply a potential mechanism for HIV-1-associated neuropathogenesis in the cART era.

Keywords: HIV-1 gp120, glutamate, cortical neuron, sEPSC, NMDA receptor, neuromodulation

Introduction

Human immunodeficiency virus type 1 (HIV-1) infection continues to be a major global public health issue. Individuals infected with HIV-1 often develop neurological symptoms including cognitive impairment and motor disturbances collectively termed as HIV-1-associated neurocognitive disorders (HAND) [1, 2]. Although the introduction of combined antiretroviral therapy (cART) has significantly decreased the severe form of the HAND mild neurocognitive impairments remain prevalent, due to virus persistence in the brain at low levels often in a latent or restricted manner and reduced cART penetration into the brain [3-6]. In contrast to frank neuronal injury and loss seen in neuropathology of severe form HAND, more subtle changes on synaptic dendrites and neuronal circuitry are believed to drive the HAND in the era of cART [7-10]. The mechanisms by which HIV-1 causes synaptodendritic alteration in the brain are not fully understood and neurotoxicity in HIV-1-infeceted brain is believed to be, at least in part, mediated by viral proteins and other bioactive molecules released from infected cells. Among the viral proteins is HIV-1 envelope glycoprotein 120 (gp120).

The gp120 molecule, the main HIV-1 coat protein, is part of the outer layer of the virus. In addition to its facilitation of HIV-1 entry into the host cells via CD4 receptors along with CCR5 and CXCR4 receptors, gp120 can be shed off from viral membrane and/or released from infected brain cells [11, 12], and accumulates in the cerebrospinal fluid and brain tissue in significant amounts [13, 14]. Soluble gp120 is a potent neurotoxin at concentrations ranging from picromolar to nanomolar *in vitro* [13, 15].

It is toxic to neural cells both *in vitro* [16, 17] and *in vivo* [18, 19]. Transgenic animals expressing gp120 developed neurodegeneration and cognitive impairment [20, 21]. Studies have shown that gp120 on the one hand induces neuronal and synaptodendritic dysfunction and injury [22-24] and on the other hand causes immune activation and resultant production of neurotoxic molecules leading to the development of HAND [12, 25, 26]. The neurocognitive impairment and pathophysiological alterations observed in HIV-1-infected individuals can be attributed to direct and/or indirect effects of gp120.

The mechanisms underlying gp120-associated neurotoxicity are multifaceted including, but are not limited to, its activation of neuronal NMDA receptor (NMDAR) [27-29] and inhibition of glutamate (Glu) uptake by astrocytes [30-32]. In the mammalian CNS, Glu is the principal excitatory neurotransmitter that plays a crucial role in mediating cognitive and behavior functions [33, 34]. Dysregulation of excitatory synaptic transmission may impair higher brain functions such as memory formation and behavior. We hypothesize that the presence of soluble gp120 in the brain may dysregulate excitatory synaptic transmission by altering Glu function from a physiological molecule to a pathophysiological substance. To test this hypothesis, we studied effects of gp120 and Glu on NMDAR-mediated spontaneous excitatory postsynaptic current ($sEPSC_{NMDAR}$) and dendritic spine morphology in rat cortical neuronal cultures. Our results showed that gp120 and Glu, each alone at low concentrations, had no significant effects on $sEPSC_{NMDAR}$ and dendritic spines, but increased the $sEPSC_{NMDAR}$ frequency, decreased numbers of dendritic spines when tested in combination. The observed results may have implications for HIV-1-associated neurocognitive decline in the era of cART.

Materials and methods

Materials

Full-length HIV-1_{IIIB} gp120 was purchased from Immunodiagnostics, Inc. (Woburn, MA). Aliquots of gp120 were kept as 100 nM stock solution at -80°C. The stock solution was diluted to desired concentrations with artificial cerebrospinal fluid (ACSF) 2-5 min before test. All other chemicals, unless otherwise specified, were purchased from Sigma-Aldrich (St. Louis, MO).

Animals

Pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) and maintained under ethical guidelines for care of laboratory animals at the University of Nebraska Medical Center. Animals were housed at constant temperature (22°C) and relative humidity (50%) under a regular light-dark cycle (light on at 7 am and off at 5 pm) with free access to food and water. All animal-use procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of University of Nebraska Medical Center (IACUC # 19-085-07-FC).

Cortical neuronal culture

Neurons were isolated from cortex tissue of E18 fetal Sprague-Dawley rats. Dissected tissue was incubated with 0.25% trypsin and 200 U DNAase contained in Hank's Buffered Salt Solution (HBSS) at 37°C for 15 min, and the digestion was stopped by addition of fetal bovine serum (FBS) at 10%. After centrifuging, the remaining tissue suspension was passed through nylon mesh with pore diameter size of 100 μm and 40 μm. Isolated cells were then suspended in neurobasal media (Gibco, Thermo Fisher) supplemented with 2% B27, 1% penicillin/streptomycin, 0.2% fetal bovine serum (FBS), and 0.25 mM L-glutamine (Invitrogen, Thermo Fisher), and seeded either in 60 mm dishes at 2.5×10^6 cells/dish, 0.2×10^6 cells/well in 12 well plates containing 15 mm diameter coverslips, or in 48-well plates at 0.05×10^6 cells/well. All dishes, coverslips and plates were pre-coated with poly-D-lysine (1 mg/ml). Cultures were maintained in supplemented neurobasal media for 8-12 days with half media change every 4 days. The purity of neuronal cells was > 90% as determined by staining with microtubule-associated protein-2 antibody (MAP-2: 1:1000, Chemicon International, Inc., Temecula, CA) and anti-NeuN antibody (1:500, Abcam, Waltham, MA).

Electrophysiology

Whole-cell recording was conducted with a patch-clamp amplifier Axopatch 200B (Molecular Devices, San Jose, CA). The extracellular solution was an artificial cerebrospinal fluid (ACSF)

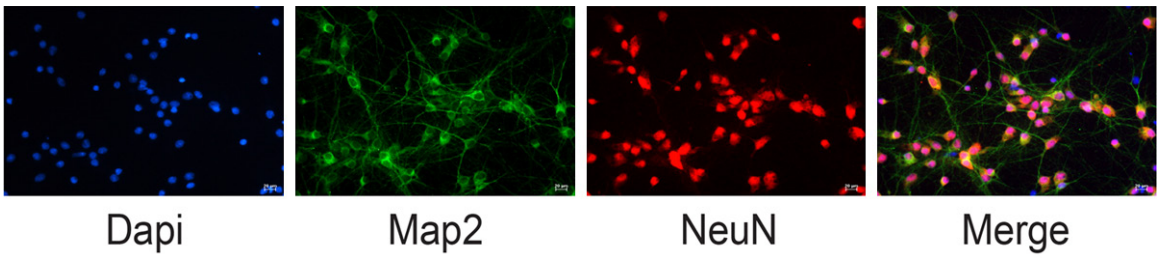

Figure 1. Determination of neuronal purity by MAP2 and NeuN staining. After 10-14 days in culture cells were incubated with anti-MAP2 (green) and Anti-NeuN (red) antibody, then the second antibodies Alexa Fluor-488 and Alexa Fluor-594 were added. Cells with color in blue were stained with Dapi. Neuronal purity was > 90%. Magnification, ×200.

contained (in mM) NaCl 125, KCl 3.5, $CaCl_2$ 3.0, $MgCl_2$ 0.2, NaH_2PO_4 1.25, $NaHCO_3$ 26, Glucose10.0. $sEPSC_{NMDAR}$ was recorded in the presence of 5 μM NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)-quinoxaline, a highly selective AMPA receptor antagonist) and 50 μM picrotoxin. Patch electrodes were filled with (in mM) K-gluconate 125, K-methysulphate 17.5, NaCl 10, HEPES 10, EGTA 0.5, GTP 0.2, K_2ATP 2.0. All experiments were performed at room temperature (22-23°C). During experiments, the cultured neurons were perfused with oxygenated (bubbled with 95% O_2 and 5% CO_2) ACSF at a constant flow rate of 2 mL/min. After formation of a high resistance seal (> 1 GΩ) and subsequent rupture of cell membrane, the neurons were voltage-clamped at -60 mV. Junction potential, series resistance were corrected using amplifier circuitry and cell capacitance was compensated (~70%). Electrical signals were filtered at 1 kHz and digitized at 5 kHz, displayed on an oscilloscope and computer monitor, digitized with Digidata 1440A digitizer (Molecular Devices) and stored on a PC computer. Data acquisition and analysis were carried out using pClamp 10 software (Molecular Devices) and data processing, graphing and statistical analysis were made using Origin 2019b (OriginLab, Northampton, MA).

Dendrite morphology and spine number quantification

Primary cortical neurons grown on poly-D-lysine-coated coverslips were treated with gp120 and/or Glu in the presence or absence of an NMDAR antagonist MK801 (20 μM) [35]. After 24 h treatment, cells were washed in phosphate buffered saline (PBS) (5 min, 3 times), then fixed in 4% paraformaldehyde in PBS (pH 7.4) for 1 h at room temperature and then blocked in 10% normal goat serum and 0.1% Triton X-100 for 30 min at room temperature. Cells were incubated with primary antibody anti-MAP2 (rabbit, 1:1000) diluted in PBS overnight at 4°C, followed by application of AlexaFluor 488 (1:500) secondary antibody (Invitrogen) in PBS for 1 h. After a final wash in PBS, coverslips were mounted in ProLong Gold antifade reagent with DAPI (Molecular Probes, Eugene, OR). Cells were visualized by Zeiss LSM 510 META NLO microscope in a 100× oil-immersion objective. The numbers of spines on selected secondary dendritic branch (50 μm in length) were counted manually.

Statistical analyses

The amplitude and occurrence of $sEPSC_{NMDAR}$ recorded in a time window of 120 s were analyzed and averaged using Clampfit 10 software. The numbers of dendritic spines on the secondary branch (50 μm in length) were counted. All data are expressed as means ± SEM and graphed using Origin2019b software. Statistical analyses were done using one-way ANOVA or student t-tests. A minimum p value of 0.05 was estimated as the significance level for all tests.

Results

Purity of rat cortical neuronal cultures

To study gp120 modulation of Glu effects synaptic dendrites we first examined cortical neuronal purity using MAP2 and NeuN staining. It was shown that neuronal cells were > 90% in each batch of cultures employed for this study (**Figure 1**).

Effects of gp120 and Glu on $sEPSC_{NMDAR}$

Gp120 was toxic to rodent neurons *in vitro* [36] and *in vivo* [37]. Its neurotoxic activity can be blocked by NMDAR antagonists [38], indicating

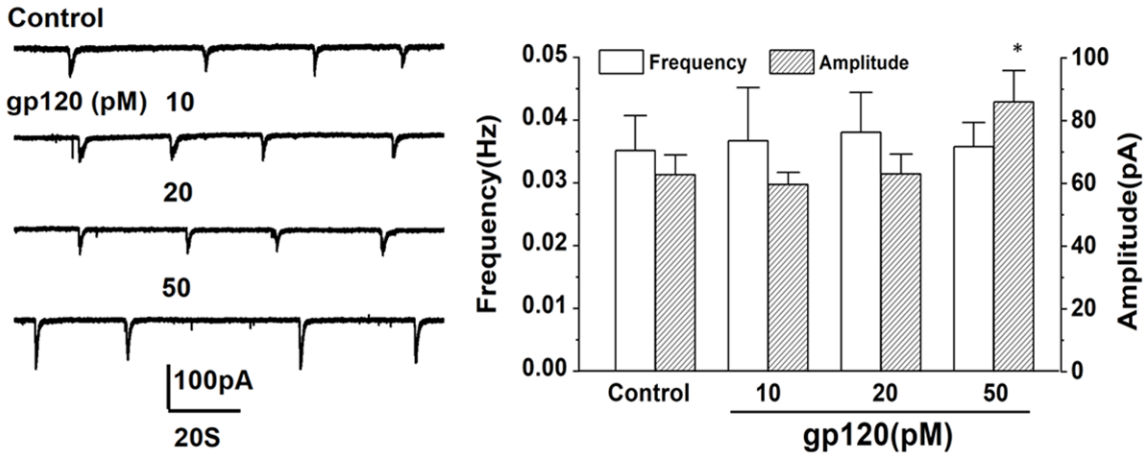

Figure 2. The effect of gp120 on $sEPSC_{NMDAR}$ at different concentrations (10 pM, 20 pM, 50 pM). Left panel: Traces of $sEPSC_{NMDAR}$ recorded under gap-free configuration from a neuron at different experimental conditions as indicated. Right panel: Group data from six neurons showed that the average frequency and peak amplitude of $sEPSC_{NMDAR}$ were not altered by gp120 at the concentrations of 10 pM and 20 pM tested ($P > 0.05$). At 50 pM, gp120 significantly increased amplitude ($P < 0.05$), but not frequency.

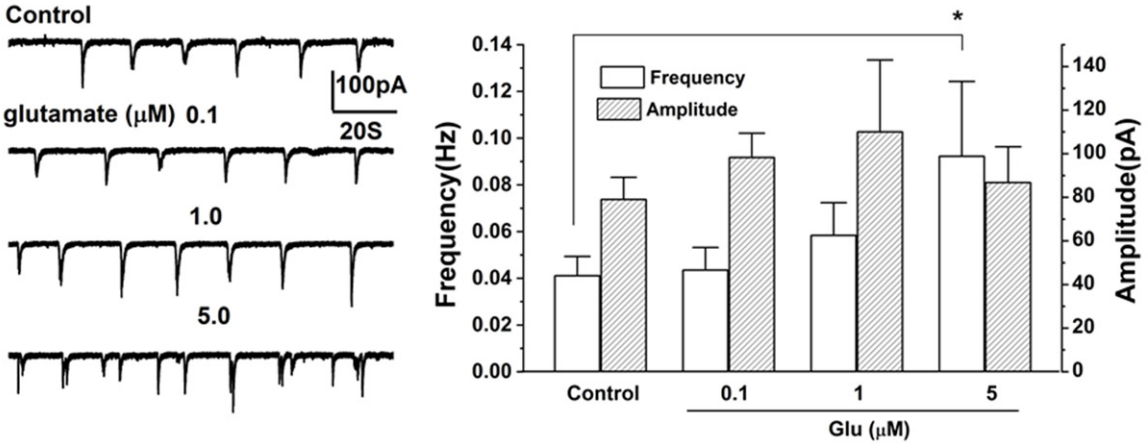

Figure 3. Effect of Glu on $sEPSC_{NMDAR}$ at different concentrations (0.1 μM, 1 μM, 5 μM). Left panel displays the traces of $sEPSC_{NMDAR}$ recorded under different experimental conditions as indicated. Right panel shows the average frequency and peak amplitude of $sEPSC_{NMDAR}$ recorded from six neurons. Note that Glu had no significant effects on $sEPSC_{NMDAR}$ amplitude ($P > 0.05$, n = 6), but increased $sEPSC_{NMDAR}$ frequency at higher concentration (5 μM, $P < 0.05$, n = 6).

an involvement of NMDAR in gp120-associated neuropathophysiology. To examine influence of gp120 on Glu in HIV-1-infected brain, we first studied individual effects of gp120 and Glu on $sEPSC_{NMDAR}$ at low concentrations. Application of gp120 (10, 20, 50 pM) alone had no significant effects on $sEPSC_{NMDAR}$ frequency (control, 0.035 ± 0.006 Hz vs. 10 pM, 0.037 ± 0.008 Hz; 20 pM, 0.038 ± 0.006 Hz; 50 pM, 0.036 ± 0.004 Hz; $P > 0.05$, n = 6). In contrast, gp120 alone had no effects on $sEPSC_{NMDAR}$ amplitude at concentrations of 10 and 20 pM (control, 62.8 ± 6.3 pA, vs. 10 pM, 59.6 ± 3.9; 20 pM, 63.0 ± 6.3; $P > 0.05$, n = 6), but increased $sEPSC_{NMDAR}$ amplitude at 50 pM (86.0 ± 10.0 pA vs. 62.8 ± 6.3 pA in control, $P < 0.05$, n = 6) (**Figure 2**). Application of Glu (0.1, 1.0, 5.0 μM) alone showed that it increased the $sEPSC_{NMDAR}$ frequency only at higher concentration (5 μM) (control, 0.041 ± 0.008 Hz vs. 0.1 μM, 0.043 ± 0.009 Hz; 1 μM, 0.058 ± 0.014 Hz; $P > 0.05$, n = 6; 5 μM, 0.092 ± 0.032 HZ; $P < 0.05$, n = 6), but had no significant effects on $sEPSC_{NMDAR}$ amplitude (control, 79.1 ± 10.2 pA vs. 0.1 μM, 98.3 ± 11.1 pA; 1 μM, 110.2 ± 33.0 pA; 5 μM, 86.8 ± 16.4 pA; $P > 0.05$, n = 6) (**Figure 3**).

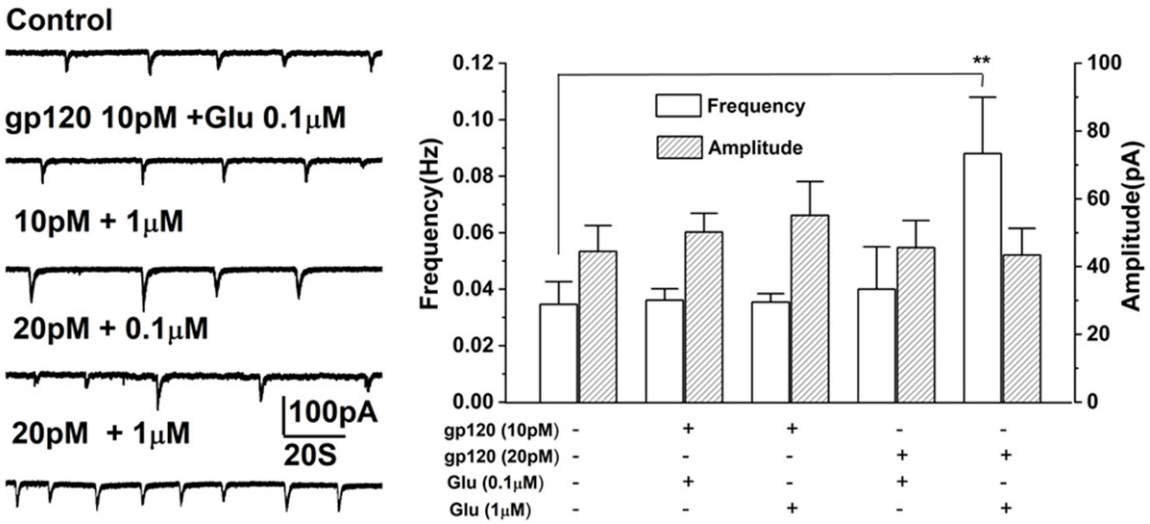

Figure 4. Gp120 (20 pM) potentiation of Glu (1 μM) effects on $sEPSC_{NMDAR}$. As gp120 (10 pM, 20 pM) and Glu (0.1 μM, 1 μM) each alone had no apparent effects on $sEPSC_{NMDAR}$. They were tested in four different combinations to explore any augment effects on $sEPSC_{NMDAR}$. The results showed that gp120 (20 pM) significantly augmented Glu (1 μM) effects on $sEPSC_{NMDAR}$ frequency. Thus, 20 pM gp120 and 1 μM Glu were used for subsequent studies. $^{**}P < 0.01$, n = 6.

Gp120 potentiation of Glu effects on $sEPSC_{NMDAR}$

As gp120 (10 pM, 20 pM) and Glu (0.1 μM, 1 μM) each along had no significant effects on $sEPSC_{NMDAR}$ frequency and amplitude, we next tested their combined effects on $sEPSC_{NMDAR}$. Application of 10 pM gp120 + 0.1 μM Glu, 10 pM gp120 + 1 μM Glu, 20 pM gp120 + 0.1 μM Glu and 20 pM gp120 + 1 μM Glu failed to alter $sEPSC_{NMDAR}$ amplitude (Control, 44.5 ± 7.6 pA vs. 10 pM gp120 + 0.1 μM Glu, 50.2 ± 5.5 pA; 10 pM gp120 + 1 μM Glu, 55.1 ± 10.0 pA; 20 pM gp120 + 0.1 μM Glu, 45.6 ± 8.0 pA; 20 pM gp120 + 1 μM Glu, 43.4 ± 7.9 pA, P > 0.05, n = 6). However, while 10 pM gp120 + 0.1 μM Glu, 10 pM gp120 + 1 μM Glu and 20 pM gp120 + 0.1 μM Glu had no significant effects on $sEPSC_{NMDAR}$ frequency (Control, 0.0347 ± 0.008 Hz vs. 10 pM gp120 + 0.1 μM Glu, 0.0361 ± 0.004 Hz; 10 pM gp120 + 1 μM Glu, 0.0354 ± 0.003 Hz; 20 pM gp120 + 0.1 μM Glu, 0.042 ± 0.015 Hz, P > 0.05, n = 6), 20 pM gp120 was found to potentiate 1 μM Glu-associated $sEPSC_{NMDAR}$ frequency (Control, 0.0347 ± 0.008 Hz vs. 20 pM gp120 + 1 μM Glu, 0.088 ± 0.020 Hz) (**Figure 4**). The alteration of $sEPSC_{NMDAR}$ frequency suggests a presynaptic site of action.

Blockade of gp120 potentiation of Glu effects on $sEPSC_{NMDAR}$ by T140

HIV-1 gp120 interacts with neuronal CXCR4 receptors which are functionally coupled with NMDA receptors [39, 40]. To examine the involvement of CXCR4 receptors in gp120 potentiation of Glu effects on $sEPSC_{NMDAR}$, we tested effects of T140, a CXCR4 receptor antagonist [41], on gp120 enhancement of Glu effects on $sEPSC_{NMDAR}$. As shown in **Figure 5**, the addition of 100 nM T140 alone did not alter the frequency of $sEPSC_{NMDAR}$ (Control, 0.040 ± 0.006 Hz vs. 100 nM T140, 0.039 ± 0.005 Hz, P > 0.05, n = 8). In contrast, it reversed gp120-associated frequency increase when applied in combination (Control, 0.040 ± 0.006 Hz; gp120 + Glu, 0.095 ± 0.020 Hz, T140 + gp120 + Glu, 0.042 ± 0.009 Hz, P < 0.01 vs. control, P < 0.01 vs. t140 + gp120 + Glu, n = 8). The amplitude of $sEPSC_{NMDAR}$ was not influenced either by T140 alone or by combined application of T140 with gp120 and Glu (Control, 30.8 ± 9.1 pA; T140, 45.2 ± 8.6 pA; gp120 + Glu, 32.9 ± 10.2 pA; T140 + gp120 + Glu, 38.1 ± 7.3 pA; P > 0.05, n = 8) (**Figure 5**).

Neurotoxic property of Gp120 potentiation of Glu on $sEPSC_{NMDAR}$

HIV-1 gp120 was shown to cause synaptodendritic arbor injury [42] and synapse loss [43, 44]. To examine whether gp120 potentiation of Glu effects on $sEPSC_{NMDAR}$ was toxic to neuronal synaptic dendrites we analyzed the number of spines on the secondary dendrites (50 μm in length) on cultured cortical neurons. Our results showed that treatment of cortical neuronal cul-

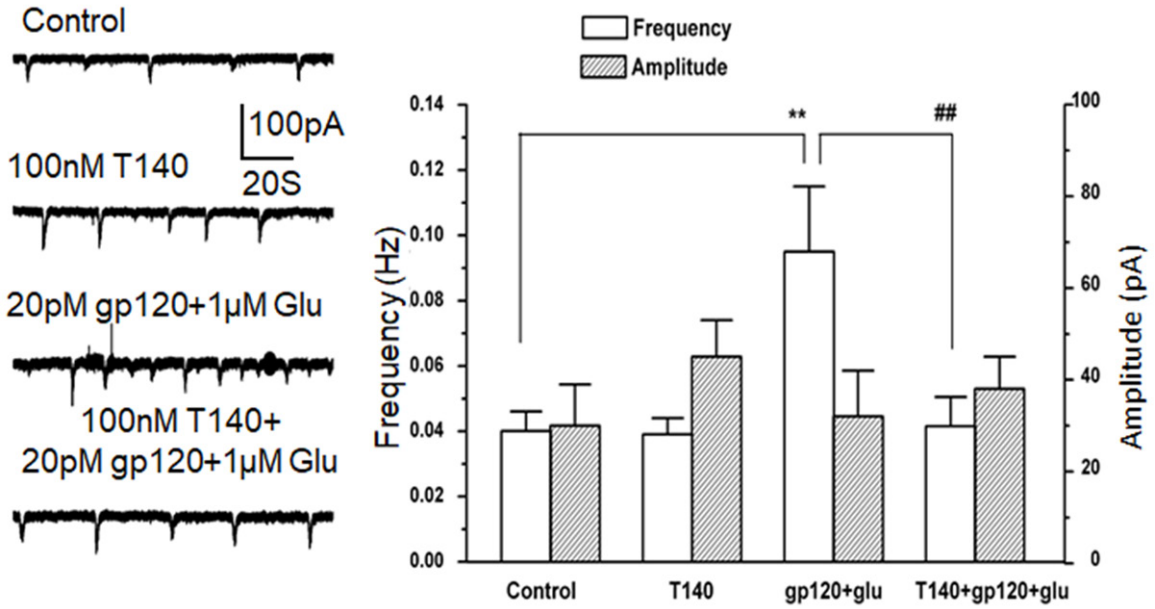

Figure 5. Blockade of gp120 augmentation of Glu-associated increase of $sEPSC_{NMDAR}$ frequency by T140 (100 nM), a CXCR4 receptor. As showed in both traces and bar graph co-application of gp120 (20 pM) and Glu (1 μM) produced a significant increase of $sEPSC_{NMDAR}$ frequency (but not amplitude). The increase of $sEPSC_{NMDAR}$ frequency was reversed by addition of T140 (100 nM) to the ACSF. Addition of T140 *per se* to ACSF had no apparent effect on $sEPSC_{NMDAR}$. These results suggest an involvement of CXCR4 receptors in gp120- and Glu-associated increase of $sEPSC_{NMDAR}$ frequency. $^{**}P < 0.01$, $^{\#\#}P < 0.01$, n = 8.

tures with gp120 for 24 h produced a concentration-dependent decrease in the number of spines (**Figure 6**). When treated with gp120 at 10, 20 and 50 pM, the average numbers of spines were 27.00 ± 1.00, 22.67 ± 2.53 and 23.69 ± 2.58 respectively. Compared with the number of spines of 26.67 ± 2.51 in control (untreated) neurons, the differences were not statistically significant (n = 8, P > 0.05). In contrast, when treated with gp120 at 100 pM and 200 pM, the number of spines were significantly reduced to 15.68 ± 2.54 and 11.67 ± 2.08 respectively in comparison with the number of spines observed in control group (n = 8, P < 0.01; **Figure 6**).

In a different set of experiments, the cultured neuronal cells were treated with different concentrations of Glu (0.1, 1, 5 uM) and resultant changes on the dendritic spines were also assayed. Addition of Glu to neuronal cultures for 24 h slightly reduced the numbers of spines on the secondary dendrites to 30.7 ± 1.5, 30.0 ± 2.13 and 30.3 ± 1.5, respectively. Compared with the numbers of spines on the secondary dendrites of 32.3 ± 2.5 in control group, the difference was not statistically significant (n = 8, P > 0.05, **Figure 7**). However, a significant decrease (n = 8, P < 0.05) on the numbers of spines was observed when Glu was applied to the cultured neurons at the concentration of 10 μM (18.6 ± 3.5 vs. 32.3 ± 2.5 in control, **Figure 7**).

As shown in **Figures 6** and **7**, 20 pM gp120 and 1 μM Glu each alone had no significant effects on the number of spines, we tested their modulatory or synergistic effects on the dendritic spines. The number of dendritic spines were 23.7 ± 1.1 and 25.0 ± 2.6 when neuronal cultures were treated with 20 pM gp120 and 1 μM Glu respectively. Compared with the control (26.0 ± 1.0), the differences had no statistical significance (n = 8, P > 0.05). However, the number of spines were significantly reduced to 11.3 ± 2.1 (n = 8, P < 0.05) when applied in combination, demonstrating gp120 potentiation of Glu neurotoxicity in cortical neuronal cultures. Such a potentiation was blocked by addition of an NMDAR antagonist MK801 (20 μM) to the culture media, indicating an involvement of NMDAR in gp120 potentiation of Glu-associated neurotoxicity (n = 8, **Figure 8**).

Discussion

Although the neurotoxic effects of HIV-1 gp120, mediated at least in part via activation of neu-

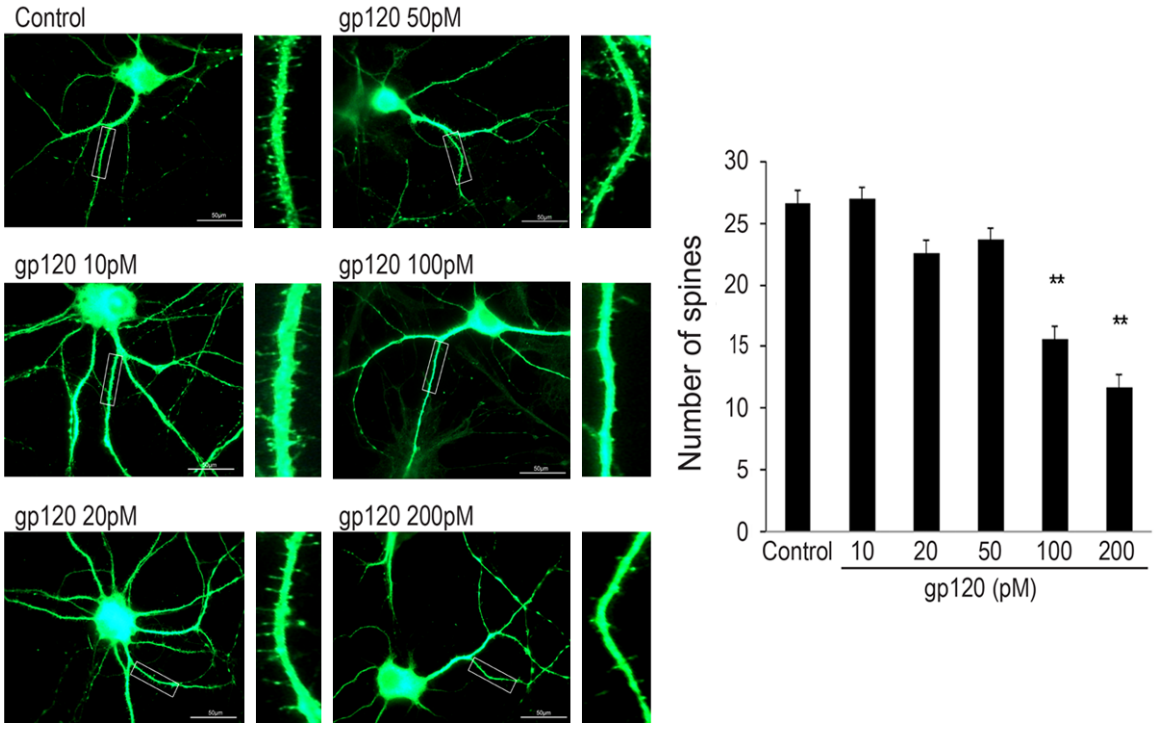

Figure 6. The concentration-dependent effects of gp120 on the number of spines on the neuronal secondary dendrites. The number of spines were manually counted on each neuron in a selected segment (50 μM in length) of a secondary oblique dendrite (marked by a rectangle in white) protruding from its parental dendrite. The left columns of photomicrographs displayed single MAP2-labeled cortical neurons treated with different concentrations of gp120 as indicated. The enlarged secondary dendrites on which dendritic spines were counted were shown vertically right to their neuronal photomicrographs correspondently. The right bar graph shows average numbers of spines counted on selected segments of secondary dendrites from cortical neurons with different treatments as shown in photomicrographs. Note that no significant change on the numbers of spines were observed when treated with 20 pM of gp120. However, gp120 significantly reduced numbers of spines at concentrations 100 pM and 200 pM. $^{**}P < 0.01$ vs. control. Scale bar equals 50 μm.

ronal NMDARs, are well studied, few investigations have focused on its modulation of Glu effects in neuronal synapses *in vitro* or in HIV-1-infected brain, especially in the era of cART. The objective of his study was to test the hypothesis that low concentrations of soluble viral protein gp120, which is present in the brains of HIV-1-infected patients treated with cART, may dysregulate neuronal synaptic activity by modulation of Glu function from a physiological molecule to pathophysiological substance. To this end, we examined the modulatory effects of low concentrations of gp120 on Glu-associated $sEPSC_{NMDAR}$ and dynamic changes of dendritic spines in rat cerebrocortical neuronal cultures. Our results showed that gp120 (20 pM) and Glu (1 μM), at low concentrations, had no significant effects on $sEPSC_{NMDAR}$ when each were tested alone. In contrast, gp120 potentiated Glu-mediated increase of $sEPSC_{NMDAR}$ frequency and worsened Glu-associated decrease of dendritic spines while applied in combination. The gp120-associated potentiation of Glu effects was significantly attenuated by either a CXCR4 receptor antagonist T140 or an NMDAR antagonist MK801, suggesting an involvement of chemokine receptor CXCR4 and NMDAR in gp120 modulation of Glu effects on synaptic dendrites.

It is widely accepted that Glu plays an important role in the pathogenesis of HAND [25, 45]. Gp120 incites Glu receptor-mediated neurotoxicity and neurodegeneration [25]. Studies have shown that gp120 induced hippocampal injury in neonatal rats was blocked by CPP, a potent and selective NMDAR antagonist [37]. Gp120 was also found to potentiate NMDA- and Glu-evoked noradrenaline release at rat hippocampal and cortical noradrenergic nerve terminals

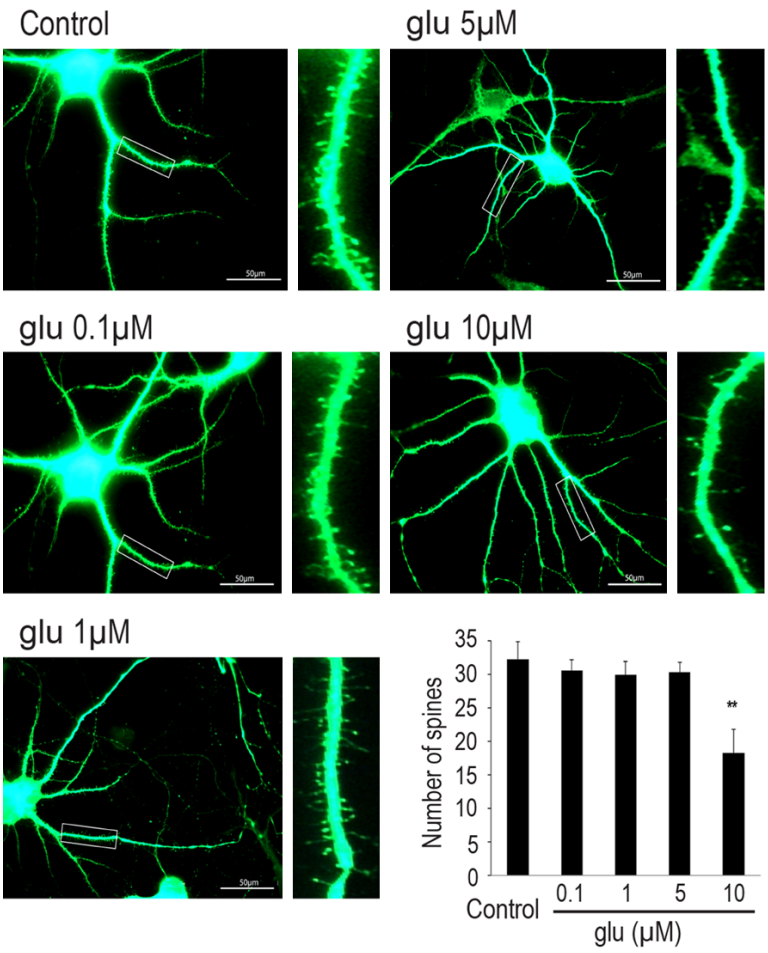

Figure 7. The effect of different concentrations of Glu on the number of spines on the secondary dendrite. Representative photomicrographs showed single MAP2-labeled cortical neurons treated with different concentrations of Glu as indicated and the magnified segment of secondary dendrite examined on each neuron was shown vertically right to each neuronal photomicrograph. The quantification of spine numbers was shown in the bottom right. Note that Glu at a lower concentration (1 μM) had no significant effect on dendritic spine numbers. It decreased the number of spines at higher concentration (10 μM). $^{**}P < 0.01$ vs. control. Scale bar equals 50 μm.

[46]. As NMDAR activation is crucial in excitotoxic activity [47], these results indicate that gp120 interplays with NMDARs resulting in neuronal injury. Consistent with these results, we observed that gp120 potentiated Glu-associated increase of $sEPSC_{NMDAR}$ occurrence and decreased the number of dendritic spines. The augment effects occurred at low concentrations of gp120 and Glu, which had no apparent effects when each was tested alone but altered these parameters significantly while tested in combination, demonstrating a modulatory effect of gp120 on Glu effects via NMDARs.

Spontaneous synaptic events modulate spike firing [48], modify dendritic spine structure [49] and regulate dendritic protein synthesis [50]. Change in sEPSC frequency is widely believed to be associated with alteration of presynaptic function. Our results showed that gp120 potentiated Glu-associated increase of $sEPSC_{NMDAR}$ frequency, suggesting a presynaptic site of action for gp20. Since chemokine receptor CXCR4 is expressed in the presynaptic terminals [51] and gp120 binds to CXCR4 [52], the above suggestion was further supported by experimental results that gp120-mediated increase of $sEPSC_{NMDAR}$ frequency was blocked by T140, a CXCR4 blocker [53]. In addition to CXCR4, NMDAR is also expressed in presynaptic terminals [54]. Thus, it was not surprising that we observed gp120 enhancement of Glu-associated increase of $sEPSC_{NMDAR}$ frequency because gp120 interacts with NMDARs [29, 55] and because CXCR4 and NMDAR are functionally coupled at nerve endings [39].

Excitatory synapses have distinct dendritic spines at the postsynaptic sites. These dendritic spines receive most of the excitatory glutamatergic input directly from apposing presynaptic terminals in the CNS [56-59]. Spontaneous released Glu and synaptic events regulate the dynamic of dendritic spines and maintain the stability of synaptic networks [60-63]. Dysregulated enhancement of spontaneous release Glu could be detrimental to dendritic spines resulting in the alteration of dendrite morphology and the decrease of spine numbers [63, 64]. Indeed, the morphological and physiological alterations in response to NMDAR activation have been observed, including a loss of spines, dendritic focal swelling, cytoskeletal degradation and a depression of

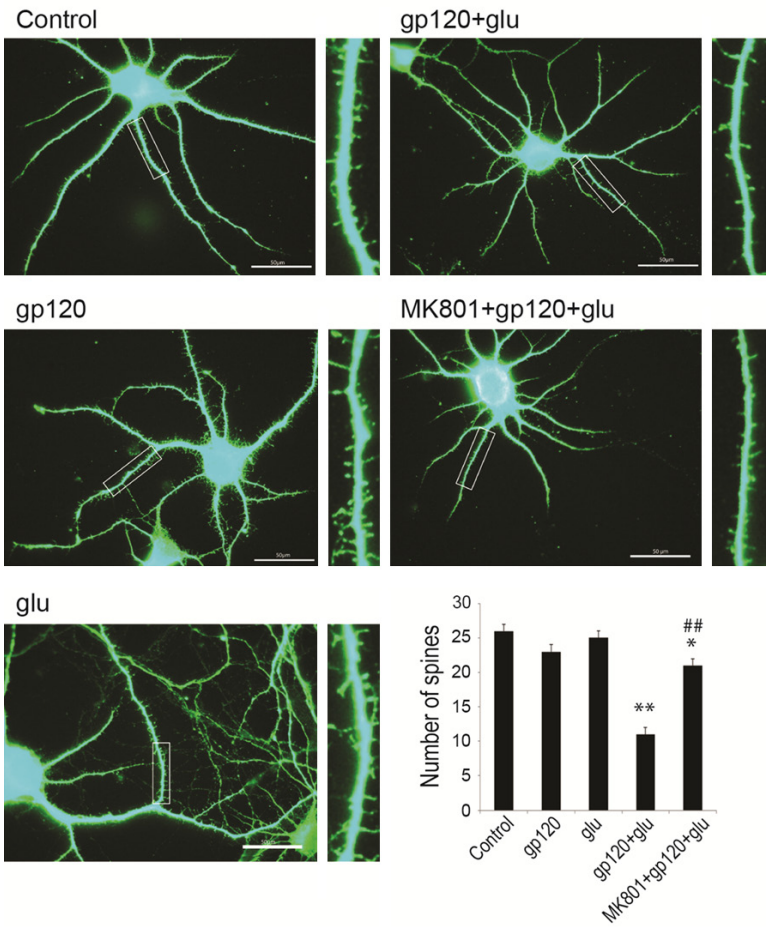

Figure 8. Synergic effects of gp120 and Glu on dendritic spines. As gp120 (20 pM) and Glu (1 μM) had no apparent effects when tested each alone (**Figures 6** and **7**), we tested their effects on dendritic spines in combination. Gp120 was found to augment Glu-associated reduction on the number of spines when applied to neuronal cultures in combination. Such a reduction was blocked by an NMDAR antagonist MK801, suggesting an involvement of NMDARs in gp120- and Glu-associated reduction of dendritic spines. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control, $^{\#\#}P < 0.01$ vs. gp120 + Glu.

neurotransmission. In this study, we observed gp120 enhancement of Glu-associated increase of $sEPSC_{NMDAR}$ occurrence and decreased the number of dendritic spines. Such an enhancement of spontaneous occurrence of Glu-associated $sEPSC_{NMDAR}$ by gp120 may contribute, at least in part, to HIV-1-associated neurological complications seen in patients treated with cART, because altered dendrite morphology is considered as an early sign of neural injury that occurs in acute neurologic and chronic neurodegenerative diseases [65].

It is worth pointing out that the concentration of gp120 used in this study was physiologically relevant as determined in our previous study [66]. The rationale for using physiologically relevant concentration is to mimic the disease condition, a mild form of HAND which is prevalent in the era of cART with low levels of viral replication in the brain. Although the actual *in vivo* plasma and brain tissue concentrations of gp120 in chronically HIV-1-infected patients remain unclear in the cART era, the levels detected in the serum of HIV-infected individuals prior to cART era were 1-8 nM [15]. Given that 1 nM of gp120 is ~0.12 μg/ml [13], the soluble gp120 concentrations were estimated between 120 and 960 ng/ml [14]. More higher levels of gp120 were also detected in secondary lymphoid organs of individuals with chronic viral infection ranging from 500 ng/ml to 5 μg/ml when all forms of gp120 were added, including soluble, cell- and virion-associated gp120 [14, 67]. The concentration of gp120 used in this study was 20 pM which was below physiological concentrations detected by Oh et al. [15]. The Glu concentration of employed in study was 1 μM, which was within the physiological range (0.02-20 μM) of the tonic basal concentrations of Glu in the extracellular space outside of synaptic cleft [68-70]. Thus, the results obtained by using physiologically relevant concentrations of gp120 and Glu may have implications for HAND pathogenesis in the era of cART that lower levels of viral replication may not cause neuronal and synaptic damage, but may induce neuronal and synaptic dysfunction by modulating the action of Glu by turning its role from physiology to pathophysiology.

In summary, the results showed that low concentrations of gp120, when applied in combination with low concentrations of Glu, potentiated Glu-mediated increase of $sEPSC_{NMDAR}$ frequency in rat cerebrocortical neuronal cul-

tures and altered the dynamic of dendritic spines. The observed effects were blocked or attenuated by either a CXCR4 receptor blocker T140 or an NMDAR blocker MK801, suggesting chemokine receptor CXCR4 and NMDAR were involved in gp120-associated modulation of $sEPSC_{NMDAR}$ occurrence and consequent dynamic changes on dendritic spines. As virus persists in brain in a latent and restricted manner in the cART era, the soluble gp120, shed off from virions or released from infected cells, may modulate the role of endogenous Glu from physiological molecule to a pathophysiological substance, a potential mechanism for HIV-1-associated neuropathogenesis in the era of cART.

Acknowledgements

The authors thank Mr. Reed Felderman for reading the manuscript. This work was supported by NIH grants R01NS077873 and R01DA050540.

Disclosure of conflict of interest

None.

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