Published in final edited form as:
J Neurovirol. 2006 June ; 12(3): 211–218. doi:10.1080/13550280600848373.

Human immunodeficiency virus gp120-induced apoptosis of human neuroblastoma cells in the absence of CXCR4 internalization

Giuseppe Bardi, **Rajarshi Sengupta**, **Muhammad Z Khan**, **Jeegar P Patel**, and **Olimpia Meucci**
Department of Pharmacology and Physiology, Drexel University College of Medicine, Philadelphia, Pennsylvania, USA

Abstract

The chemokine receptor CXCR4 functions as human immunodeficiency virus (HIV)-1 coreceptor and is involved in acquired immunodeficiency virus (AIDS) neuropathogenesis. CXCR4 is expressed by most cell types in the brain, including microglia, astrocytes, and neurons. Studies have shown that the HIV envelope protein gp120 binds to neuronal CXCR4 and activates signal transduction pathways leading to apoptosis. However, the natural CXCR4 ligand (CXCL12) has been referred to induce both neuronal survival and death. Here the authors used flow cytometry to determine whether gp120 and CXCL12 differ in their ability to induce CXCR4 internalization in the human neuroblastoma cells SH-SY5Y, which constitutively express CXCR4. As expected, increasing concentration of CXCL12 reduced surface expression of CXCR4 in a time- and concentration-dependent manner. Conversely, $gp120_{IIIB}$ (monomeric or oligomeric, in presence or absence of soluble CD4) did not change CXCR4 membrane levels. Similar results were obtained in a murine lymphocyte cell line (300-19) stably expressing human CXCR4. Nevertheless, $gp120_{IIIB}$ was still able to activate intracellular signaling and proapoptotic pathways, via CXCR4. These results show that $gp120_{IIIB}$ toxicity and signaling do not require CXCR4 internalization in SH-SY5Y cells, and suggest that the viral protein may alter normal CXCR4 trafficking thus, interfering with activation of prosurvival pathways.

Keywords

cell death; chemokine; CXCL12; CXCR4; GPCRs; signaling

Introduction

The chemokine receptor CXCR4 is involved in the neurological complications of acquired immunodeficiency syndrome (AIDS) (Kaul *et al*, 2001; Zhang *et al*, 2003b). CXCR4 is the specific receptor for the chemokine CXCL12 (previously known as SDF-1), which orchestrates the development of many areas of the brain as well as other tissues (Lazarini *et al*, 2003; Tran and Miller, 2003; Zou *et al*, 1998). CXCR4 can also bind the human immunodeficiency virus (HIV) envelope protein gp120 that induces apoptotic cell death in primary neurons and cell lines (Bachis and Mocchetti, 2004; Catani *et al*, 2000; Hesselgesser *et al*, 1997; Kaul and Lipton, 1999; Mattson *et al*, 2005; Meucci and Miller, 1996). Despite a convincing role of gp120 in the apoptosis of neurons, the effects of CXCL12 on neuronal survival remain controversial.

Address correspondence to Olimpia Meucci, MD, PhD, Department of Pharmacology and Physiology, Drexel University College of Medicine, 245 North 15th Street, NCB Room 8804, Philadelphia, PA 19102, USA. E-mail: olimpia.meucci@drexel.edu.

In vivo studies indicate that the CXCR4/CXCL12 pair is crucial to neuronal development and proliferation, and a number of *in vitro* evidence favors a prosurvival action of this chemokine (Chalasani *et al*, 2003; Khan *et al*, 2003; Meucci *et al*, 1998). However, studies from other authors suggest that CXCL12 can also be neurotoxic (Kaul and Lipton, 1999). A recent report demonstrated that only a cleaved form of the chemokine—produced by the action of matrix metalloproteinases under pathological conditions, namely HIV encephalitis—is responsible for cell death (Zhang *et al*, 2003a). Interestingly, cleaved CXCL12 does not bind to CXCR4 as well as the full-length protein. This is in agreement with our previous studies showing that recombinant CXCL12 promotes neuronal survival and that differences in the intracellular signaling activated by CXCL12 and gp120 exist. Specifically, our data show that, although gp120 is able to stimulate mitogen-activated protein (MAP) kinases, and to induce Ca^{2+} rise in neurons, it is unable to activate prosurvival pathways, such as the phosphoinositide-3 kinase/(PI3K)/AKT pathway, which is necessary for chemokine neurotrophic action (Khan *et al*, 2004; Meucci *et al*, 1998). Moreover, gp120 and CXCL12 also differ in their regulation of specific cell cycle proteins involved in neuronal survival, namely p53, Rb, and E2F (Khan *et al*, 2003, 2005), which are directly and indirectly modulated by AKT. This could depend on differences in the intrinsic efficacies of the two CXCR4 ligands (Khan *et al*, 2004) and/or the presence of multiple receptor conformations (Baribaud *et al*, 2001; Zhou *et al*, 2002). Furthermore, based on recent evidence suggesting that receptor internalization may regulate G protein-coupled receptor (GPCR)-mediated signaling in various ways (Lefkowitz and Shenoy, 2005), we hypothesized that gp120 and CXCL12 may also diverge in their ability to induce CXCR4 internalization. Indeed, studies on alpha-thrombin receptor (a member of the GPCR family) have shown that the expression of a dominant negative beta-arrestin 1 (a group of intracellular proteins that promote receptor endocytosis and recycling) inhibits the rapid activation of the PI3K/AKT pathway without affecting the extra cellular signal-regulated kinase (ERK) pathway (Goel and Baldassare, 2002; Goel *et al*, 2002; Lefkowitz and Shenoy, 2005).

In order to determine whether differences in intracellular signaling and biological outcomes between CXCL12 and gp120 are associated with alterations in receptor trafficking, we studied the effect of the viral protein on CXCR4 internalization in the human neuroblastoma cell line SH-SY5Y by flow cytometry. Though other studies have previously reported the effect of gp120 on CXCR4 internalization, the data are quite controversial (Badr *et al*, 2005; Bodner *et al*, 2003; Fernandis *et al*, 2002, 2003; Tarasova *et al*, 1998), and the correlation between internalization, signaling, and toxicity have not been addressed. Furthermore, most of the previous studies were performed on cells transfected with CXCR4, whereas SH-SY5Y cells constitutively express CXCR4. This is a crucial aspect, considering that the signal transduction machinery may be quite different in cells that do not normally express CXCR4. The results reported here show that, despite the effect of the viral protein on cell signaling and survival, treatment of cells with $gp120_{IIIB}$ (alone or in complex with CD4) did not affect surface expression of CXCR4. These findings suggest that gp120-induced signaling and neurotoxicity do not require receptor internalization.

Results

Several studies have reported the neurotoxic effect of gp120 *in vitro* and *in vivo* and the involvement of chemokine receptors in this process (Catani *et al*, 2000; Hesselgesser *et al*, 1998; Kaul and Lipton, 1999; Meucci and Miller, 1996; Pandey and Bolsover, 2000; Zheng *et al*, 1999). In line with these reports, treatment of SH-SY5Y cells with $gp120_{IIIB}$ (200 pM) resulted in significant, albeit moderate, cell death (Figure 1), which was blocked by pretreatment with AMD3100 (100 ng/ml). This result confirms the involvement of CXCR4 in $gp120_{IIIB}$-induced death of SH-SY5Y cells.

As the gp120 is expressed on the HIV virus as a trimeric complex, we asked whether gp120-induced toxicity was higher in cultures treated with an oligomeric form of the protein. Furthermore, as binding of HIV to the chemokine receptor is regulated by CD4, we tested the action of $gp120_{IIIB}$/CD4 complexes on the neuroblastoma cells. However, comparable levels of cell death were induced by monomeric and oligomeric $gp120_{IIIB}$ either in the presence or absence of CD4 (Figure 1B). Of note, dependence of $gp120_{IIIB}$ on CD4 has been reported to be a cell type-specific event (Bodner *et al*, 2003). Treatment of SH-SY5Y cells with CXCL12 (20 nM) or AMD3100 (100 ng/ml) alone did not affect basal cell survival ($P > 0.05$ versus control), as expected (Figure 1B).

Changes in cell surface expression of CXCR4 induced by $gp120_{IIIB}$ (or CXCL12) were studied by flow cytometry. SH-SY5Y cells were incubated for 60 min at 37°C with increasing concentrations of either ligand, ranging from 20 pM to 20 nM (Figure 2). The samples were stained with a phycoerythrin-conjugated mouse monoclonal antibody as described in the methods section, and analyzed by fluorescence-activated cell sorting (FACS). To quantify differences in receptor expression, we measured the mean fluorescence intensity after every treatment and evaluated the percentage of CXCR4 on the cell surface of treated cells in comparison to the untreated cells. As expected, CXCL12 reduced surface expression of CXCR4 in a time- and dose-dependent manner, whereas $gp120_{IIIB}$ (up to 20 nM) did not change CXCR4 level (Figure 2). Incubation with 20 nM CXCL12 for 60 min led to a reduction of CXCR4 staining greater than 50% (Figure 2B).

To determine whether longer treatments were required to observe receptor internalization by $gp120_{IIIB}$, SH-SY5Y cells were incubated with the ligands up to 12 h. In agreement with the previous experiments, $gp120_{IIIB}$ (200 pM) did not affect CXCR4 surface expression at any time point observed (Figure 2C). Similar results were obtained with a high concentration of $gp120_{IIIB}$ (20nM, up to 4 h) (data not shown). On the contrary, CXCL12 (20 nM) internalized the receptor reaching the maximal level within the first 2 h. After this time the receptor expression gradually returned towards basal level reaching about 80% at 12 h (Figure 2C). This surface expression dynamics likely depends on ligand degradation (which reduces ligand-induced internalization) and recycling of the receptor (which helps restoring the receptor membrane pool) (Neel *et al*, 2005).

Similar results were obtained with the oligomeric$gp120_{IIIB}$, which was unable to induce internalization of the chemokine receptor on SH-SY5Y neuroblastoma cells (Figure 3A). Also, binding of $gp120_{IIIB}$ to equimolar concentrations of human soluble CD4 did not change CXCR4 expression on the surface of treated cells (Figure 3A). We then tested a gp120 from a different HIV strain, i.e., the $gp120_{SF2}$. Treatment with 20 nM $gp120_{SF2}$ at 37°C for 60 min (Figure 3A) or 4 h (data not shown) also did not alter CXCR4 expression on the surface of SH-SY5Y cells. Finally, to determine whether this phenomenon was limited to neuroblastoma cells, we used a mouse pre-B-cell line stably transfected with human CXCR4 (300-19/CXCR4) (Figure 3B). These cells were treated with increasing concentration of $gp120_{IIIB}$ or CXCL12 for 60 min (Figure 3B). Experiments with the oligomeric $gp120_{IIIB}$ (20 nM) were also performed (Figure 3C). Altogether, these studies showed that, though CXCL12 induces a dose-dependent internalization of CXCR4, reaching almost 50% of receptor internalization at a concentration of 2 nM, $gp120_{IIIB}$ was unable to induce CXCR4 internalization in the mouse pre-B cells. This is shown by the complete overlay of the traces of untreated cells (blue) and cells treated with either monomeric-$gp120_{IIIB}$ (orange) or oligomeric-$gp120_{IIIB}$ (green) (Figure 3C).

The results of the flow cytometry studies could raise concerns about the ability of gp120 to signal through the chemokine receptor on SH-SY5Y cells. Thus, we tested the ability of the viral protein to activate CXCR4-dependent pathways, such of ERK1/2 phosphorylation,

caspase activation or other apoptotic pathways (Garden *et al*, 2002; Khan *et al*, 2005; Meucci *et al*, 1998). As previously reported in different cells (Khan *et al*, 2004; Lazarini *et al*, 2000; Meucci *et al*, 1998), CXCL12 and $gp120_{IIIB}$ are both able to induce phosphorylation of ERK1/2 in SHSY5Y cells (Figure 4A), with peak responses generally observed at 5 min for both ligands (data not shown). This effect was completely blocked by pretreatment of cells with the CXCR4 specific inhibitor AMD3100 (100 ng/ml) (Figure 4). The HIV protein also stimulated, in an AMD3100-dependent manner, caspase 3 cleavage (Figure 1A) and E2F1 activity (Shimizu *et al*, submitted). These results demonstrate the presence of a functional chemokine receptor on the surface of SH-SY5Y neuroblastoma cells and show that $gp120_{IIIB}$-induced cell death and signaling do not require CXCR4 internalization in the neuroblastoma cells.

Discussion

Chemokine receptors expressed on the cell surface undergo a basal level of internalization and degradation in absence of specific ligand (Signoret *et al*, 2000). A certain amount of receptors on the cell membrane is maintained by new synthesis and recycling mechanisms. Internalization of chemokine receptors can be enhanced by ligand binding leading to a clathrin-mediated or lipid raft/caveolae-dependent endocytosis (Neel *et al*, 2005). This agonist-mediated receptor internalization represents an important physiological feedback mechanism that protects the cell against acute and chronic over-stimulation. The signaling through CXCR4 in brain-derived cells can result in opposite biological events, such as survival or apoptosis (Chalasani *et al*, 2003; Kaul and Lipton, 1999; Khan *et al*, 2004; Meucci *et al*, 1998). Activation of these pathways may depend on the nature of the ligand and the subsequent intracellular events (Khan *et al*, 2005; Zhang *et al*, 2003a). The present study suggests that CXCR4 internalization may also contribute to the different effects of CXCR4 on cell survival. The fact that gp120 does not induce CXCR4 internalization emphasizes a potential role of CXCR4 internalization in the activation of prosurvival pathways—although this still remains to be proven. However, previous studies showing that CXCL12, but not gp120, activates the PI3K/AKT pathway (Khan *et al*, 2004) support this hypothesis. In addition, studies from other investigators indicate that beta-arrestins (which mediate receptor internalization) are instrumental in the coupling of GPCRs and tyrosine kinases receptors to antiapoptotic pathways, including AKT (Goel and Baldassare, 2002; Povsic *et al*, 2003; Revankar *et al*, 2004). Thus, pathways regulated by AKT (such as the Rb/E2F pathway) could be altered as well (Khan *et al*, 2003).

The inability of gp120 to induce CXCR4 internalization was also reported in a CXCR4-transfected cell line (Wang *et al*, 1998). These authors found that a specific anti-CD4 antibody was required for the internalization of the gp120/CXCR4/CD4 complex, and suggest that CXCR4 endocytosis is secondary to CD4-dependent signaling. Indeed, our results in SH-SY5Y cells and those from other investigators in hematopoietic $CD34^+$ cells demonstrate that soluble CD4 is not sufficient for gp120 to trigger CXCR4 internalization, whereas gp120 causes CXCR4 internalization in CD4+ lymphocytes (Aiuti *et al*, 1999; Fernandis *et al*, 2002). Altogether, these data suggest that gp120-induced CXCR4 endocytosis is strictly dependent on the cellular expression of CD4, at least in certain cells. As neurons do not generally express the CD4 molecule, it is possible that gp120 does not cause CXCR4 internalization in neurons *in vivo*. This hypothesis is also supported by our previous studies (Khan *et al*, 2004) showing that pretreatment of $CD4^-/CXCR4^+$ cells with $gp120_{IIIB}$ did not abolish calcium responses stimulated by the physiological CXCR4 agonist (CXCL12), suggesting that the viral protein is unable to induce proper CXCR4 down-regulation despite that it activates several intracellular responses (Meucci *et al*, 1998, and this study). However, a recent confocal microscopy study reported a time-dependent internalization of recombinant gp120 in cerebellar granule neurons (Bachis *et al*, 2003). The authors find that internalization of biotin-conjugated gp120 is blocked by brain-derived neurotrophic factor, which also prevents gp120 neurotoxicity and reduces

CXCR4 expression, i.e., CXCR4 total protein levels. The effect of gp120 on CXCR4 internalization/surface expression, however, is not reported in their study. Moreover, gp120 internalization was not observed in glial cells (Bachis *et al*, 2003), which also express CXCR4, thus questioning whether this is a CXCR4-dependent event (Lazarini *et al*, 2003). Thus, the apparent discrepancy with our findings could be due to various scenarios, including differences in the cell types and experimental approaches, the reported binding of gp120 to other molecules expressed on the neuronal surface, such as glycosphingolipids (Cook *et al*, 1994; Long *et al*, 1994), and/or the expression of unknown CD4-like molecules in granule neurons. Alternatively, the HIV envelope protein could enter the cell via ligand-independent chemokine receptor internalization (Neel *et al*, 2005; Signoret *et al*, 2000), which would be consistent with the kinetics of gp120 accumulation in cerebellar granule neurons (Bachis *et al*, 2003). On the other hand, the dynamics of CXCR4 expression on the membrane of SH-SY5Y cells treated with CXCL12 (Figure 2) is a typical example of ligand-induced receptor endocytosis, a common and reversible mechanism of desensitization of chemokine receptor and GPCRs in general (Ferguson, 2001; Neel *et al*, 2005).

In conclusion, our findings show that gp120-induced signaling and toxicity is independent of CXCR4 internalization. Thus, gp120 and CXCL12 differ in their interaction with CXCR4 at multiple levels, which may also include receptor trafficking. However, it remains to be established whether this phenomenon is responsible for the altered coupling of CXCR4 to survival pathways upon stimulation by the HIV envelope protein.

Materials and methods

Cells cultures

SH-SY5Y human neuroblastoma cells were obtained from ATCC and cultured in 45% minimum essential medium, 45% Ham's F-12 medium, 10% fetal bovine serum (FBS) containing 50 μg/ml gentamycin. Medium was replaced every 3 days. Mouse pre-B 300-19 cells stably transfected with human CXCR4 were a kind gift from Dr. Bernhard Moser (Institute of Cell Biology, University of Bern, Switzerland). These cells were cultured in RPMI 1640 supplemented with 10% FBS, 1% nonessential amino acids, 1mM sodium pyruvate, 0.05 mM β-mercaptoethanol, 50 U/ml penicillin, 50 mg/ml streptomycin, 2 mM glutamine, and 1.5 μg/mL puromycin.

Flow cytometry

SH-SY5Y or 300-19/CXCR4 cells were treated with CXCL12 or gp120s as indicated in the appropriate figures for different times at 37°C. After treatments, cells were washed and resuspended in cold Ca^{2+}-free phosphate-buffered saline (PBS), and then incubated in PBS supplemented with 10% horse serum (30 min on ice). Cells were then resuspended in FACS buffer (1% bovine serum albumin (BSA)/PBS) and incubated (30 min on ice in the dark) with 12.5 μg/ml (5 μg/ml for 300-19/CXCR4 cells) phycoerythrin (PE)-conjugated antibodies (FAB173P; R&D System). We used a mouse monoclonal anti-human CXCR4-PE and a mouse IgG2B-PE as the isotype control. After incubation, cells were washed and fixed with 1% paraformaldehyde in FACS buffer. Samples acquisition was performed with a FACS Calibur (BD).

Survival

Cell death was evaluated 24 h after treatment. Hoechst 33342 (2 μg/ml) was used to evaluate differences between normal and apoptotic nuclei (Meucci *et al*, 1998). Additionally, cleaved caspase 3 staining with an antibody that specifically recognizes cleaved/active caspase 3 (80 ng/ml; Cell Signaling) was also used to identify apoptotic cells as described previously (Khan *et al*, 2005). Six microscopic fields per coverslip were counted and three coverslips/treatment

were used for each experiment. Analysis of the staining has been performed using Metamorph (Universal Imaging) software.

Western Blots

Following drug treatment, cells were washed with ice-cold PBS, scraped with lysis buffer (150 mM NaCl, 50 mM Tris, 0.5% Na deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 10 mM $Na_4P_2O_7$, 5 mM EDTA, 1% Triton X, 5 μg each of aprotinin, leupeptin, and pepstatin/1 mM 4-(2 aminoethyl)benzeresulfonyl fluoride HCE (AEBSF)/1 mM vanadate], and incubated for 30 min on a rotor at 4°C. After centrifugation at 20,800 × g for 10 min, the protein concentration of the supernatants was determined by the bicinchoninic acid protein assay (PIERCE). Equal amout of protein (40 μg) were loaded in each lane. Proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes for immunoblotting. After blotting for pERK (11.25 ng/ml), membranes were stripped and reprobed for total ERK (1.25 ng/ml). Both antibodies are from Cell Signaling Technology. Horseradish peroxidase (HRP)-conjugated Protein A (1:20000; Sigma) was used to resolve the bands. An image acquisition and analysis system, from Alpha Innotech (FluorChem 8900) was used for detection of chemiluminescent bands and densitometry analysis. Intensity levels from total ERK bands were used to normalize phosphoprotein signals and compensate for possible variations in protein loading. Data are reported as mean ± SEM.

Reagents

Unless otherwise specified, tissue culture media are from Gibco-Invitrogen and other general reagents are from Sigma. CXCL12 was purchased from PeproTech (NJ). Recombinant HIV-1 $gp120_{IIIB}$ (both monomeric and oligomeric) was from ImmunoDiagnostics. Stock solutions of gp120s and CXCL12 were prepared and stored as previously described (Meucci *et al*, 1998). AMD3100 was obtained from Sigma and dissolved in sterile water before further dilution in culture medium. gp120-sCD4 binding was achieved after 30 min incubation at 37°C of equimolar amount of the two reagents as previously described by (Bodner *et al*, 2003). Recombinant soluble CD4 was from Intracel Corporation.

Statistical analysis

One-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test was used for CXCR4 internalization and survival experiments. Paired *t* test was used to compare differences in the band densities of immunoblots.

Acknowledgements

The following reagent was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 SF2 gp120 from Chiron Corporation. This work was supported by the NIH grants DA15014 and DA19808 to OM. JP was partially supported by an NRSA fellowship (DA-020234).

Abbreviations used

HIV, Human Immunodeficiency Virus; AIDS, acquired immune deficiency syndrome; gp120, glycoprotein 120KDa; GPCRs, G protein-coupled receptors; PI3K, phosphoinositide-3 kinase; AKT, serine/threonine kinase AKT/PKB; ERK, extracellular signal-regulated kinase.

References

Aiuti A, Turchetto L, Cota M, Cipponi A, Brambilla A, Arcelloni C, Paroni R, Vicenzi E, Bordignon C, Poli G. Human CD34(+) cells express CXCR4 and its ligand stromal cell-derived factor-1.

Implications for infection by T-cell tropic human immunodeficiency virus. Blood 1999;94:62–73. [PubMed: 10381499]

Bachis A, Major EO, Mocchetti I. Brain-derived neurotrophic factor inhibits human immunodeficiency virus-1/gp120-mediated cerebellar granule cell death by preventing gp120 internalization. J Neurosci 2003;23:5715–5722. [PubMed: 12843275]

Bachis A, Mocchetti I. The chemokine receptor CXCR4 and not the *N*-methyl-D-aspartate receptor mediates gp120 neurotoxicity in cerebellar granule cells. J Neurosci Res 2004;75:75–82. [PubMed: 14689450]

Badr G, Borhis G, Treton D, Moog C, Garraud O, Richard Y. HIV type 1 glycoprotein 120 inhibits human B cell chemotaxis to CXC chemokine ligand (CXCL) 12, CC chemokine ligand (CCL)20, and CCL21. J Immunol 2005;175:302–310. [PubMed: 15972662]

Baribaud F, Edwards TG, Sharron M, Brelot A, Heveker N, Price K, Mortari F, Alizon M, Tsang M, Doms RW. Antigenically distinct conformations of CXCR4. J Virol 2001;75:8957–8967. [PubMed: 11533159]

Bodner A, Toth PT, Oh SB, Lu M, Tran PB, Chin RK, Ren D, Miller RJ. CD4 dependence of gp120IIIB-CXCR4 interaction is cell-type specific. J Neuroimmunol 2003;140:1–12. [PubMed: 12864967]

Catani MV, Corasaniti MT, Navarra M, Nistico G, Finazzi-Agro A, Melino G. gp120 induces cell death in human neuroblastoma cells through the CXCR4 and CCR5 chemokine receptors. J Neurochem 2000;74:2373–2379. [PubMed: 10820198]

Chalasani SH, Baribaud F, Coughlan CM, Sunshine MJ, Lee VM, Doms RW, Littman DR, Raper JA. The chemokine stromal cell-derived factor-1 promotes the survival of embryonic retinal ganglion cells. J Neurosci 2003;23:4601–4612. [PubMed: 12805300]

Cook DG, Fantini J, Spitalnik SL, Gonzalez-Scarano F. Binding of human immunodeficiency virus type I (HIV-1) gp120 to galactosylceramide (GalCer): relationship to the V3 loop. Virology 1994;201:206–214. [PubMed: 8184533]

Ferguson SS. Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. Pharmacol Rev 2001;53:1–24. [PubMed: 11171937]

Fernandis AZ, Cherla RP, Chernock RD, Ganju RK. CXCR4/CCR5 down-modulation and chemotaxis are regulated by the proteasome pathway. J Biol Chem 2002;277:18111–18117. [PubMed: 11877445]

Fernandis AZ, Cherla RP, Ganju RK. Differential regulation of CXCR4-mediated T-cell chemotaxis and mitogen-activated protein kinase activation by the membrane tyrosine phosphatase, CD45. J Biol Chem 2003;278:9536–9543. [PubMed: 12519755]

Garden GA, Budd SL, Tsai E, Hanson L, Kaul M, D'Emilia DM, Friedlander RM, Yuan J, Masliah E, Lipton SA. Caspase cascades in human immunodeficiency virus-associated neurodegeneration. J Neurosci 2002;22:4015–4024. [PubMed: 12019321]

Goel R, Baldassare JJ. beta-Arrestin 1 couples thrombin to the rapid activation of the Akt pathway. Ann NY Acad Sci 2002;973:138–141. [PubMed: 12485850]

Goel R, Phillips-Mason PJ, Raben DM, Baldassare JJ. alpha-Thrombin induces rapid and sustained Akt phosphorylation by beta-arrestin1-dependent and -independent mechanisms, and only the sustained Akt phosphorylation is essential for G1 phase progression. J Biol Chem 2002;277:18640–18648. [PubMed: 11901145]

Hesselgesser J, Halks-Miller M, DelVecchio V, Peiper SC, Hoxie J, Kolson DL, Taub D, Horuk R. CD4-independent association between HIV-1 gp120 and CXCR4: functional chemokine receptors are expressed in human neurons. Curr Biol 1997;7:112–121. [PubMed: 9024623]

Hesselgesser J, Taub D, Baskar P, Greenberg M, Hoxie J, Kolson DL, Horuk R. Neuronal apoptosis induced by HIV-1 gp120 and the chemokine SDF-1 alpha is mediated by the chemokine receptor CXCR4. Curr Biol 1998;8:595–598. [PubMed: 9601645]

Kaul M, Garden GA, Lipton SA. Pathways to neuronal injury and apoptosis in HIV-associated dementia. Nature 2001;410:988–994. [PubMed: 11309629]

Kaul M, Lipton SA. Chemokines and activated macrophages in HIV gp120-induced neuronal apoptosis. Proc Natl Acad Sci U S A 1999;96:8212–8216. [PubMed: 10393974]

Khan MZ, Brandimarti R, Musser BJ, Resue DM, Fatatis A, Meucci O. The chemokine receptor CXCR4 regulates cell-cycle proteins in neurons. J NeuroVirol 2003;9:300–314. [PubMed: 12775414]

Khan MZ, Brandimarti R, Patel JP, Huynh N, Wang J, Huang Z, Fatatis A, Meucci O. Apoptotic and antiapoptotic effects of CXCR4: is it a matter of intrinsic efficacy? Implications for HIV neuropathogenesis. AIDS Res Hum Retroviruses 2004;20:1063–1071. [PubMed: 15585097]

Khan MZ, Shimizu S, Patel JP, Nelson A, Le MT, Mullen-Przeworski A, Brandimarti R, Fatatis A, Meucci O. Regulation of neuronal P53 activity by CXCR4. Mol Cell Neurosci 2005;30:58–66. [PubMed: 16005638]

Lazarini F, Casanova P, Tham TN, De Clercq E, Arenzana-Seisdedos F, Baleux F, Dubois-Dalcq M. Differential signalling of the chemokine receptor CXCR4 by stromal cell-derived factor 1 and the HIV glycoprotein in rat neurons and astrocytes. Eur J Neurosci 2000;12:117–125. [PubMed: 10651866]

Lazarini F, Tham TN, Casanova P, Arenzana-Seisdedos F, Dubois-Dalcq M. Role of the alpha-chemokine stromal cell-derived factor (SDF-1) in the developing and mature central nervous system. Glia 2003;42:139–148. [PubMed: 12655598]

Lefkowitz RJ, Shenoy SK. Transduction of receptor signals by beta-arrestins. Science 2005;308:512–517. [PubMed: 15845844]

Long D, Berson JF, Cook DG, Doms RW. Characterization of human immunodeficiency virus type 1 gp120 binding to liposomes containing galactosylceramide. J Virol 1994;68:5890–5898. [PubMed: 8057468]

Mattson MP, Haughey NJ, Nath A. Cell death in HIV dementia. Cell Death Differ 2005;12(Suppl 1): 893–904. [PubMed: 15761472]

Meucci O, Fatatis A, Simen AA, Bushell TJ, Gray PW, Miller RJ. Chemokines regulate hippocampal neuronal signaling and gp120 neurotoxicity. Proc Natl Acad Sci U S A 1998;95:14500–14505. [PubMed: 9826729]

Meucci O, Miller RJ. gp120-induced neurotoxicity in hippocampal pyramidal neuron cultures: protective action of TGF-beta1. J Neurosci 1996;16:4080–4088. [PubMed: 8753870]

Neel NF, Schutyser E, Sai J, Fan GH, Richmond A. Chemokine receptor internalization and intracellular trafficking. Cytokine Growth Factor Rev 2005;16:637–658. [PubMed: 15998596]

Pandey V, Bolsover SR. Immediate and neurotoxic effects of HIV protein gp120 act through CXCR4 receptor. Biochem Biophys Res Commun 2000;274:212–215. [PubMed: 10903920]

Povsic TJ, Kohout TA, Lefkowitz RJ. Beta-arrestin1 mediates insulin-like growth factor 1 (IGF-1) activation of phosphatidylinositol 3-kinase (PI3K) and antiapoptosis. J Biol Chem 2003;278:51334–51339. [PubMed: 14534298]

Revankar CM, Vines CM, Cimino DF, Prossnitz ER. Arrestins block G protein-coupled receptor-mediated apoptosis. J Biol Chem 2004;279:24578–24584. [PubMed: 15051714]

Signoret N, Pelchen-Matthews A, Mack M, Proudfoot AE, Marsh M. Endocytosis and recycling of the HIV coreceptor CCR5. J Cell Biol 2000;151:1281–1294. [PubMed: 11121442]

Tarasova NI, Stauber RH, Michejda CJ. Spontaneous and ligand-induced trafficking of CXC-chemokine receptor 4. J Biol Chem 1998;273:15883–15886. [PubMed: 9632631]

Tran PB, Miller RJ. Chemokine receptors: signposts to brain development and disease. Nat Rev Neurosci 2003;4:444–455. [PubMed: 12778117]

Wang JM, Ueda H, Howard OM, Grimm MC, Chertov O, Gong X, Gong W, Resau JH, Broder CC, Evans G, Arthur LO, Ruscetti FW, Oppenheim JJ. HIV-1 envelope gp120 inhibits the monocyte response to chemokines through CD4 signal-dependent chemokine receptor down-regulation. J Immunol 1998;161:4309–4317. [PubMed: 9780207]

Zhang K, McQuibban GA, Silva C, Butler GS, Johnston JB, Holden J, Clark-Lewis I, Overall CM, Power C. HIV-induced metalloproteinase processing of the chemokine stromal cell derived factor-1 causes neurodegeneration. Nat Neurosci 2003a;6:1064–1071. [PubMed: 14502291]

Zhang K, Rana F, Silva C, Ethier J, Wehrly K, Chesebro B, Power C. Human immunodeficiency virus type 1 envelope-mediated neuronal death: uncoupling of viral replication and neurotoxicity. J Virol 2003b;77:6899–6912. [PubMed: 12768009]

Zheng J, Thylin MR, Ghorpade A, Xiong H, Persidsky Y, Cotter R, Niemann D, Che M, Zeng YC, Gelbard HA, Shepard RB, Swartz JM, Gendelman HE. Intracellular CXCR4 signaling, neuronal apoptosis and neuropathogenic mechanisms of HIV-1-associated dementia. J Neuroimmunol 1999;98:185–200. [PubMed: 10430052]

Zhou N, Luo Z, Luo J, Fan X, Cayabyab M, Hiraoka M, Liu D, Han X, Pesavento J, Dong CZ, Wang Y, An J, Kaji H, Sodroski JG, Huang Z. Exploring the stereochemistry of CXCR4-peptide recognition and inhibiting HIV-1 entry with D-peptides derived from chemokines. J Biol Chem 2002;277:17476–17485. [PubMed: 11880384]

Zou YR, Kottmann AH, Kuroda M, Taniuchi I, Littman DR. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature 1998;393:595–599. [PubMed: 9634238]

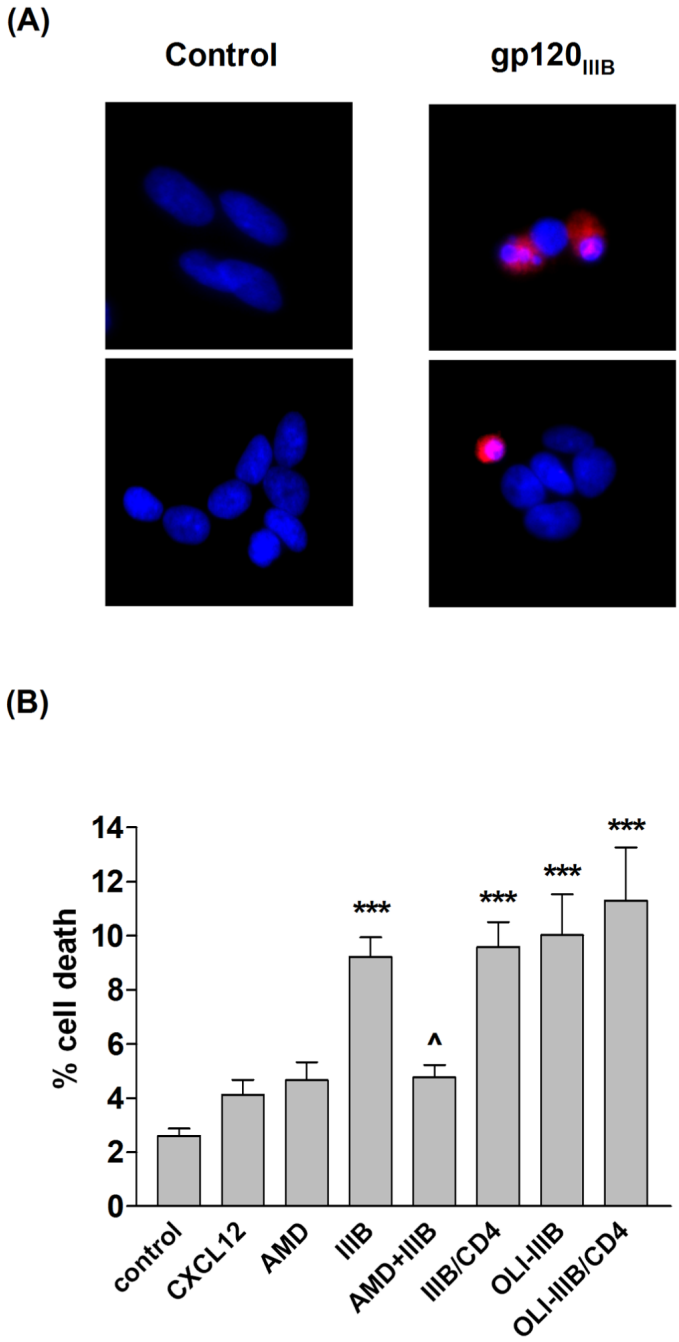

Figure 1.

HIV gp120 induces cell death in SH-SY5Y cells. SH-SY5Y neuroblastoma cells were serum starved for 48 h and treated with monomeric or oligomeric $gp120_{IIIB}$ (200 pM), monomeric or oligomeric $gp120_{IIIB}$/CD4 complexes (200 pM), or CXCL12 (20 nM) for the following 24 h at 37°C. Two groups of samples were pretreated for 15 min at 37° C with 100 ng/ml of AMD3100 and subsequently treated with vehicle or monomeric $gp120_{IIIB}$ (200 pM) for 24 h at 37°C. At the end of the treatment, cells were fixed, stained for cleaved caspase 3 (red in **A**), and counted. The graph shows percentage of dead cells (mean ± SEM) from three independent experiments (**B**). Six microscopic fields per coverslip were counted and three

coverslips per treatment were used for each experiment. (*** $P < 0.001$ gp120s versus control; ^ $P < 0.001$, $gp120_{IIIB}$ versus AMD3100 + $gp120_{IIIB}$).

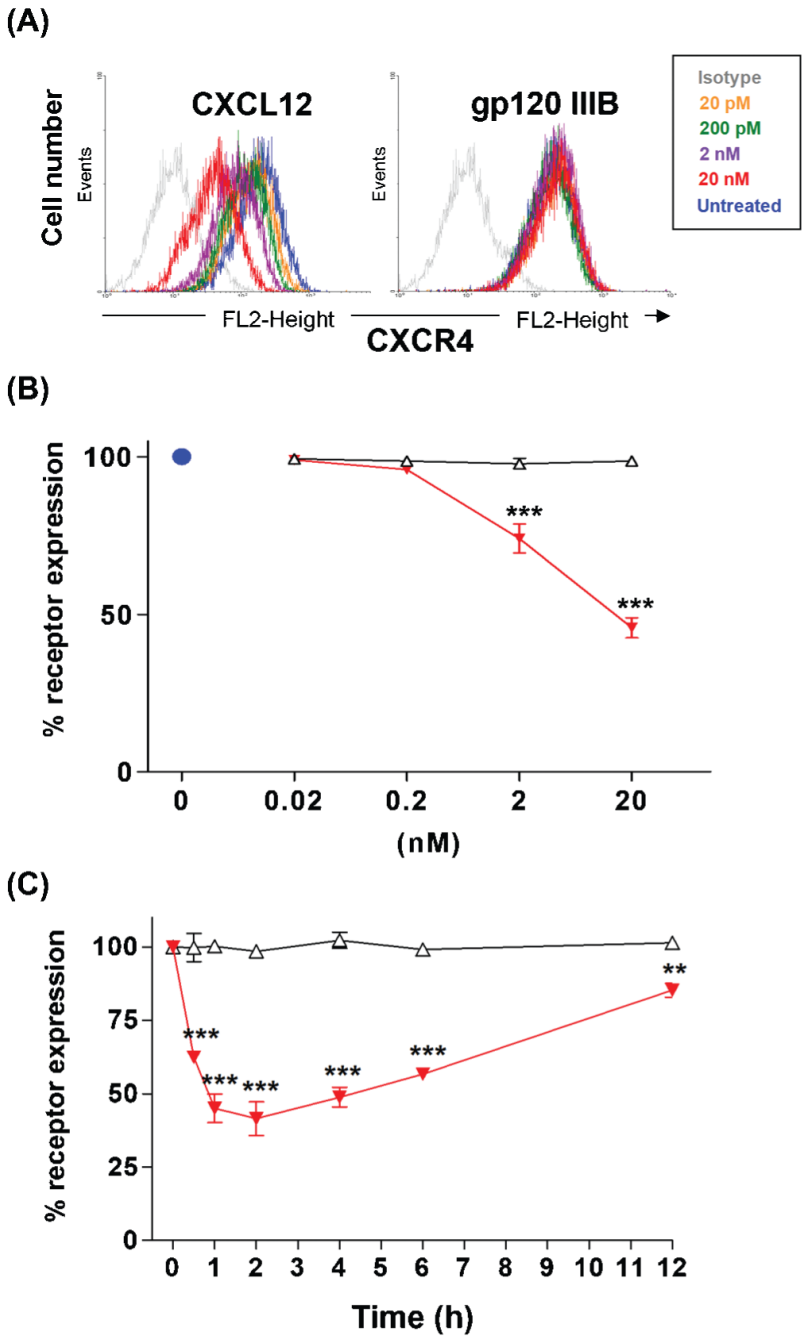

Figure 2.

HIV gp120 is unable to induce CXCR4 internalization in SH-SY5Y cells. SH-SY5Y neuroblastoma cells were incubated with different concentrations of CXCL12 and $gp120_{IIIB}$ (20 pM to 20 nM). The relative cell surface expression of CXCR4 was determined by flow cytometry after staining with PE-conjugated monoclonal anti-human CXCR4 antibody. (**A**) Representative traces from experiments in which cells were incubated with the two ligands for 60 min. (**B**) The average of three independent experiments reported as the mean fluorescence intensity of CXCL12-treated (*red filled triangle*) and $gp120_{IIIB}$-treated (*open triangles*) groups, compared to untreated cells (100%, *blue dot*). Some of the error bars of $gp120_{IIIB}$ treated cells are not clearly visible in the graph and thus reported here as well: at 0.02 nM, 99.37% ± 1.26%;

at 0.2 nM, 98.63% ± 1.23%; at 2 nM, 97.8% 1.72%; at 20 nM, 98.67% ± 1.30%. (**C**) Data from time-course experiments (from 30 min to 12-h) after treatment with 200 pM $gp120_{IIIB}$ (*open triange*) or 20 nM CXCL12 (*filled red triangles*). The graph represents the averages of three independent experiments ± SEM. (treatments versus control, ** $P < 0.01$; *** $P < 0.001$).

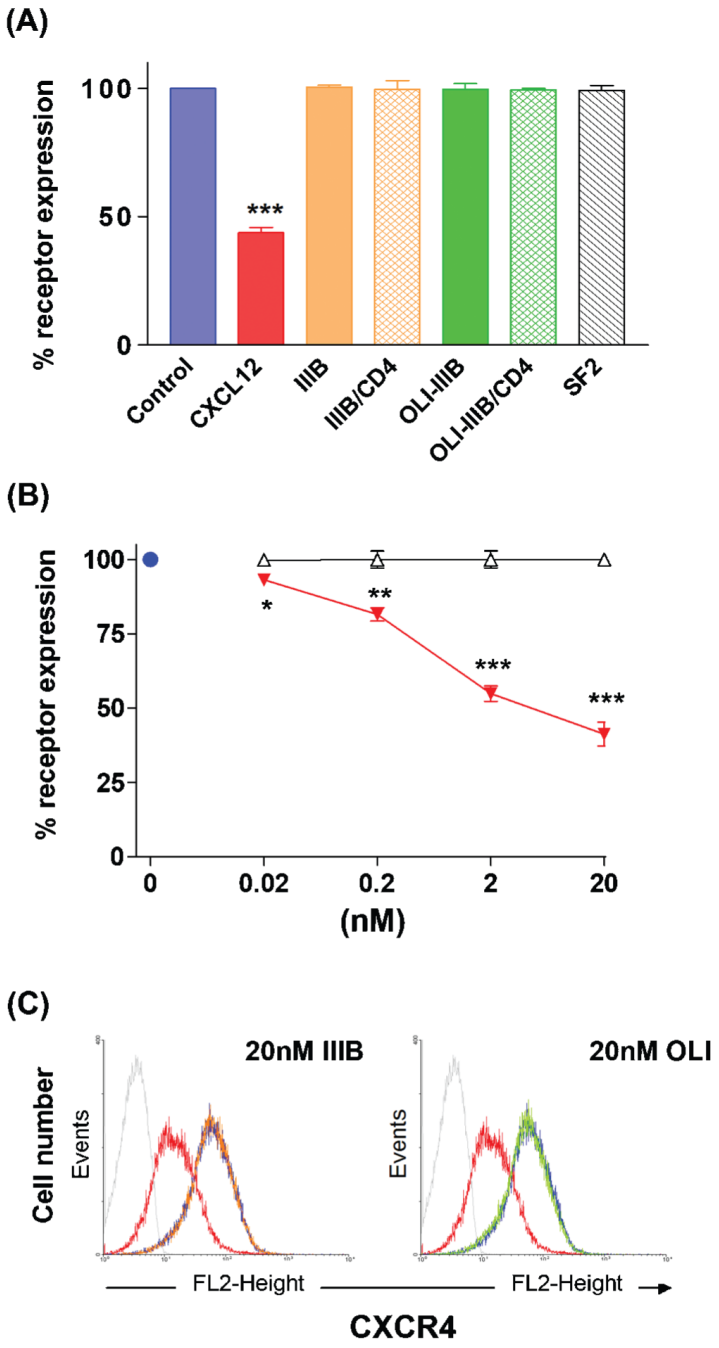

Figure 3.

-HIV $gp120_{IIIB}$/CD4 complex does not affect CXCR4 expression. (**A**) CXCR4 cell surface expression on SH-SY5Y cells after incubation for 60 min at 37°C without (control) or with 20 nM of the indicated ligands. Bars represent the averages of two to four independent experiments ± SEM. (**B**) 300-19/CXCR4 cells were incubated for 60 min at 37°C with increasing concentrations of $gp120_{IIIB}$ (*open triangles*) or CXCL12 (*red filled triangles*). The percentage of receptor expression from two independent experiments as compared to control cells is shown (mean ± SEM) (treatments versus control, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). (**C**) Representative traces from a FACS analysis experiment in 300-19/CXCR4 cells (60 min at 37°C). A total of three independent experiments with both monomeric and oligomeric

$gp120_{IIIB}$ as well as the CXCL12 control were performed; untreated cells (*blue trace*), 20 nM monomeric-$gp120_{IIIB}$ (*orange trace*), 20 nM oligomeric-$gp120_{IIIB}$(*green trace*), 20 nM CXCL12 (*red trace*). The gray trace represents the antibody isotype control.

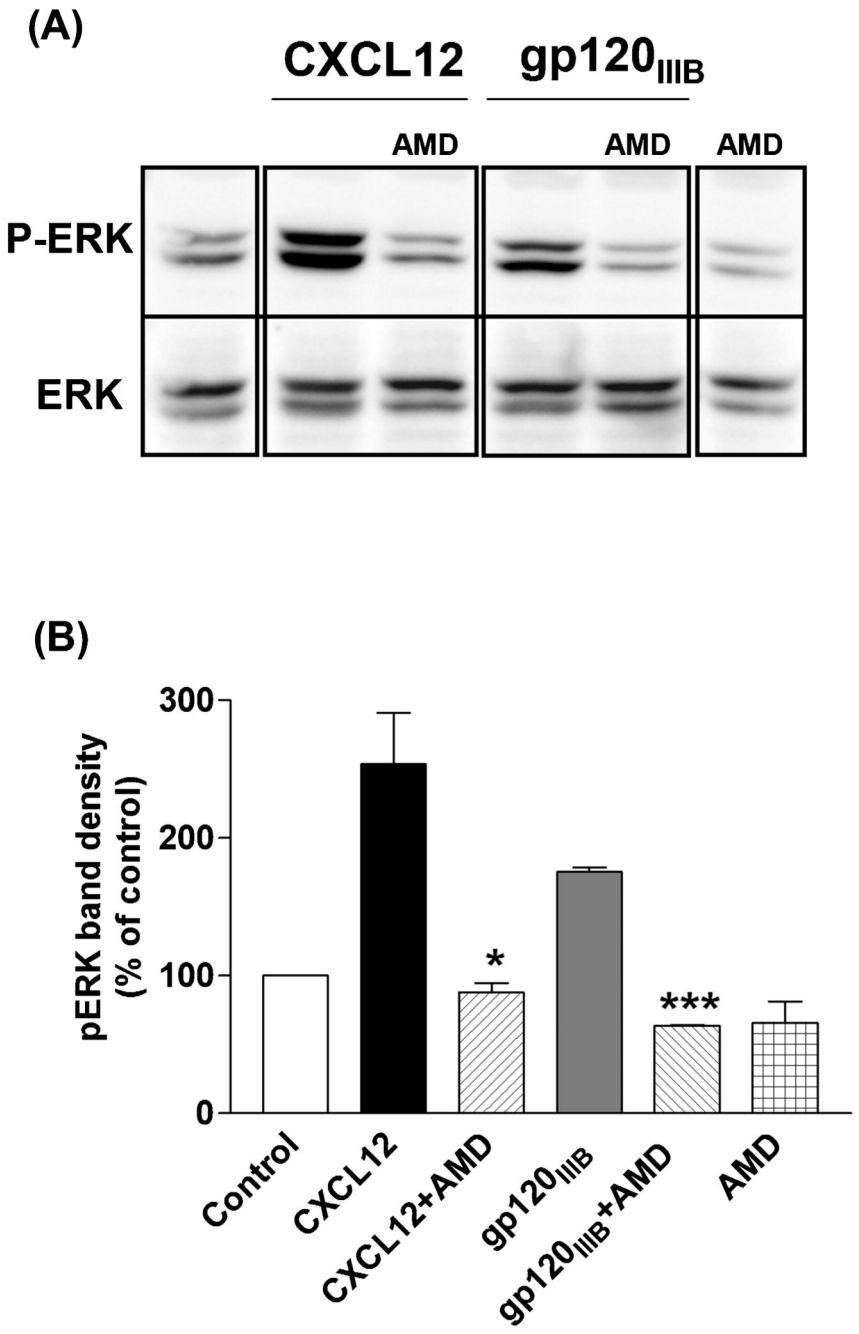

Figure 4.

HIV $gp120_{IIIB}$signals through CXCR4 on SH-SY5Y cells. Pretreatment of SH-SY5Y cells with the specific CXCR4 antagonist AMD3100 (100 ng/ml) abolishes CXCL12- (2 nM, 5 min) and $gp120_{IIIB}$- (200 pM, 5 min) induced phosphorylation of ERK (**A**). (**B**) The densitometry analysis of the bands (mean ± SEM) of three independent experiments (CXCL12 + AMD versus CXCL12,*P** < 0.05; $gp120_{IIIB}$ + AMD versus $gp120_{IIIB}$, *** *P* < 0.001).